THE USE OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND MEASURES OF CATTLE TEMPERAMENT TO PREDICT FEEDLOT PERFROMANCE OF COMMERCIAL BEEF CATTLE

by

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ABSTRACT

The objective of this study was to identify small molecule metabolites in a serum sample taken at entry into the feedlot that can predict performance, and animal health. One-hundred and thirty-one Angus x Simmental steers from a single ranch were sampled at a commercial feedlot. Blood samples for metabolite analysis, chute score, exit velocity, and blood lactate concentration for temperament classification were collected in addition to feedlot performance data and carcass quality measurements. The GLM and LSM procedures of SAS were used to evaluate differences between temperament classifications. Steers were divided into three exit velocity classifications with fast animals having exit velocity greater than one standard deviation from the mean and slow animals having exit velocities lower than one standard deviation from the mean. Forty metabolites were quantified using 1H NMR Spectroscopy from serum. Metaboanalyst was used to analyze serum metabolites and phenotypic values using one way- ANOVA, PCA, PLS-DA, and a permutation test to cross validate. Data was normalized and scaled. No metabolites were predictive of any of the animal health metrics collected. Five metabolites were different in exit velocity class at $p < 0.01$ (methanol, isopropanol, lactate, isobutyrate, and pyruvate). Similarly, 7 metabolites were different between chute score classes at $p < 0.01$ (methanol, isobutyrate, creatinine, dimethyl sulfone, hippurate, isopropanol, and succinate). Furthermore, several metabolites in serum at entry in the feedlot were related to carcass quality metrics: back fat (urea and 2-hydroxyisobutyrate at $p < 0.01$), a trend for prediction of quality grade at $p = 0.068$, carcass value $p = 0.085$. The relationship between serum metabolites, feedlot performance traits, and eventual carcass quality warrants further research to elucidate the roles these metabolites play during the feedlot period and in predicting carcass merit.

CHAPTER ONE

INTRODUCTION

Metabolic processes are an integral part controlling homeostasis. Understanding the complex relationships of energy, fat, and protein metabolism would allow us to address problems that are associated with stressors related to nutrition, disease, reproduction, social interactions and changes to environment. The understanding of metabolism would increase the efficiency of production, decrease the incidence of disease, and develop proper management for cattle in a feedlot setting. Entry into the feedlot is a very challenging time for cattle due to all the stressors that are associated with the process. Cattle that are entering into a feedlot have been transported, mixed with animals of an unknown background. There are roughly 30,000 feedlots across the United States with 14.4 million cattle (Nation Cattlemen's Beef Association, 2017). Feedlots are used to efficiently feed cattle to a desirable weight and degree of fatness. Typical feedlot diets start with a high roughage diet, and progressively move towards a high concentrate, grain, diet. Most of the feedlots in the United States are in the Midwest, or in areas where corn production is high.

Feedlot illness results in 1-5% mortality rate and 15-45% morbidity rate (Kelly et al., 1986). Rates of mortality and morbidity change depending on overall general animal health. Bovine respiratory disease (BRD) is the predominant source of feedlot mortality and morbidity and is a complicated disease involving several pathogens and stressors which costs the beef industry in excess of $4 billion annually (Grandin, 1997). Animal health is of major concern as many studies have shown that healthier more docile animals perform better in a feedlot (Fell et al., 1999; Voisinet et al., 1996; Burrow and Dillon, 1997). Fell et al., 1999, reported that 5 out of

the 12 nervous animals were taken to the hospital pen for respiratory disease while no calm animals were treated. Cattle temperament also influences growth (Burrow and Dillon, 1997), carcass grading, meat quality (Voisinet et al., 1996), and feedlot performance Boles2012). This research is based on a single blood collection from 143 steers on entry into the feedlot and the objective was to evaluate the predictive ability of serum metabolites for animal health phenotypes. Specific measurements of feedlot performance and carcass characteristics such as ADG, RFI, days on feed, dry matter intake (DMI), hot carcass weights, yield grade, quality grade, marbling score, rib eye area (REA) and carcass value were also examined. Nuclear magnetic resonance spectroscopy (NMR) was used for serum metabolite profiling. NMR metabolic profiling can give us a look into protein, lipid, and carbohydrate metabolism; thus, opening new avenues for the study of long-term effects of stress and environment. If NMR metabolic profiling can identify a potential suite of metabolites that can distinguish cattle based on growth curve, production traits, or carcass traits it will add to our understanding of the effects of BRD and aid in identification and treatment of BRD in the feedlot. Furthermore, it will provide producers with a potential tool to more efficiently manage feedlot cattle.

CHAPTER TWO

LITERATURE REVIEW

Feedlot Systems in the United States

Use of Feedlots

There are roughly 30,000 feedlots across the United States with 14.4 million cattle (National Cattlemen's Beef Association, 2017). Cattle feeding at a large scale was first introduced in the early 60's, when the demand for higher quality beef in larger quantities emerged. Feedlots require governmental approval to be owned and operated. Feedlots are used to efficiently feed cattle to a targeted weight and degree of fatness. Furthermore, feedlot diets start with a high roughage diet and progressively move towards a high concentrate. With the high demand for gain most feedlots in the United States are in the Midwest, or in areas where corn production is high. Furthermore, with feedlots moving to areas where grain production is high transportation costs were reduced. The use of feedlots allowed a single concentrated area to finish a large quantity of animals that were marketable for slaughter faster and easier. Historical data indicates (Historical Grading Volumes BEEF, 2017), in the 1960 s 7,000 million pounds of beef was graded in the US. Ten years later that number almost doubled to 13,927 million pounds; in 2015 there were 23,720 million pounds graded. The increase in poundage was due to directed selection decisions and increased use of technologies including nutrition, and implants to increase pounds produced per animal.

Feedlot Facilities

Health and welfare of animals is important to the production of high-quality carcasses. Research has shown that shade, water, and environmental factors can impact animal health (Hulbert et al., 2011; Hammond et al., 1996; and Waynert et al., 1998). Development of housing and feedlot facilities requires integration of space, shelter, feed, water, waste management and handling facilities. Poor cattle performance can be attributed to poor conditions, such as muddy pens, harsh winds, and wet resting areas (Mader et al., 1999). Summertime temperatures, humidity, and solar radiation cause discomfort and/or death of cattle (Curtis, 1981; Morrison and Prokop, 1983; Lefcourt and Adams, 1996). Long periods of high heat reduce feed intake and increase expenditure of energy to maintain homeostasis (NRC, 1981). Shade and water can mitigate the effects of heat on animals in hot environments (Bond et al., 1997; Blackshaw and Blackshaw, 1994; Mader et al., 1997b). The primary purpose of shade is to protect the animal from intense, direct solar radiation. A simple shade structure can reduce radiant heat on an animal by 30% or more by intercepting direct solar radiation (Bond et al., 1967). Beede and Collier (1986), were able to find that the physical protection from solar radiation with artificial or natural shade offers one of the most immediate and cost-effective approaches of enhancing productivity of ruminants.

Feedlot Nutrition

Balanced diets and water are important to animal welfare but is also vital for optimizing cattle performance. Beef cattle are finished to a desirable weight and degree of fatness in feedlots. High energy diets allow these animals to produce more muscle and fat at a faster rate. Typical finishing periods are 100 days or more, where heifers and steers obtain weights of 1000

to 1400 pounds (Smith et al., 1997). Furthermore, studies have shown that type of diets or nutrition available determines how quickly they finish. Fernadez at el. (1999) showed that steers fed a conventional feeding program to carcass endpoint at 163 days, while organic fed steers took 225 days to reach the same carcass endpoints.

Increased average daily gain (ADG), dry matter intake (DMI), and decreased residual feed intake (RFI) are associated with animals that are more efficient (Nkrumah et al., 2006). increased animal efficiency results in less resources required to reach market weight and increases profitability.

Feedlot Performance

Feedlot performance measures include average daily gain (ADG) to Dry Matter Intake (DMI), Residual Feed Intake (RFI), Average Daily Gain (ADG), Days on Feed, Dry Matter Intake (DMI), Hot Carcass Weights (HCW), Yield Grade (YG), Quality Grade (QG), Marbling score (MARB), Rib Eye Area (REA), and carcass value.

Residual feed intake (RFI) is defined as the actual feed intake minus the expected feed intake of each animal, with expected intake determined by growth rate and metabolic body weight (Sanz et al., 2004). RFI is calculated utilizing individual intake data and is relative to a contemporary group. Research has shown that there is considerable individual animal variation in feed intake above and below that expected or predicated on the basis of size and growth.

Average daily gain (ADG) is a value that shows the weight gain of an animal per day. It is obtained by dividing how much an animal weighs by the period of time spent accomplishing the desired weight. Furthermore, ADG is an average meaning that the number you calculate will

be an average of the amount of weight that animal gains each day and is not an exact number for the number of pounds gained each day (Ohio State University Extension, 2011).

Hot carcass weight or sometimes called carcass weight is referring to the hot or un-chilled weight of the carcass. This is directly after harvest and the removal of the head, hide, blood and visera. On average the hot carcass weight will be approximately 60-64 percent of the live animal slaughter weight, but this can vary greatly from one animal to another. Hot carcass weight is used to determine yield grade. Yield grade is defined as the percentage of closely trimmed, boneless, retail cuts from the carcass. Yield grade is calculated utilizing HCW, REA, fat thickness between the 12th and 13th rib and kidney, pelvic and heart fat (KPH). Fat thickness is measured at the 12th rib at a distance ¾ of the way from the spinal column towards the ventral side of the carcass with a metal ruler divided into 0.1-inch increments. The rib eye area is the area of the longissimus muscle measured between the 12th and 13th rib using a standard grid that had 10 dots per square inch and dots completely surrounded by lean were the ones counted. The square inches measured with the grid. Internal fat or KPH is estimated as a percentage of the carcass weight (USDA 2017 United States Standards for Grades of Carcass Beef).

Quality grade is a composite evaluation of physiological maturity and marbling expected to influence meat palatability (USDA 2017 United States Standards for Grades of Carcass Beef). These factors include carcass maturity, firmness, texture, and color of lean, and the amount of distribution of marbling within the lean. Marbling score or marbling is a subjective measure of intramuscular fat in the longissimus dorsi. Graders evaluate the amount and distribution of marbling in the ribeye muscle at the cut surface after the carcass has been ribbed between the 12th and 13th ribs (Hale et al., 2013). Each degree of marbling is divided into 100 subunits. The

marbling categories are: Abundant (AB which correlates to quality grade prime+ in young animals), Moderately Abundant (MAB which correlates to quality grade prime in young animals), Slightly Abundant (SLAB which correlates to quality grade prime- in young animals), Moderate (MD which correlates to Choice+ in young animals), Modest (MT which correlates to choice in young animals), Small (SM which correlates to choice- in young animals), Slight (SL which correlates to both Select+ (50-100) and Select- (00-49)), and Traces (TR which correlates to Standard+ (34-100) and Standard (00-33). After a degree of maturity and marbling has been determined, these two factors are combined to arrive at the final quality grade.

Carcass Value is determined by the animal's weight, USDA quality grade, yield grade, and absence of defects such as dark-cutting and bruises (Tatum et al., 2015). Carcass Value will change depending on the grid the carcass is sold on.

Cattle Stress

Perception of Environment

Previous experience and perception of the environment influences animal behavior. The typical "Fight of Flight" response is triggered in an animal when the animal perceives something in the environment has changed or is dangerous. There are multiple ways an animal perceives the environment, mostly through sensory stimuli (Waynert et al., 1999; Lanier et al., 2000).

Cattle are particularly sensitive to auditory stimuli (Grandin, 1993; Grandin, 1998; Waynert et al., 1999; Lanier et al., 2000). Stimuli that were most effective for eliciting a startled response from cattle were intermittent, high-pitched sounds, and sudden movements. Previous work has shown that reducing any sort of loud stimuli; vocalization from humans or noise from machinery and equipment while handling cattle resulted in calmer, more easily handled animals

Grandin, (1998); Waynert et al., (1999); Lanier et al., (2000). Waynert et al. (1999) reported that sounds made by humans while handing cattle had a greater effect on heart rate and reactivity than equipment sounds such as gates banging. Pajor et al. (1999) also found that shouting at cows while moving them was associated with animal vocalization, an indication of animal stress. Both of these studies concluded that heart rate and vocalization in cattle increased with human-associated noise, concluding that an auditory stimulus in a cattle's environment has a large impact on the animal's overall stress response. Grandin, (1993; 1998) reported that cattle do get accustomed to sounds in their environment over time.

Cattle also respond to visual stimuli. Cattle possess 360-degree panoramic vision which gives them the ability to detect movement behind their bodies without moving their heads (Grandin, 1980). Furthermore, cattle have 25 to 50 degrees binocular vision but possess very poor depth perception (Grandin, 1980). Due to their lack of depth perception, slatted-type sunshades, casting a zebra striped shadow pattern, tended to cause balking or refusal to move forward in cattle. Grandin (1980) concluded that proper management of light and the minimization of changing light pattern, encouraged compliance in cattle during handling (Grandin 1980).

Cattle also respond to olfactory stimuli. Pfister et al. (1990) found that animals differed in their response to the presence of predator odors during feeding. The animals that were more sensitive to olfactory stimuli would take more time assessing the stimuli before consuming food. Grandin (1975) found that cattle would stop and sniff blood when present. This response occurred when animals were entering chutes where animals had been castrated or when enter the

stunning chutes at the harvest facility. Thus, the olfactory system influenced the animals' perception of their environment and how previous exposure can cause changes in behavior.

The perception of environment by the animal can lead to physiological and behavioral responses, which can be imprinted on the brain (Grandin 1997; Cooke et al., 2013). Memories, whether positive or negative determines how the animal will respond to subsequent stimuli. Stimuli identification encodes for a cascade of neuroendocrine signals allowing animals to respond to repeated stimuli and change or maintain homeostasis after response to stimuli (Aguilera, 1994).

Neuroendocrine Factors

In order to survive in any environment, animals must maintain homeostasis. Homeostasis is the ability to maintain a constant internal environment in response to changes. In order to maintain homeostasis in an ever-changing environment cattle require constant behavioral, endocrine, and autonomic adaptations in order to respond to external and internal stressors presented by the environment. There is a cascade of stress responses that are controlled by the hypothalamic-pituitary-adrenal axis (HPA). During the cascade by the hypothalamic-pituitary-adrenal axis neuroendocrine secretions come from the brain to the adrenal gland where synthesis and secretion of glucocorticoids and catecholamines takes place. A direct correlation to the cascade affect is the animal secreting the adrenal steroid hormone, cortisol in response to stress. Curley et al., (2008) reported that heifers with differing temperaments had different reactions to exogenous HPA (CRH, ACTH) hormones. Additionally, limbic system and amygdala involvement determine speed and severity of the stress response which is ultimately determined by prior experiences stored in the hippocampus (Medina et al., 2002; Cooke et al., 2013).

Hypothalamus

The Hypothalamus or "Master Gland" as it was referred to by early researcher because of its involvement in the maintenance of homeostasis (Senger, 2012). Two specific hypothalamic nuclei that play a large roll in stress response are the paraventricular nuclei and the supraoptic nuclei. These nuclei and associated axons are important in the secretions of CRH, ACTH and vasopressin during a stress response (Aguilera 1998, Hadley and Levine, 2006).

Corticotropin-Releasing Hormone

Corticotropin-releasing hormone (CRH) is the first neurohormone identified from the hypothalamus. CRH starts the neuroendocrine-endocrine cascade of the physiological response to stress which ultimately restores homeostatic conditions via down-stream secretion of glucocorticoids (Aguilera, 1998; Cook, 2004; Hadley and Levine, 2006). CRH neurons distributed in dorsal, lateral, and medial-ventral parvicellular systems and act on the sympathoadrenal system stimulating glucocorticoid secretion from the adrenal gland (Aguilera, 1998; Cook, 2004)

The use of electro-chemical probe technology has given researchers the ability to rapidly measure CRH response within the brain during a stress related event. Cook (2002) expressed that as stress increased in sheep CRH active neurosecretory cell activity in the amygdala also increased, foreshadowing the repetitive stress potentiated responsiveness of CRH from the amygdala. Furthermore, a feedback mechanism was elucidated involving circulating glucocorticoids reacting to the secretion of CRH in the PVN (Aguilera, 1998; Cook, 2002, 2004). Glucocorticoids react to the amygdala preemptive response to stressors, reflecting the role of the CRH on the pituitary gland and the release of ACTH (Cook, 2002).

Pituitary Gland

The pituitary gland is composed of two distinct lobes, the anterior and posterior. The hypothalamic-hypophyseal portal coming from the capillary beds in the median eminence provide a vascular link between the pituitary gland and the central nervous system. When CRH is secreted into the hypothalamic-hypophyseal portal, corticotropes within the anterior pituitary, synthesize and secrete ACTH (Page, 1982; Hadley and Levine, 2006). ACTH enters the adenohypophysis of the pituitary vein and is systematically released into the peripheral blood system. ACTH stimulates the adrenal glands to secrete glucocorticoids, mainly cortisol, in response to a stress stimulus.

Cortisol

Steroidogenic cells found in the adrenal cortex are important for both biological and metabolic processes. Lipid droplets supply cholesterol for the synthesis of cortisol (Hadley and Levine, 2006). In homeostatic conditions, cortisol follows a circadian rhythm. In cattle, the highest concentration of cortisol is released around 0800 and decreases throughout the day and evening until cortisol begins to rise again around midnight (Gatti et al., 2009). There is an increase in the concentration of cortisol when the animal perceives a stressor, and a stimulus is released to alter physiological and metabolic conditions. The rise in cortisol is in preparation for the “Fight or Flight” response (Cook, 2004; Gatti et al., 2009). Furthermore, cortisol is released in response to an elevation of epinephrine. An increased in epinephrine is involved in regulating visceral functions, which can lead to increased blood flow to muscles, increased heart rate, and pupil dilation.

Cortisol levels rise in response to increased epinephrine and is responsible for the animal's ability to respond to a stressful stimulus. When CRH and ACTH are secreted, the concentration of cortisol rises, which allows for the increased synthesis and activation of glycolytic and gluconeogenic enzymes in the liver to provide energy for brain and muscle function (Gatti et al., 2009; Cooke, 2014). In short-term the release of cortisol is beneficial to provide energy needed to respond to a stressful stimulus, but in long-term, elevated cortisol causes a catabolic effect on skeletal muscle and adipose tissue (Gatti et al., 2009; Fazio et al., 2012; Cooke, 2014). Following proteolysis and lipolysis, the subsequent amino acids and free fatty acids become gluconeogenic within the liver and produce glucose that is responsible for bodily functions (Gatti et al., 2009).

In other livestock species, very similar responses have been seen. Sheep exposed to predator stress, Corticotropin-releasing hormone and cortisol concentrations increased in the amygdala and blood (Cook, 2002). Cook (2002) also showed that with immunosensor-based micro-dialysis probes in the amygdala, detected two CRH peaks. The first peak of CRH in the amygdala is in response to predation, while the second peak of CRH follows the peak cortisol concentration in the amygdala. Cook (2002), recorded an inverse change in the peaks of CRH in the amygdala with increased exposure to predation. Furthermore Cook (2002), found that sheep under predation and exposure to additional stress (electrical shock) had exaggerated peaks of CRH and cortisol, expressing a fear conditioned response.

To conclude, high chronic cortisol concentration can impair growth and performance. This is apparent when the temperament of the animal becomes flightier, and/or with the presence of a conditioned fear response. Moreover, there is a costly metabolic expense to supply more

energy in response to elevated cortisol. Short-term secretion of cortisol is essential for homeostasis, but when excessive secretion occurs there is catabolism of muscle and adipose tissue. Identifying and sorting cattle based on degree of temperament could result in an opportunity to minimize the stress response during handling and enhance growth, efficiency, and carcass quality of cattle.

Whole Body Response

Research has indicated that temperament has an impact on carcass and meat quality. Constant exposure to stress leads to excessive catabolic effects due to high concentration of catecholamines. The high concentration of catecholamine results in negative effects on cattle in growth, and marbling (Jones and Marchello, 1983; Forkman et al., 2007). Catabolism is the breakdown of nutrients or body tissues, thus the reason for lower growth, marbling and carcass quality during a catabolic state. Researchers have shown that as temperament of an animal becomes more aggressive or excitable, there is a higher incidence of bruising and high ultimate pH resulting in dark cutters (Fordyce et al., 1988b; Voisinet er al., 1997a; Petherick et al., 2002; Vogel et al., 2011). Since the elevated catecholamines result in increased heart rate, measuring heart rate may provide a way to assess an animal's response to stress. Bulita et al. (2011) reported increased heart rate during transport, with a return to resting heart rate following unloading. Furthermore, Burdick et al. (2011b) reported that an increased exit velocity and pen score was related to higher concentrations of epinephrine and cortisol. Additionally, bulls with more flighty temperaments had higher rectal temperatures than calmer bulls. Gruber (2010) evaluated rectal temperature, and serum cortisol in Bos Taurus steers and heifers with physical

handling and chute restraint. Results demonstrated that steaks from animals with higher stress response had increased Warner-Bratzler shear force (WBS)

Temperament Measurements

Temperament is defined as a way in which an individual reacts to a novel or challenging situation (Grandin, 1998; Reale et al., 2000; Ferguson et al., 2006). Many studies have shown that calmer, more even-tempered animals perform better in a feedlot setting because there is less energy spent on fighting and restless behavior. Calmer animals perform better in the feedlot. Researchers have shown that as temperament of an animal becomes more aggressive or excitable, there is a higher incidence of bruising and dark cutting (Fordyce et al., 1988b; Voisinet et al., 1997a; Petherick et al., 2002; Vogel et al., 2011). Gardner1999) Since cattle temperament may influence growth, carcass grade, meat quality and the number of dark cutters, and feedlot performance quantifying temperament of cattle from weaning to slaughter for production and selection decisions are important (Gruber et al., 2010; Café et al., 2011; Turner et al., 2011; Boles et el., 2015).

Cattle handled quietly are less likely to respond negatively and are less likely to become ill or perform poorly (Smith et al., 1997).

Early attempts to classify temperament in cattle were subjective. The subjective measurements consisted of evaluating the animal's behavior in the chute or pen resulting in a chute score or pen score. Tulloh (1961) used a 1- 6 score with 1 described as "animal is quiet both in body language and vocalization" and the other extreme 6 described as "animal is aggressive with body language and vocalization". Grandin (2003) later refined the scale to be a 5-point scale to assess the animal in a chute. This scoring system has been adopted by the Beef

Improvement Federation; 1 – animal is calm vocally and stands still, 2 – slightly restless with little vocalization, 3 – restless and shakes the chute, 4 – vigorously shaking the chute continuously, and 5 – struggling violently, rearing, twisting of the body with lots of vocalization. Chute scoring is a common evaluation method for evaluating temperament, however, as it is a subjective scoring system there are concerns about the repeatability between observers (Grandin 2003). Another concern in cattle is misscoring animals that freeze when restrained instead of showing more volatile behavior in evaluations that include restraints (Burrow and Corbet, 2000).

Other, less subjective measurements of cattle temperament have been developed. Exit velocity was originally referred to as flight distance, but as equipment advanced exit velocity came to be defined as the rate at which an animal traveled a standard distance (Fordyce et al., 1982; Ferguson et al., 2006). Furthermore, Curley et al. (2006) reported that blood cortisol levels were related to how fast an animal left the handling chute. Thus, suggesting exit velocity was a viable tool to evaluate stress during livestock handling (Boles et al., 2015). Burdick et al., (2011) also reported an increase in exit velocity as days of age increased in Brahman calves from 21 days of age through 56 days postweaning and that the more temperamental calves had persistent increases over time.

Fordyce et al. (1988a) reported that horned cattle temperament was lower than polled cattle. This finding lead to suggesting that Bos Taurus shorthorn cattle tended to be more docile than Bos indicus Brahman cattle. The conclusion that Bos Taurus cattle had lower temperament measures than Bos Indicus cattle has been documented by many researchers (Fordyce et al., 1988a; Voisinet et al., 1997b; Curley et al., 2006; Ferguson et al., 2006; Burdick et al., 2009;

Burdick et al., 2011a; Café et al., 2011). Furthermore, exit velocity is persistent (Boles et al., 2015)

Blood lactate concentration has been suggested as a method of temperament classification (Boles et al., 2015; Williams et al., 2019). Boles and co-workers reported a significant correlation between chute score and blood lactate as well as a significant correlation between blood lactate and exit velocity. Additionally, Williams et al. (2016) reported that exit velocity could be predicted 7 out of 10 times with a combination of blood lactate concentration and rectal temperature.

Feedlot Health

Feedlot Disease/Bovine Respiratory Disease (BRD)

Bovine respiratory disease (BRD) is the most common and costly disease of feedlot cattle in the United States, and the consequences of BRD result in losses of up to US$291/animal with individual net returns decreased based on the number of treatments (Smith, 1998; NAHMS, 2000a, Smith, 2009; USDA, 2013; Fulton et al., 2005, Brooks et al., 2011; Cernicchiaro et al., 2012). Bovine respiratory disease is a multifactorial disease involving several pathogens and stress interactions and costs the beef industry in excess of $4 Billion annually (Grandin, 1997). Schneider et al., 2009, reported a decrease in carcass value of $23.23, $30.15, and $54.01 for cattle treated once, twice, or ≥3 times, respectively, compared with the carcass value for cattle that were never treated. Additionally, Brooks et al. (2011) reported that net returns decreased for cattle in the backgrounding and finishing phases as number of BRD treatments increased. Net returns for untreated cattle compared to cattle treated once, twice, or three times typically yielded greater net returns of $111.12, $92.51, $59.98, and $20.62, respectively per hundred weight

(Brooks2007). Identifying effective methods to accurately diagnose and control BRD is very important to the industry.

Transportation Sickness

Transportation sickness is a problem with many animals that have flighty temperaments entering the feedlot. Hulbert et al., (2010) reported that flightier bulls had greater glucose and cortisol levels than calmer bulls. Transportation stress is involved in the complex etiology of bovine respiratory disease complex (BRDC) or "shipping fever" in cattle (Galyan et al., 1999; Loerch and Fluharty. 1999). Cattle are often transported long distances. Transportation causes stress, which results from many factors, including pre-transport management, noise, vibration, novelty, social regrouping, crowding, climatic factors, restraint, loading and unloading, transit time, and feed and water deprivation (Swanson and Morrow-Tesch, 2001; Hulbert et al., 2010). Tarrant, (1990), reported that the most stressful part of transportation is the confinement of animals within a moving vehicle, which greatly increased the risk of injury and damage to the carcass and meat quality. Furthermore, cattle are social creatures, and transportation can mix animals resulting in social stress. Proudfoot et al., (2015) reported cattle that are calmer in a group of around 20 animals. Shipping of animals, regardless of distance and limiting other possible stressors, is a very stressful experience for cattle. Temperamental cattle tend to have higher cortisol levels which can impact their immune response. Cattle temperament is known to result in lower immune function and more frequent incidence of infection (Salak Johnson et al., 2007). The risk of cattle developing BRD is greatest in the days immediately following their arrival into the feedlot, and innate immune responses plays a role in resistance to BRD (Salak Johnson et al., 2007; Theurer et al., 2013).

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) was discovered in 1947 by researchers at Stanford and Harvard universities. NMR initially was used in identifying structures of small molecules. The process measures alterations in spin of electrons to help identify these small molecules. More recently, it has been crucial in the study of metabolomics, which is looking at specific metabolites in cells, biofluids, tissue and/or organisms. NMR has many benefits over traditional metabolite assays:

1) The number of metabolites identified per sample. Traditional metabolite assays could only identify one metabolite per assay, NMR metabolic profiling has the potential to identify hundreds of metabolites. Increasing metabolites identified allows researchers a better understanding of the diversity of metabolism in an individual.
2) The cost per sample. Given the amount of data generated by one sample, NMR is much more cost effective than traditional metabolite assays.
3) The time commitment. NMR metabolic profiling needs expertise to analyze samples. The time commitment to learn how to analyze the spectra produced by NMR metabolic profiling may be high, taking about 30 minutes to analyze one spectrum. After gaining experience analyzing samples, one spectra can be analyzed in 10-15 minutes. Whereas traditional metabolite assays can take anywhere from 1-6 hours for 32 samples.

Theory of Nuclear Magnetic Resonance

Protons in the nucleus are constantly spinning, similarly to electrons spinning in the outer orbit of an atom. The spin associated with the proton can be thought of as a rotation, however, this is just metaphorical term as protons are not round but more "phantom particles" (Moskowitz, 2014).

NMR Metabolic Profiling and Bovine Respiratory Disease

The complex nature of BRD makes establishing a universal 'gold standard' for BRD identification difficult. Current diagnosis methods rely on subjective visual signs of illness, often combined with rectal temperature or lung auscultation to determine if antibiotic treatment is warranted (Wohr et al., 2015). These diagnostic protocols have varying accuracy in diagnosing BRD and the search for alternative diagnosis methods are important (Farrow et al., 2009, Kilmczak et al., 2014, Hou et al., 2015). Metabolomics monitors alterations in the concentration of small metabolites in tissues and biofluids that include lipids, amino acids, vitamins and sugars (Goldansaz et al., 2017, Fiehn, 2002, Nicholson et al., 1999). Metabolite biomarkers have the potential to aid in the diagnosis of BRD. Metabolomics have been used to diagnose pneumonia and respiratory conditions in humans. L-histidine, glutamic acid and allantoin have been identified as biomarkers related to the host response to infection (Lucas et al., 2011, Laiakis et al., 2019, Gascoyne et al., 2013). As documented to date, there has been minimal research on metabolomics as a diagnostic tool for infectious diseases in cattle such as BRD. Researchers have reported between five and twelve metabolites that have differentiated healthy and BRD infected calves (Marrone, et al., 2016, Maurer et al., 2018). The use of NMR metabolic profiling could give researchers the opportunity to study the global metabolism of cattle and create a

potential panel of metabolites that may assess herd health in relation to BRD. The use of this metabolic profile of cattle would allow feedlots, ranchers, and farmers to more efficiently manage and treat potential illness. We hypothesis that the metabolite concentrations would be different in animals that entered the feedlot and underwent treatment than those that did not. The objectives were to determine if we could find metabolites in a single blood sample collected upon entry to the feedlot that would indicate whether animals would reach clinical illness or would have impaired performance in the feedlot.

CHAPTER THREE

MATERIALS AND METHODS

Animals and Housing

One-hundred and thirty-one (n = 131) feedlot cattle housed at the Chappell Feedlot (Chappell, NE) were utilized for the study. The animals were sampled at entry to the feedlot. All the animals in this study originated from the BAIR Ranch in Martindale, MT prior to being transported to the feedlot. All research procedures were conducted under a protocol approved by the Montana State University Agricultural Care and Use Committee, 2014-AA09. Following a thirty-day step-up period in the feedlot, the steers were fed a 94% concentrate ration consisting of rolled corn, beet pulp, dried distillers' grains, protein supplement, corn silage, and ground hay (dry matter basis: 14.58% CP and 0.61 Mcal/lb net energy for gain). During this stage, the steers were implanted with Component TE-S (120 mgof trenbolone acetate, 24 mg of estradiol ESP and 29 mg of tylosin tartrate). Pen average daily gains (ADG) were calculated from average entry and exit weights over time on feed, DMI came from individual pen data at closeout, as well as days on feed. Feed intake and behavior was monitored for 42 days and average daily gain, dry matter intake and residual feed intake calculated.

Daily DMI was measured using a Growsafe system (Airdrie AB). GrowSafe is a program that monitors feed intake and frequency of feeding of individual animals. GrowSafe uses a RFID which is an electronic Radio Frequency Identification (RFID) ear tag. The RFID allows the system to detect which animal is in the GrowSafe system at any given moment, and also tracks the weight of feed that disappears from the feedbunk while that animal is at the bunk.

A Polaris timer system (Farmtek Inc., Wylie, TX) was used to measure exit velocity with photo-transmitters placed 1.83 and 3.65 meters in front of chute (Williams et al. 2019). The time was converted to meters per second for statistical analysis.

The same observer assigned chute scores for all animals (BIF, 2018). Chute scores were assigned according to behavior throughout handling process, i.e., entering hydraulic squeeze chute, while measures were being collected, and exit from the squeeze chute.

Blood Sampling Procedures

While the animals were restrained in a hydraulic squeeze chute, blood was drawn for blood lactate concentration and metabolite measurements. Blood was collected via jugular venipuncture, and then a drop of blood (< 2 μL) was placed on the lactate test strip, and lactate was measured with a Statstrip Xpress® Lactate meter (Nova Biomedical, Waltham, MA). Blood samples were obtained by venipuncture of the jugular vein from each steer. Blood samples were cooled, allowed to clot and centrifuged at 3000 XG for 30 minutes and then serum was separated and stored. Plasma was centrifuged within 6 hours of collection and then the plasma was separated off and stored. Serum samples were stored in two aliquots, one at -20°C and one at -80°C until assayed for metabolites.

Residual Feed Intake Calculations

RFI was calculated as the difference between expected feed intake and actual feed intake. Expected feed intake was calculated based on rate of gain and the body weight of the animal. The statistical model is $Y = B0 + B1X1 + B2X2 + E$ where y is expected dry matter intake, B0 is the

equation intercept, B1 and B2 are the coefficients of the equation of the equation, X1 is the mid-test metabolic body weight, X2 is the average daily gain, and E is the residual. RFI is used by the feedlot industry as an estimate of efficiency. An animal with a larger negative value is considered to be more efficient while an animal with a positive value is considered to be less efficient.

The General Linear Model and Least Square Means procedure of SAS (SAS 9.4, 2014) were used to evaluate differences between temperament classifications. Pearson correlations were calculated between temperament and performance variables. Steers were divided into three exit velocity classifications with fast animals having exit velocity greater than one standard deviation from the mean and slow animals having exit velocities lower than one standard deviation from the mean.

NMR Sample Preparation

Serum samples were prepared for nuclear magnetic resonance spectroscopy as follows: Samples were thawed at room temperature after being stored at -80°C and using reagents that were kept at -20°C until used. A 1:4, 500µl Serum:1500µl Acetone solution was added to 2mL plastic, flat-cap conical vials. The resulting mixture was inverted 10 time and incubated at -20°C for 60 minutes to allow for protein precipitation, followed by sample centrifugation at 13000 xg for 30 min. Following this step, clarified supernatants were transferred to new 2.0 mL tubes and dried overnight using a Speedvac vacuum centrifuge with no heat, and stored at -80°C until further use. For NMR, dried metabolite extracts were resuspended in 600µl of NMR buffer consisting of: 25 mM of NaH_2PO_4/Na_2HPO_4, 0.4 mM of imidazole, 0.25 mM of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in 90% H_2O/10% D_2O, pH 7.0.

^{1}H NMR Spectra Acquisition and Preprocessing

Samples were spun at 13,000 rpm for 2 minutes to remove remaining debris and 500 µL transferred to a 5 mm Bruker NMR tube. One dimensional (1D) ^{1}H NMR spectra were recorded at the Montana State University NMR Core Facility at 298 K (25°C) on a Bruker 600-MHz AVANCE III solution NMR spectrometer equipped with a SampleJet™ automatic sample loading system, a 5 mm triple resonance liquid-helium-cooled TCI probe, and Topspin™ software (Bruker version 3.6). One-dimensional ^{1}H NMR experiments were performed using the Bruker '*zgesgp'* pulse sequence with 256 scans, a ^{1}H spectral window of 9600 Hz, 64K data points, and a dwell time interval of 52 µsec amounting to an acquisition time of 3.3 sec, and a 2 sec relaxation recovery delay between acquisitions (Cederholm et al. 2019).

1H NMR Data Analysis

Additional processing of 1D ^{1}H NMR spectra and metabolic profiling were performed using Chenomx NMR Suite software (version 8.1; Chenomx Inc., Edmonton, Alberta, Canada). Preprocessed '1H' NMR spectra files were imported into Chenomx and baseline correction was conducted using the automatic cubic spline function and manual breakpoint adjustment to procure a flat, well-defined baseline. All ^{1}H chemical shifts were referenced to the DSS signal (0.0 ppm), and imidazole ^{1}H NMR signals were used to make adjustments arising from slight variations in sample pH. Identification and quantitation of metabolites was performed by manually peak-based fitting 1D ^{1}H spectral patterns, intensities, and chemical shifts with reference to small molecule spectral patterns present in the Chenomx database for 600 MHz (^{1}H Larmor frequency) magnetic field strength NMR; manual adjustments were performed to attain optimal spectral pattern fits based upon metabolite intensity and peak cluster location. In addition

to serving as a chemical shift reference, the internal DSS (0.25 mM) standard was also used for metabolite quantitation. Furthermore, acetone was removed from the analysis as it was used in the sample preparation.

Data and Statistical Methods

Quantitative metabolic profiles were exported from Chenomx software as concentrations in mM. Metabolite concentrations were normalized to protein content and NMR buffer volume before multivariate statistical analysis by MetaboAnalyst 3.0 (Nicholson et al., 1999, Xia and Wishart, 2016; Blakebrough-Hall et al., 2020). After normalization, metabolite concentrations were log-transformed and auto-scaled, mean-centered and divided by standard deviation (SD), to yield a Gaussian data distribution prior to multivariate statistical analysis, which included 2D principal component analysis (PCA) and hierarchical clustering analysis (HCA). A Euclidean distance measure and Ward clustering algorithm were used when performing HCA in MetaboAnalyst.

Chemometrics

Concentrations of compounds were statistically analyzed using MetaboAnalyst 3.0 (Xia and Wishart, 2016). For multigroup analysis a one-way Analysis of Variance (ANOVA) was utilized. ANOVA determines whether the concentrations are statistically different, it is usually followed by post-hoc analyses in order to identify which of the two levels are different. Fisher's least significant difference method (Fisher's LSD) provides a preliminary overview about features that are potentially significant in discriminating the conditions under study. The analyses conducted included a Principle Component Analysis (PCA) and a partial least squares

discriminant analysis (PLS-DA). The PLS-DA model was used to visualize the data set and to accurately measure the covariance among the response. The R2 and Q2 variables were used to ensure the PLS-DA model was accurate and appropriate. The R2 variable is equal to the sum of squares captured by the model and the Q2 variable is the cross-validated vale of the R2. The PLS-DA model was used to identify any significantly different metabolites between the feedlot cattle used in this study. Variable Importance in Projection (VIP) is a weighted sum of squares of the PLS loadings taking into account the amount of explained Y-variation in each dimension. VIP scores are calculated for each component. PCA was used to distinguish the differences associated with different members of cattle in the group. A permutation test statistic was used to cross validate the effectiveness of the model. A permutation test statistic is the ability of the model to distinguish between the groups of cattle. A permutation test with a $p > 0.05$ cannot distinguish the difference between the groups of cattle examined. The last step in determining model quality was to look at the leverage plots. The leverage points identify outliers in the model and any metabolites that can be used for predictor capabilities for the model.

Data Management

No standard formulas exist for categorizing the data for Metaboanalyst 3.0 we categorized the data as follows. With RFI ranging from negative numbers to positive numbers it is important to recognize that negative numbers for residual feed intake means there is less feed needed to maintain the animal and supply energy for growth. RFI was classified as three groups. High -1.91 to -0.61, Medium -0.60 to 0.60, and Low RFI was 0.61 to 1.91 and these determinations were based on the group as a whole. The division of thirds was based on having equal replicates in each high, medium, and low category.

Dry Matter Intake was also divided up into three groups based on the group as a whole. Dry matter intake was based on total feed given to a pen of animals during the total finishing period. This data was obtained from close out data supplied by Chappell Feedlot. Animals that consumed approximately 1,880 kg of food throughout their feeding duration were considered low, 4,759 kg consumed approximately were medium and high consumed 2,293 kg based on pen intake, feed provided, and days on feed. The division of these groups were from the group as a whole and is the top, middle, and bottle third from the closeout data received from Chappell feedlot.

Average Daily Gain was broken up into categories in order to analyze the data. For average daily gain animals were divided into three groups low, medium, and high. The low category was 0.64-1.11 kg, medium was 1.12-1.57 kg, and high was 1.58-2.05 kg.

Days on feed in this study was broken into three categories: 196 days 225 and 239 days. The days on feed came from the closeout data from Chappell feedlot. The reason for the difference in the number of days of feed for some animals is due to how Chappell selects to send animals to harvest. Chappell sends animals to harvest based on ultrasound data, thus the reason there are more choice than anything else seen in this group of animals, because Chappell sends their animals to harvest at choice.

Docility scores were categorized into groups as well within the 5-point grading scale: chute score of 1-2 were docile, 3 was medium, and high temperament was 4 and above. The reason for breaking the groups was to identify variation in temperament.

Exit velocity was categorized into three groups of fast, medium and slow. This was measured in meters per second. The fast group was one standard deviation above the mean (0.4

seconds to 1.16 seconds) medium was within one standard deviation of the mean (1.3 seconds to 3.05 seconds) and then slow was one standard deviation below the mean (3.07 seconds to 4.81 seconds).

Rib eye area was measured as square inches and then converted into cm^2, and the formula for that was $cm^2 = in^2$ X 6.4516. For rib eye area there were 5 categories and there were broken up by 10 cm increments, with the first group being 70 followed by 80, 90, 100, 110 in cm^2. These measurements were divided into 10 cm increments to allow for Metaboanalyst to analyze the data with ample number of replicates.

Marbling scores were converted to numbers; 300 = slight, 400 = small, 500 = modest, 600= moderate, 700 was slightly abundant. The data was then categorized into groups based on these numbers. This was to ensure that there were enough animals in each group for analysis.

Quality Grade assigned based on the marbling scores and then divided into the categories of low Choice (Ch-), average Choice (Ch), high Choice (Ch+) and low prime (P-)

Yield Grade was reported in the closeout data. The categories of yield grade seen in this study were 1, 2, 3, and 4. There were no yield grade 5 carcasses in this study.

Hot carcass weights were also assigned to three groups light, medium, and heavy. The groups were light from 278 kg to 340 kg, medium 341 kg to 401 kg and heavy carcasses were 402 kg to 464 kg.

Value per hundred weight of the carcasses was broken up into 5 categories. These 5 categories were separated into ten dollar per hundred weight increments, $170, $180, $190, $200, and $210. This was done by taking the value of the carcass and dividing it by the number of hundred weight, which came from the close out data.

Back fat was converted from inches into centimeters by multiplying the measurement in inches by 2.54, which came from the closeout data from Chappell feedlot which were already categorized for back fat in inches. For back fat there were 11 categories that were identified, they are as follows from least to thickest back fat: 0.711, 0.813, 0.914, 1.02, 1.12, 1.22, 1.32, 1.42, 1.52, 1.63, and 1. 73cm.

Metaboanalyst 3.0 Data Analysis

Data Normalization

The normalization procedures implemented below are grouped into four categories. Sample specific normalization allows users to adjust concentrations manually based on biological inputs; row-wise normalization allows general-purpose adjustment for differences among samples; data transformation and scaling are two different approaches to make features more comparable, if these normalizations were not made then comparing the data would have been impossible. In this study we use both manual and automated corrections to achieve better results.

The normalization of the data seen here consists of a row-wise procedure, normalization by the sum to make sure best results were achieved and that the data could be compared. A data transformation of generalized log transformation was performed. Finally, data scaling using auto scaling.

One-Way ANOVA

Univariate analysis is the most common method used for exploratory data analysis. For multi-group analysis, MetaboAnalyst provides one-way Analysis of Variance (ANOVA) of data. As ANOVA only tells whether the overall comparison is significant or not, it is usually followed by the post-hoc analyses in order to identify which two levels are different. MetaboAnalyst uses two of the most commonly used methods for this purpose- Fisher's least significant difference method (Fisher's LSD) and Tukey's Honestly Significant Difference (Tukey's HSD). For this study in particular we used Fisher's LSD.

Principal Component Analysis (PCA)

PCA is an unsupervised method aiming to find the directions that best explain the variance in a data set (X) without referring to class labels (Y). A PCA is trying to locate a trend or significance in the data, without referencing the labels that we gave to the data. The data are summarized into much fewer variables called scores which are weighted average of the original variables. The weighting profiles are called loadings. The PCA analysis is performed using the PRCOMP package. The calculation is based on singular value decomposition, which allows us to possibly draw conclusions to why separation of groups is happening.

Partial Least Squares – Discriminant Analysis (PLS-DA)

PLS is a supervised method that uses multivariate regression techniques to extract metabolite information that can predict the call membership (Y) via linear combination of original variables (X). The PLS regression is performed using the PLSR function in the R pls package. The classification and cross-validation are performed using the corresponding wrapper function offered by the caret package.

To assess the significance of the class discrimination, a permutation test was performed. In each permutation of PLS-DA a model was built between the data (X) and the permuted class labels (Y) using the optimal number of components determined by cross validation for the model based on the original class assignment. MetaboAnalyst supports two types of test statistics for measuring the class discrimination. The first one is based on prediction accuracy during training. The second one is separation distance based on the ratio of the between group of sums of the squares and the within group sum of squares (B/W-ratio). If the observed test statistic is part of the distribution based on the permuted class assignments, the class discrimination cannot be considered significant.

There are two variable importance measures in PLS-DA. The first, Variable Importance in Projection (VIP) is a weighted sum of squares of the PLS loadings taking into account the amount of explained Y-Variation in each dimension. Please note, VIP scores are calculated for each metabolite. When more metabolites are used to calculate the feature of importance, the average of the VIP scores are used. The other important measure is based on the weighted sum of PLS-regression. The weights are a function of the reduction of the sums of squares across the number of PLS components. Please note, for multiple-group (more than two) analysis, the same number of predictors will be built for each group. Therefore, the coefficient of each feature will be different depending on which group you want to predict. The average of the feature coefficients is used to indicate the overall coefficient-based importance.

CHAPTER FOUR

RESULTS AND DISCUSSION

Results

The data for this study was collected on entry into Chappell feedlot, in Chappell Nebraska. The data collected was chute score, blood lactate, exit velocity, and blood collections via jugular venipuncture for NMR.

Treated Vs. Non-Treated for BRD

The first trait analyzed was treatments for BRD of treated vs non-treated. Data used was treatment cost while being at Chappell feedlot. The data normalization for treatment scores of treated vs non-treated is shown in Figure 1. Data was normalized by sum, using a row-wise procedure and then log transformed. PCA and PLSDA shown in Figure 2 and Figure 3 respectively showed little to no separation between treated vs. non-treated classifications. The PLSDA validation with permutation shown in Figure 5 generated a p-value based on permutation of $P = 0.764$ (764/1000). This shows that the model cannot predict any better than random. Figure 4 shows the number of components contributing to the difference between classifications. It indicates that there was not a viable classification based on metabolite features possible for treated vs non-treated. Figure 6 shows an ordered list of the most important features in discriminating between treated vs non-treated classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are citrate, choline, alanine, phenylalanine, and tyrosine. No patterns were evident in which features were highest or lowest concentrations by treated vs non-treated classification.

Figure 7 shows a similar analysis using a random forest approach. The four most important features identified using this method were lactate, creatinine, pyruvate, and glutamate.

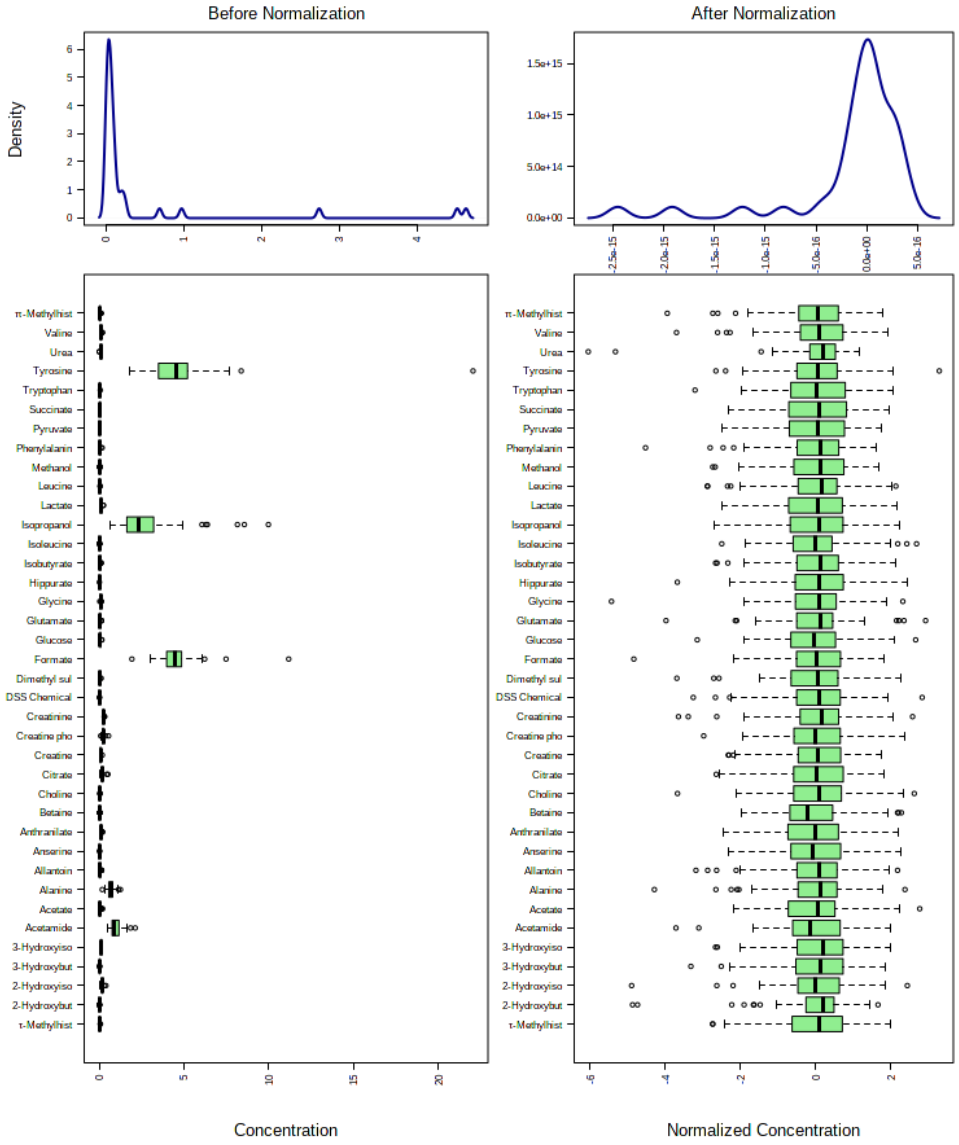

Figure 1. Treated vs Nontreated for Bovine Respiratory Disease (BRD) Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limitations. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

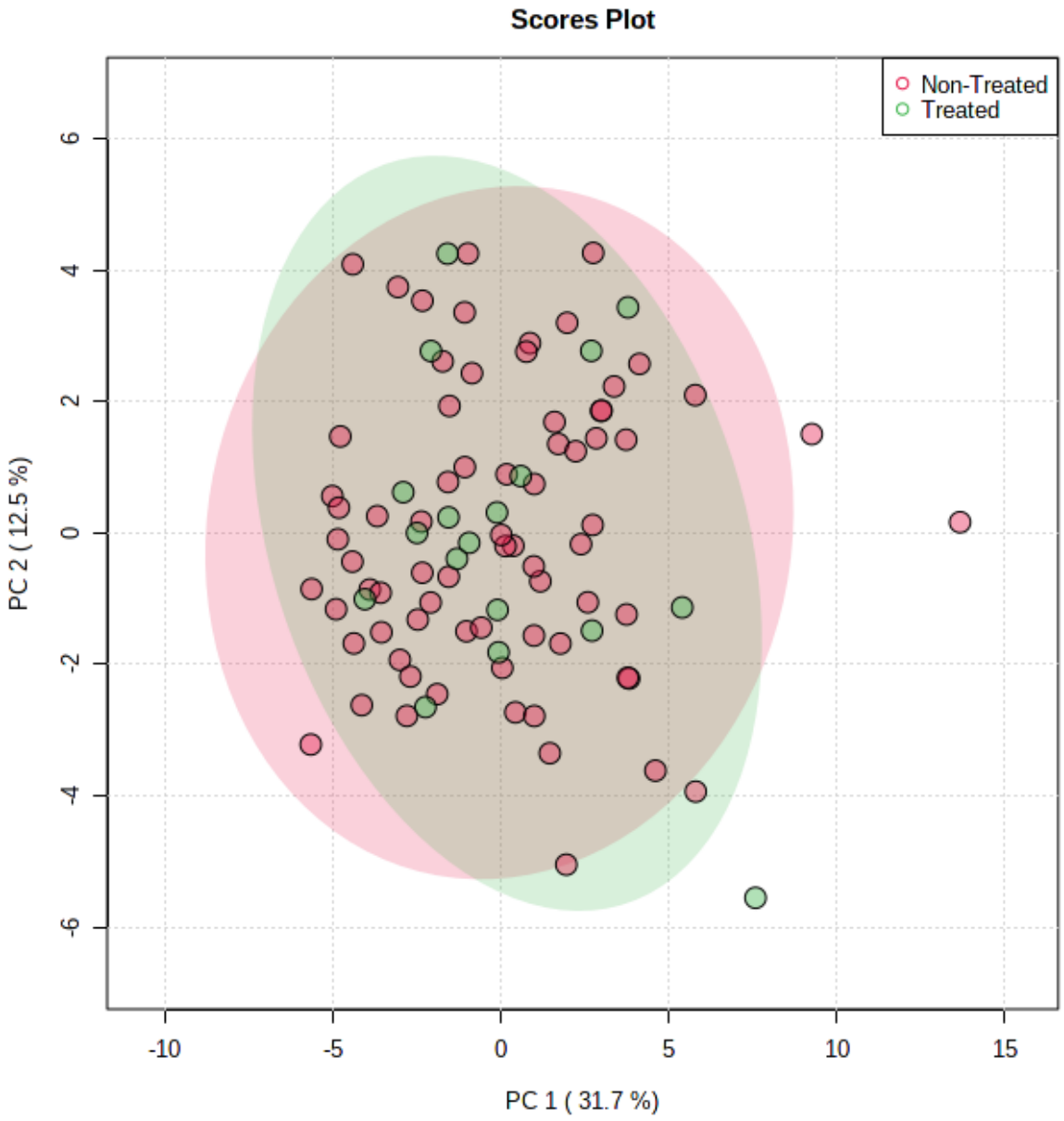

Figure 2. Significant features related to Treated vs Nontreated for BRD identified by PCA: Scores plot between the selected principal components or group classifications for treated vs nontreated. The variation explained by the first and second principal component is in parentheses. In this image there is no significant separation between the groups.

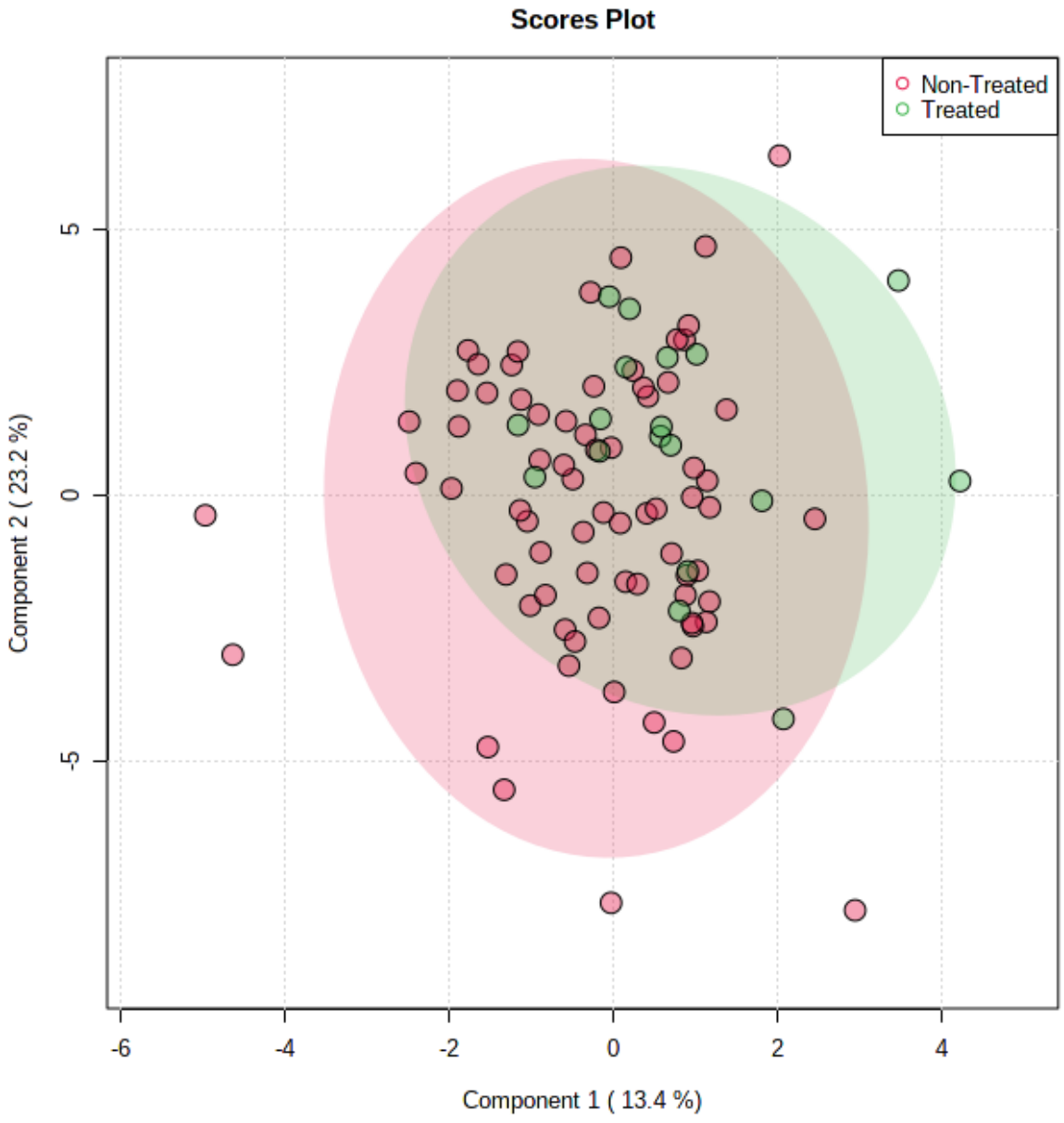

Figure 3. Significant features related to Treated vs Nontreated BRD identified by PLS-DA: Scores plot for treated vs. nontreated for BRD between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is no significant separation between the groups.

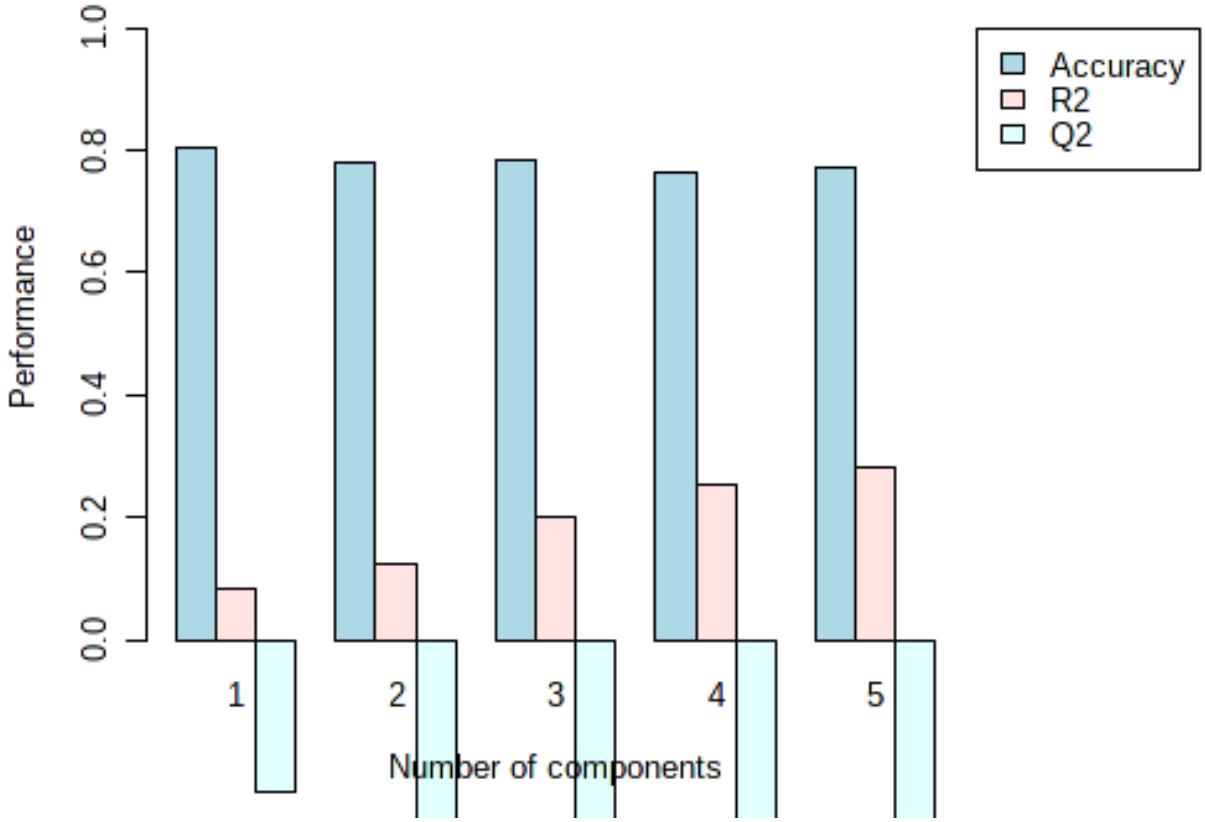

Figure 4. Significance related to Treated vs Nontreated BRD identified by Components Test: PLS-DA classification using different number of components. There is not significance.

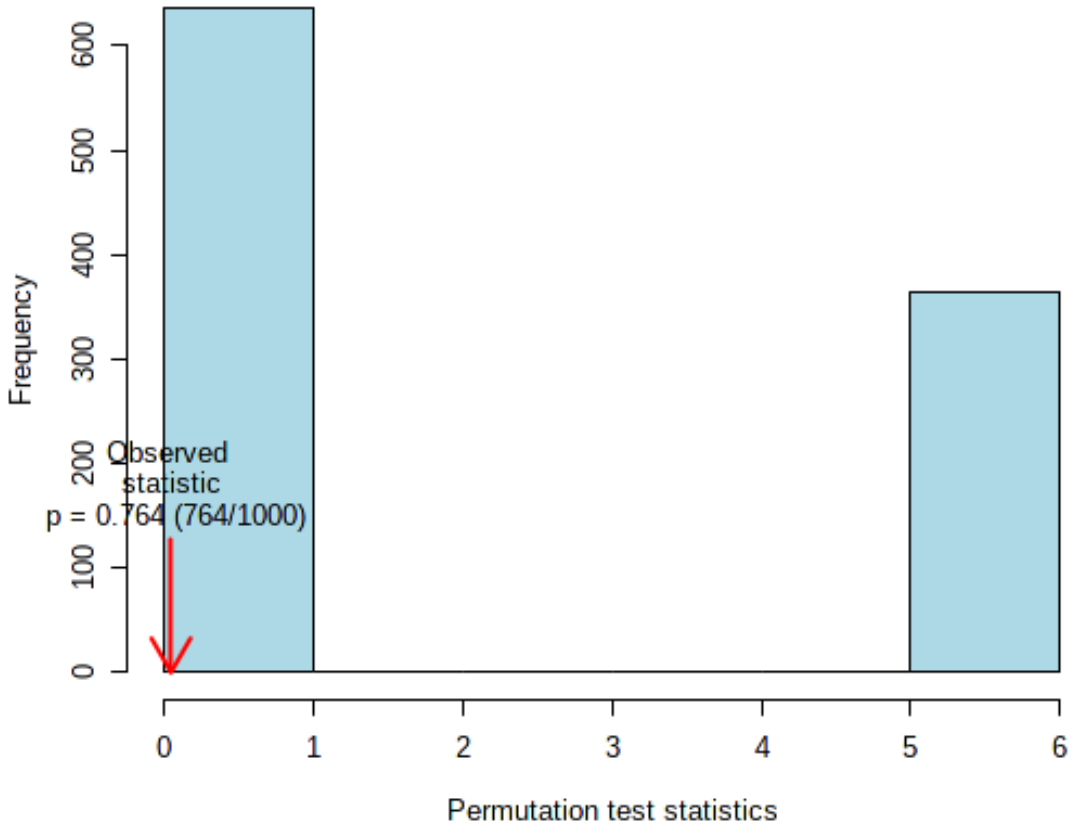

Figure 5. Significance related to Treated vs Nontreated BRD identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The p value based on permutation is p = 0.764 (764/1000). The p value shows that there is no significant evidence that the model can differentiate between the classes.

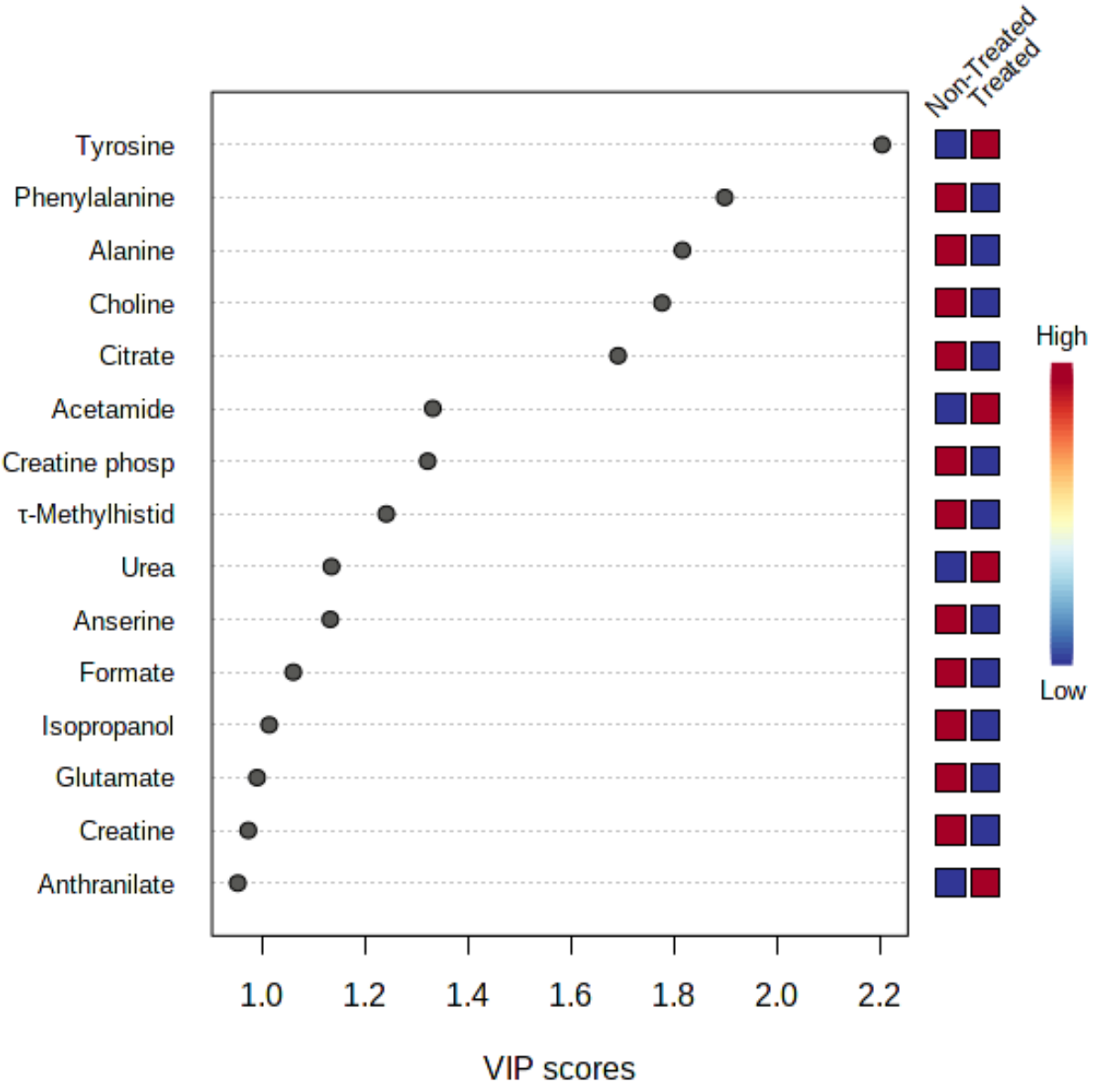

Figure 6. Significant Metabolites related to Treated vs Nontreated BRD identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

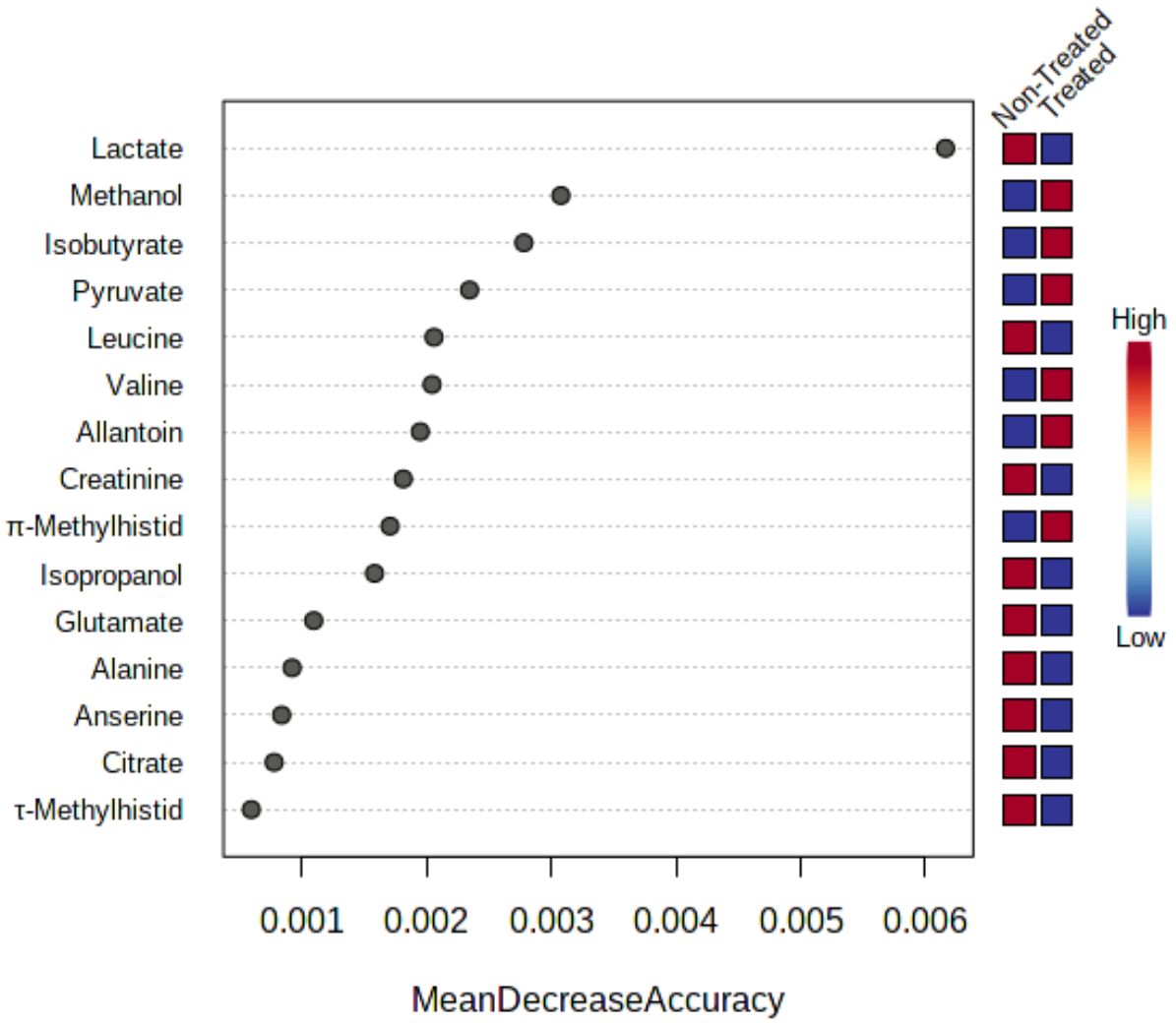

Figure 7. Significant Metabolites related to Treated vs Nontreated BRD identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Back Fat (cm)

The second trait analyzed was back fat (cm). The subcutaneous back fat was measured between the 12th and 13th rib after harvest. The data normalization for back fat is shown in Figure 8. Data was normalized by sum using a row-wise procedure and then $\log_{10}$ transformed. A one-way Analysis of Variance (ANOVA) was used to identify metabolites that could discriminate between back fat categories. Figure 9 shows the important metabolites identified by ANOVA. Table 1 shows the significant metabolites associated with back fat thickness at harvest the two metabolites that were significantly different based on the back fat categories were urea and 2-hydroxyisobutyrate. Urea is an important metabolite that is used in protein deposition and utilization of energy. PCA and PLSDA (Figure 10 and 11, respectively) did not suggest that these two metabolites could predict what back fat would be or the model could not explain the variation. The PLSDA validation (Figure 12) generated a P-value based on permutation of P = 0.541 (541/1000). This shows that the model using the metabolites could not predict back fat any better than random. Figure 13 shows the number of components that would allow the best classifications. It indicates that there is not viable classification based on metabolite features possible for back fat. Figure 14 shows an ordered list of the most important metabolites in discriminating between back fat classifications from the PLSDA analysis. Metabolites with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are hippurate, creatinine, tyrosine, and 2-hydroxyisobutrate. Hippurate is an important metabolite which is involved in catabolic processes and the differences in hippurate concentrations could lead to the differences in back fat thickness and help us understand the growth curve of these animals. Creatinine is a by-product of protein breakdown. No patterns were evident in which metabolites were highest or lowest concentrations by backfat

classification. Figure 15 shows a similar analysis using a Random forest approach. The two most important features identified using this method were leucine and 2-hydroxyisobutyrate. Leucine in utilized in protein synthesis.

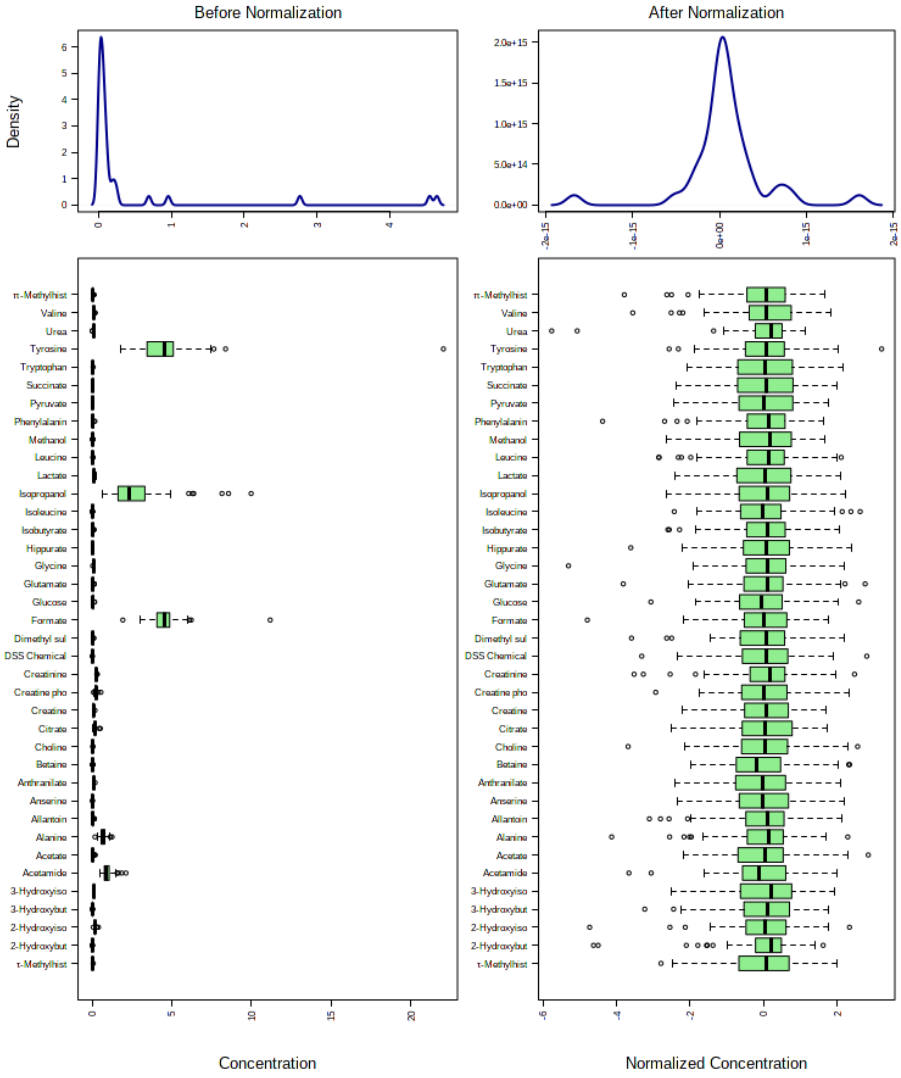

Figure 8. Back Fat Data before and After Normalization Procedures Box plots and kernel density plots show the difference in data distribution before and after normalization. The boxplots show at most 50 metabolites due to space limitations. The density plots at the top are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: autoscaling.

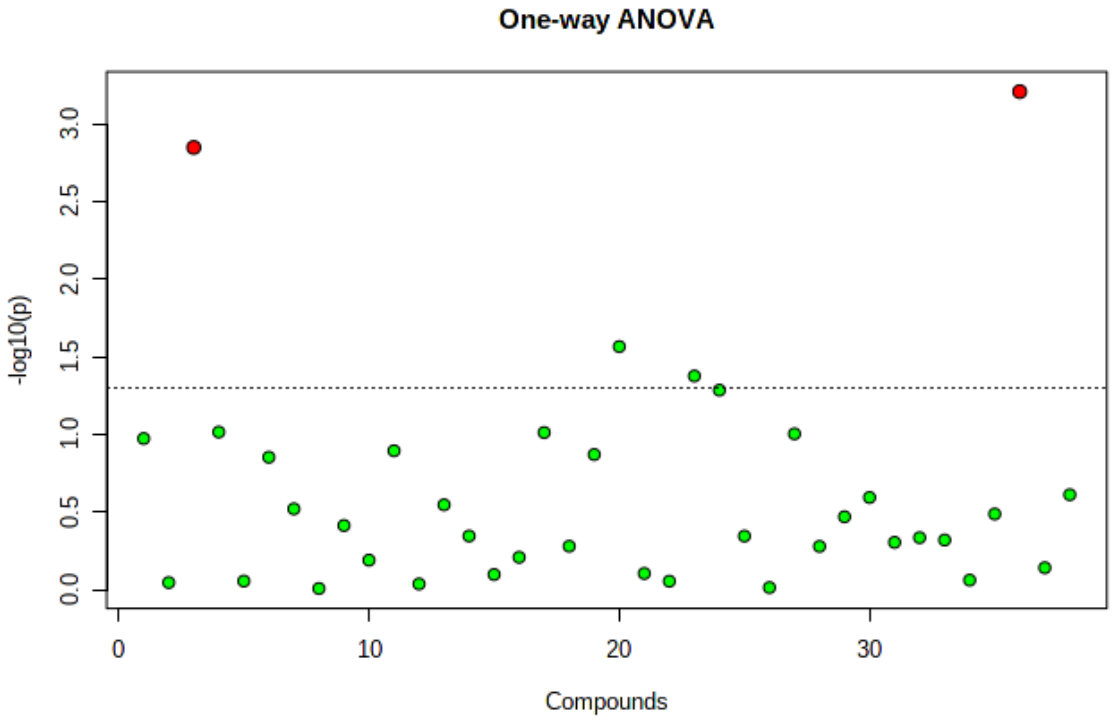

Figure 9. Significant features related to Back Fat (cm) identified by Analysis of Variance: Important metabolites identified by ANOVA for back fat categories, p-value threshold 0.05. On the x-axis are the metabolites, the y-axis is the –log10 adjustment of the P-value. The reason for some metabolites shown as green even though they are at or above the level of significance is because they were falling within the FDRs, which make them non-significant.

Compound	F.value	P-value	$Log^{10}(p)$	FDR	Fisher's LSD
Urea	3.63	0.00	3.21	0.02	0.711 – 1.63
2 – Hydroxyisobutyrate	3.32	0.00	2.85	0.03	0.711 – 0.813

Table 1. Significant features related to Back Fact(cm) identified by One-Way ANOVA and post-hoc analysis: The metabolites identified were urea and 2-Hydroxyisobutyrate.

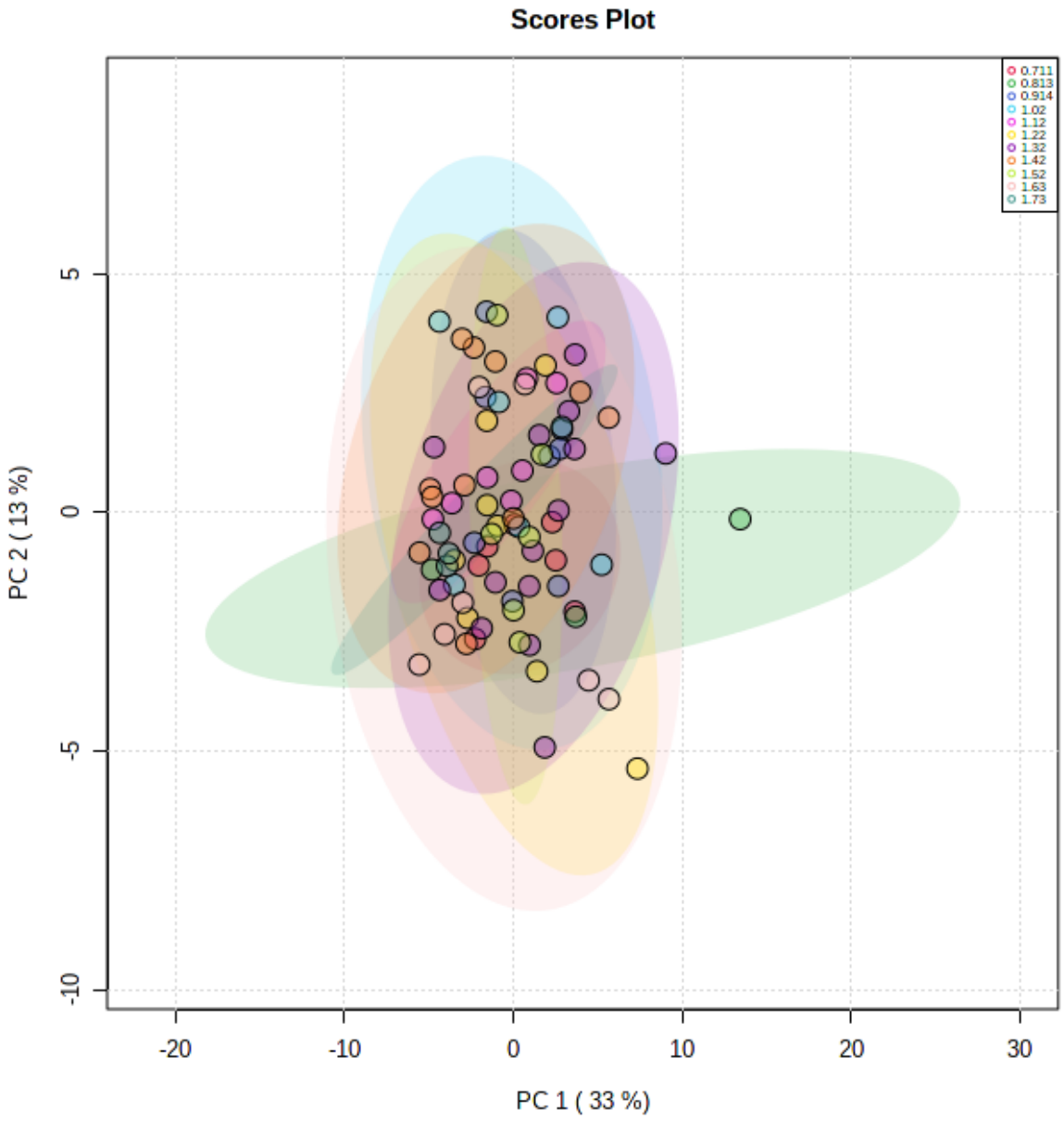

Figure 10. Significant features related to Back Fact(cm) identified by PCA: Scores plot between the selected principal components or group classifications for Back Fat (cm). The variation explained by the first and second principal component is in parentheses.

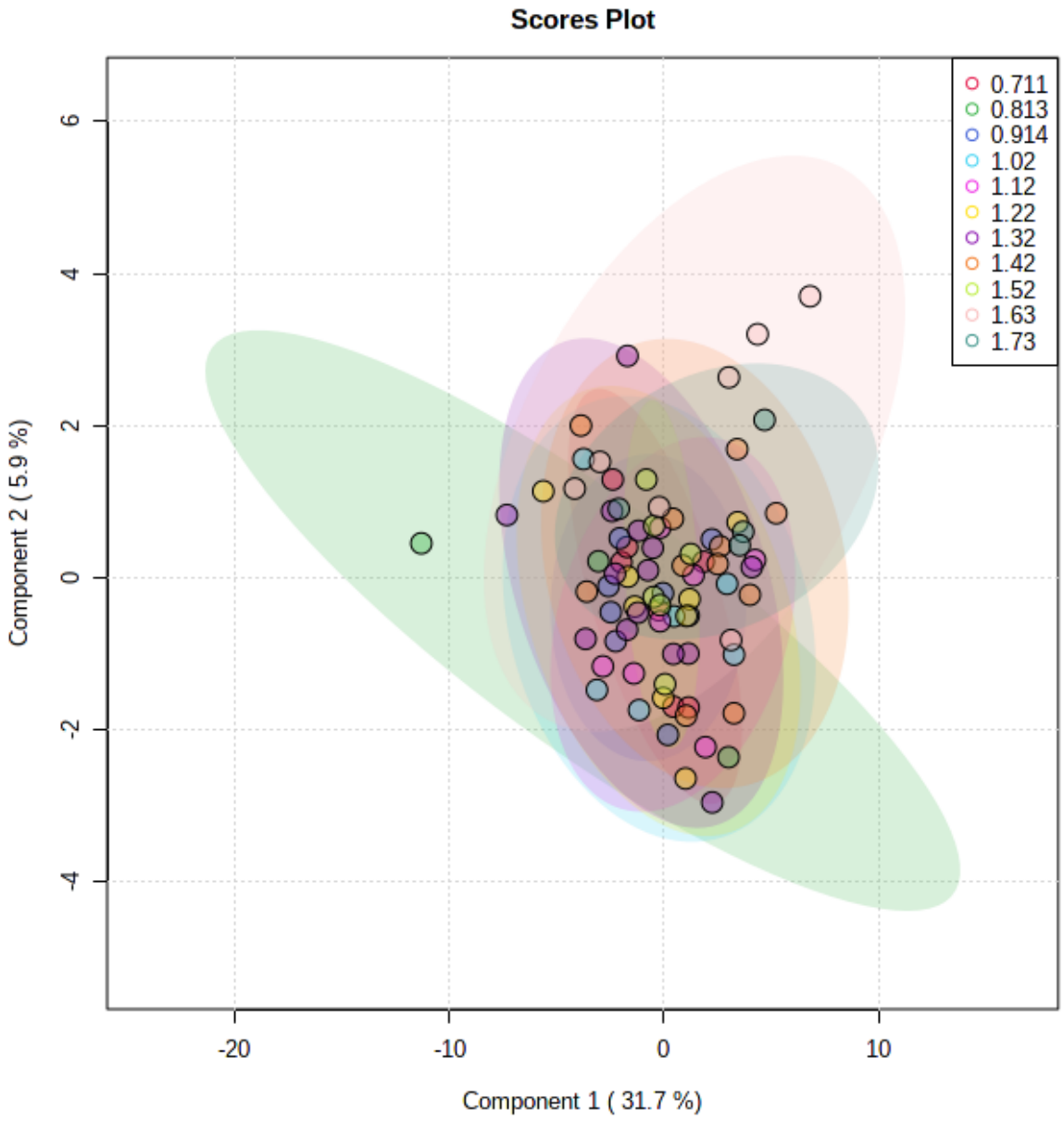

Figure 11. Significant features related to Back Fact(cm) identified by PLS-DA: Scores plot for back fat between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this imagine there is no significant separation between the groups.

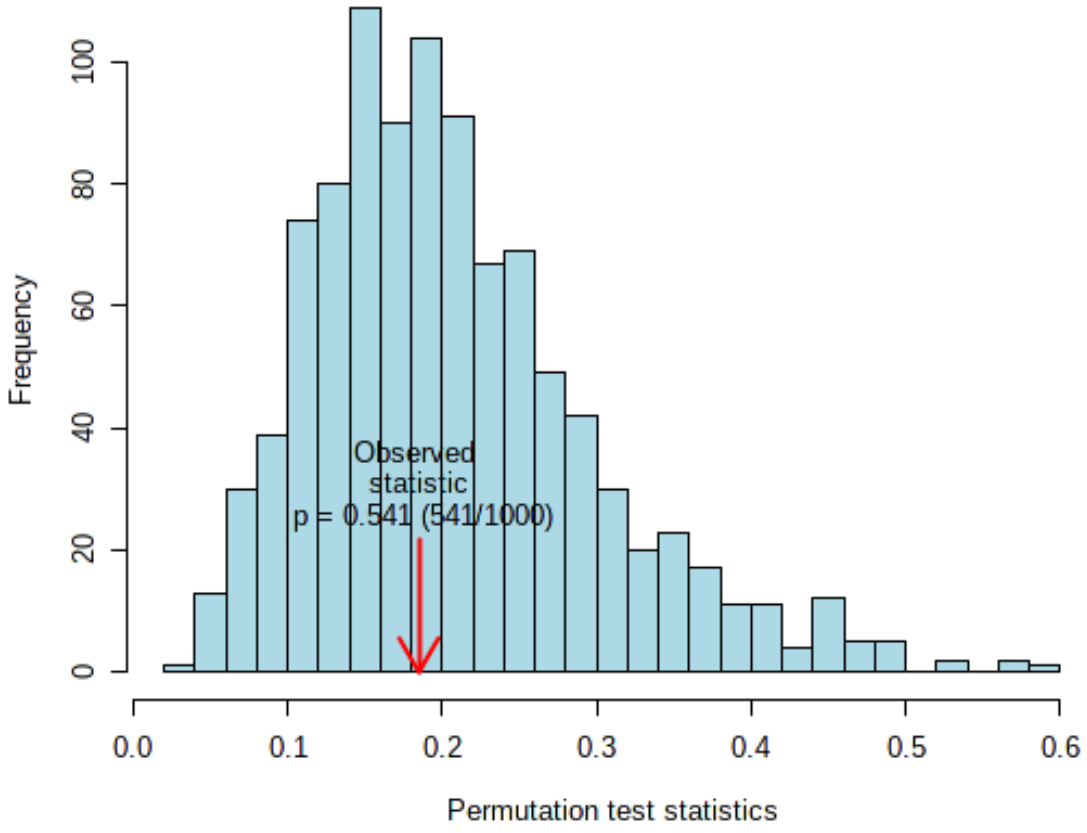

Figure 12. Significance related to Back Fact(cm) identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance for back fat. The p-value based on permutation is p = 0.541 (541/1000). This shows that the model using the significant metabolites cannot predict back fat thickness any better than random.

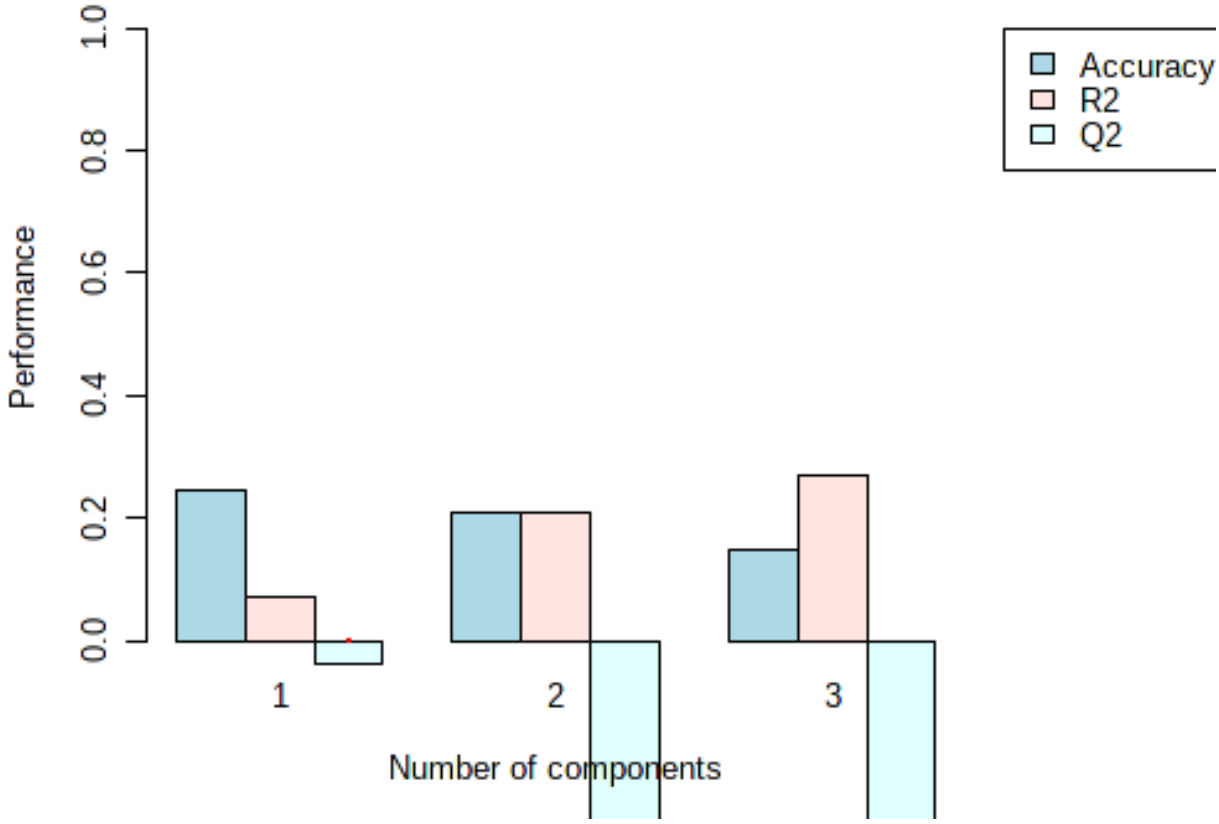

Figure 13. Significance related to Back Fact(cm) identified by Components Test: PLS-DA classification using different number of components. There is not significance.

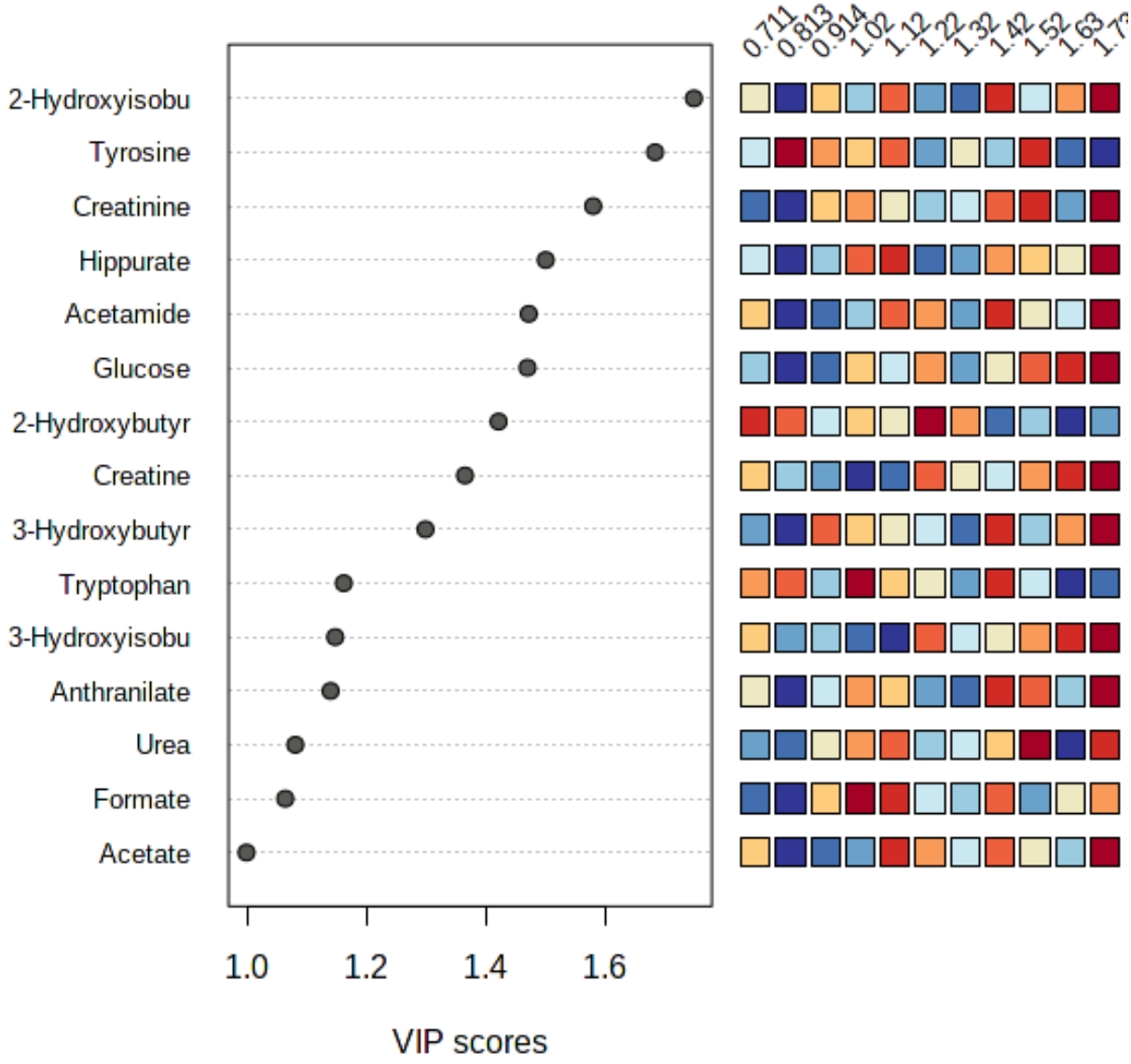

Figure 14. Significant Metabolites related to Back Fact(cm) identified by VIP Scores: Important metabolites identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each back fat group.

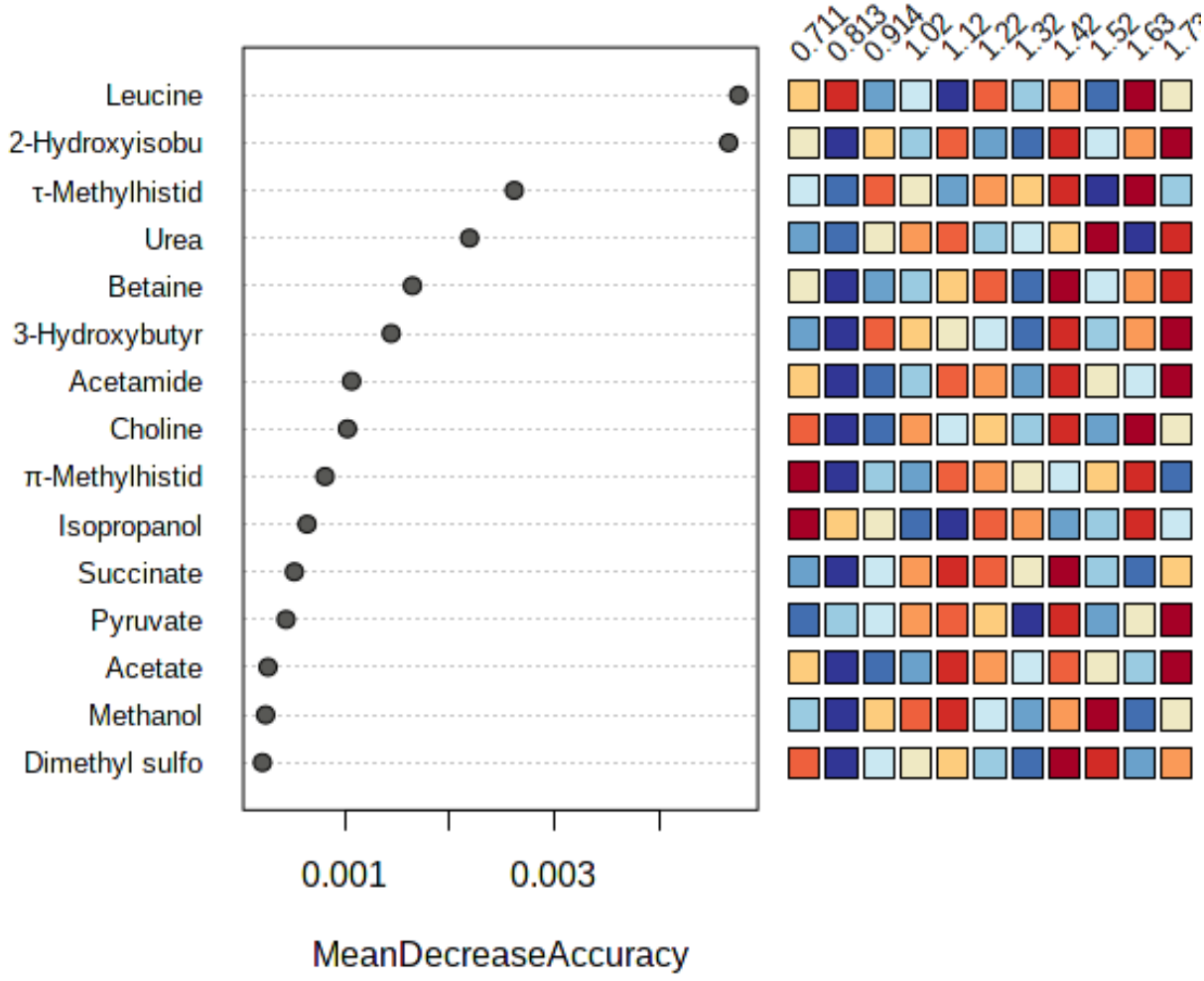

Figure 15. Significant Metabolites related to Back Fact(cm) identified by Random Forest: Significant metabolites identified by Random Forest. The metabolites are ranked by the mean decrease in classification accuracy.

Value Per Hundred Weight of Carcass

Value of carcass reported in closeout was used to determine value per hundred weight of carcass. The carcasses were then assigned to five categories based on a dollar value, the first group was $170-$179.99, followed by $180-189.99, $190-$199.99, $200-$209.99, and lastly $210-$220. Value per hundred weight data was normalized by sum, using a row-wise procedure (Figure 16) and then $\log_{10}$ transformed. A one-way Analysis of Variance (ANOVA) was first used to identify metabolites that discriminated between value per hundred weight (Figure 16), unfortunately there were none. PCA and PLSDA (Figure 17 and 18, respectively) showed a slight but not statistically significant separation between $CWT and the classified groups. The PLSDA validation with permutation (Figure 19) generated a P-value based on permutation of $P = 0.085$ (85/1000). This shows that there was a trend between the groups of value per hundred weight. Figure 19 shows the number of metabolites that would allow the best classifications. An ordered list of the most important features in discriminating between $CWT classifications from the PLSDA analysis is in Figure 21. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are succinate, 2-hydroxyisobutyrate, creatinine, isobutryrate, and formate. No patterns were evident in which metabolites were highest or lowest concentrations by $CWT classification. Figure 22 shows a similar analysis using a mean accuracy approach. The single most important feature identified using this method was anthranilate.

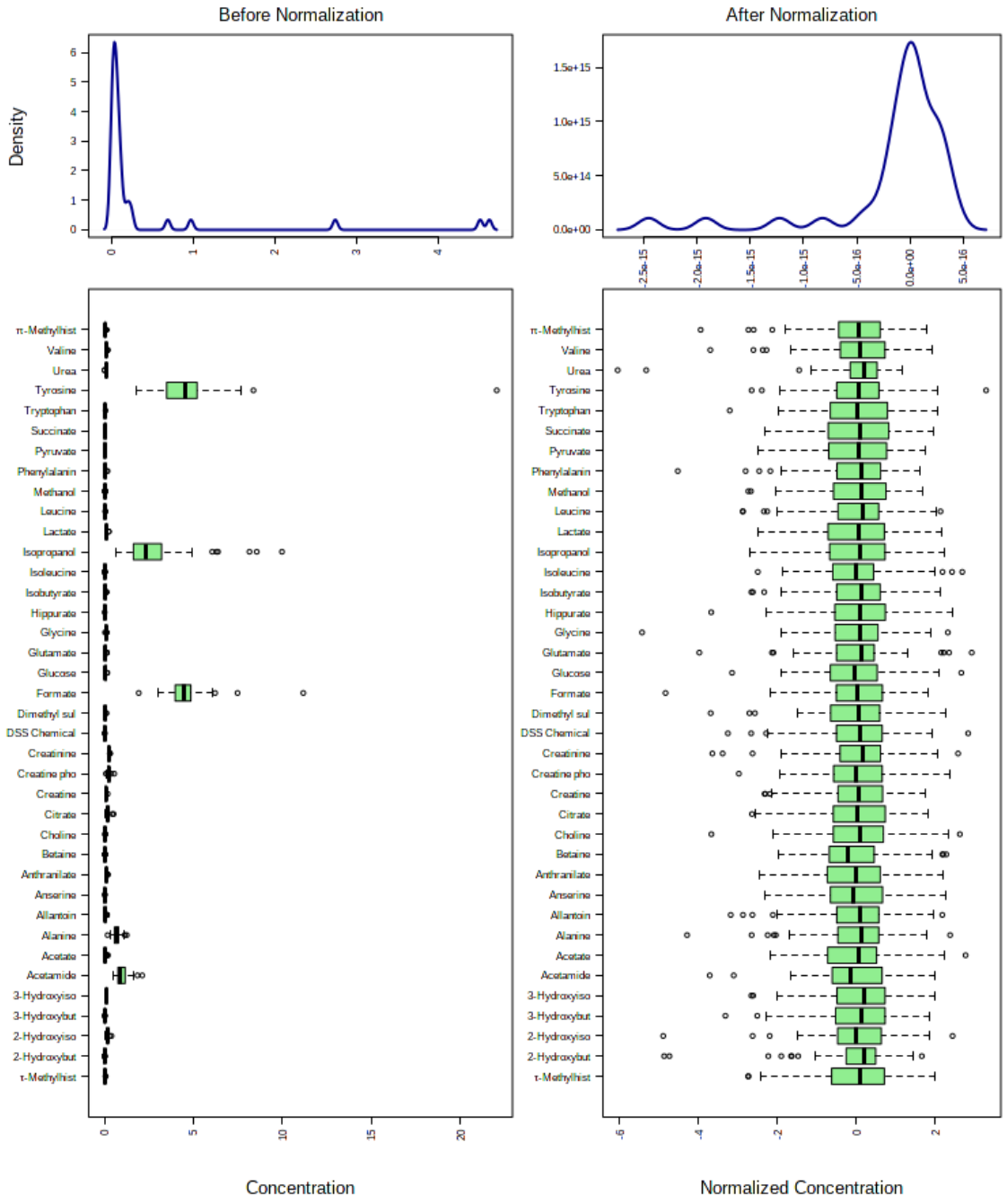

Figure 16. Value per hundred weight data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limitations. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

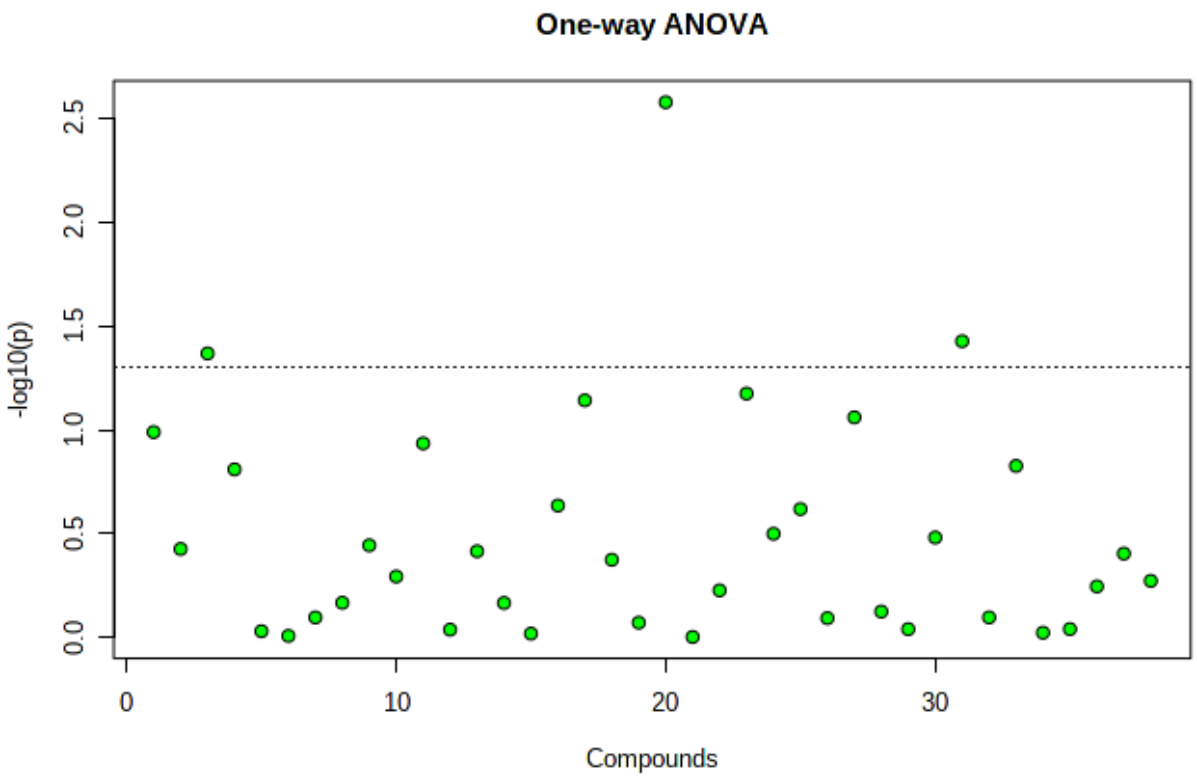

Figure 17. Significant features related to Value per Hundred Weight identified by Analysis of Variance: Important metabolites identified by ANOVA for value per hundred weight categories, p-value threshold 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10 which is the adjustment made to find the level of significance of the metabolites in this list of 39. The reason for some metabolites shown as green even though they are at or above the level of significance is because they were falling within the FDRs, which make them non-significant.

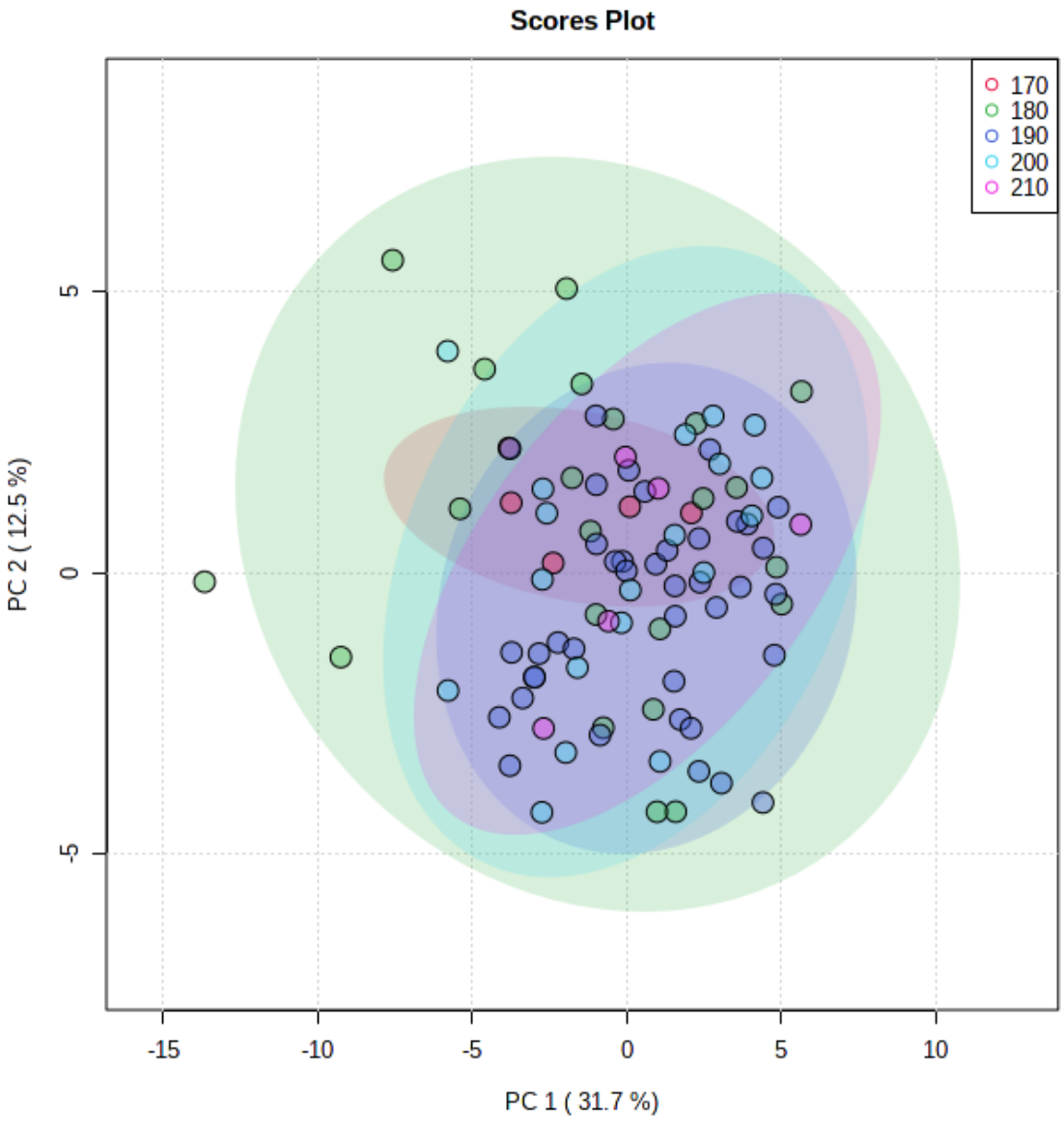

Figure 18. Significant featuers related to value per hundred wiehgit identified by PCA: Scores plot between the selected principle components or group classifications of value per hundred weight. The variation explained by the first and second principal component is in parentheses.

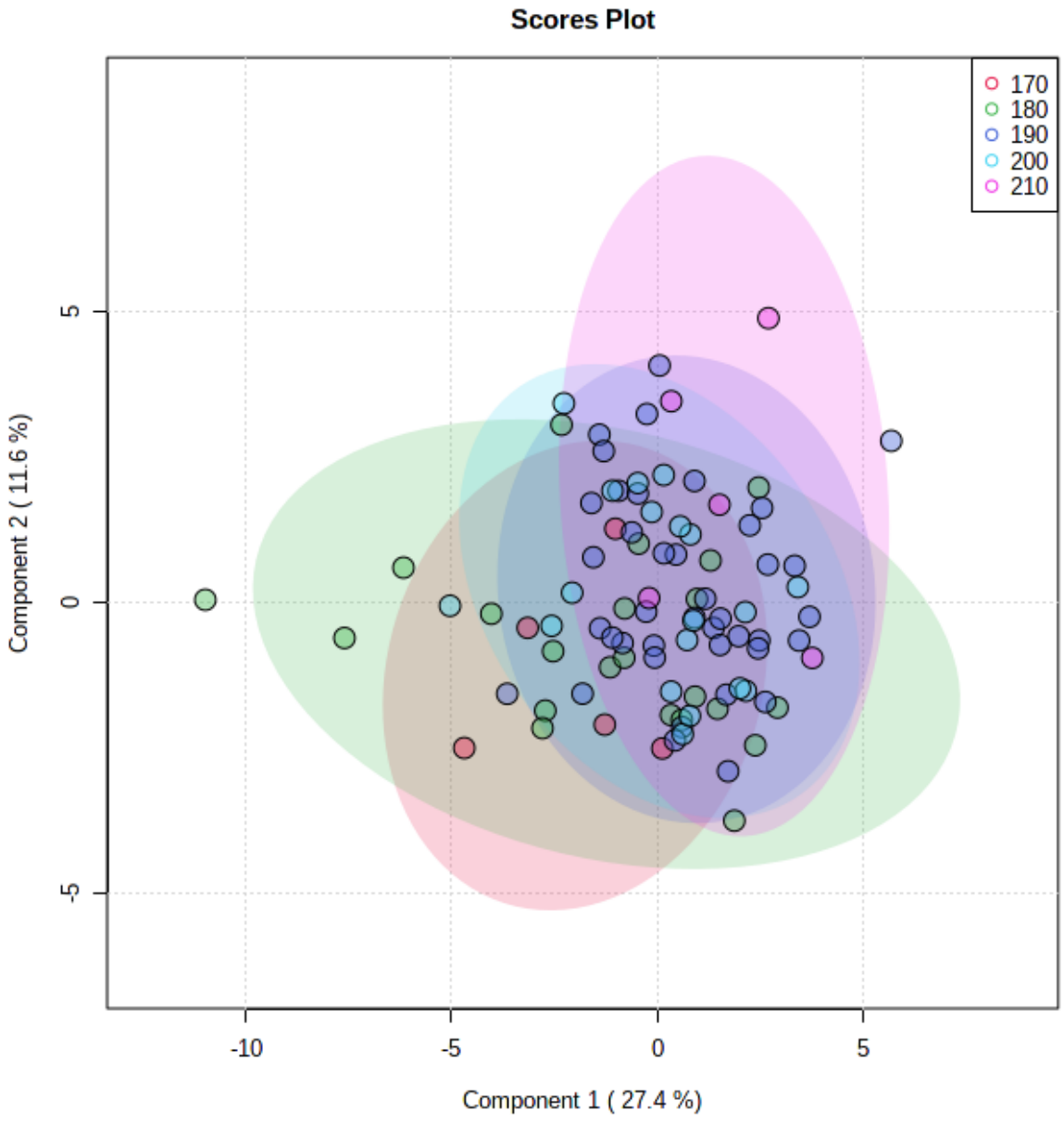

Figure 19. Significant features related to value per hundred weight identifed by PLS-DA: Scores plot for value per hundred weight between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is slight separation between the groups.

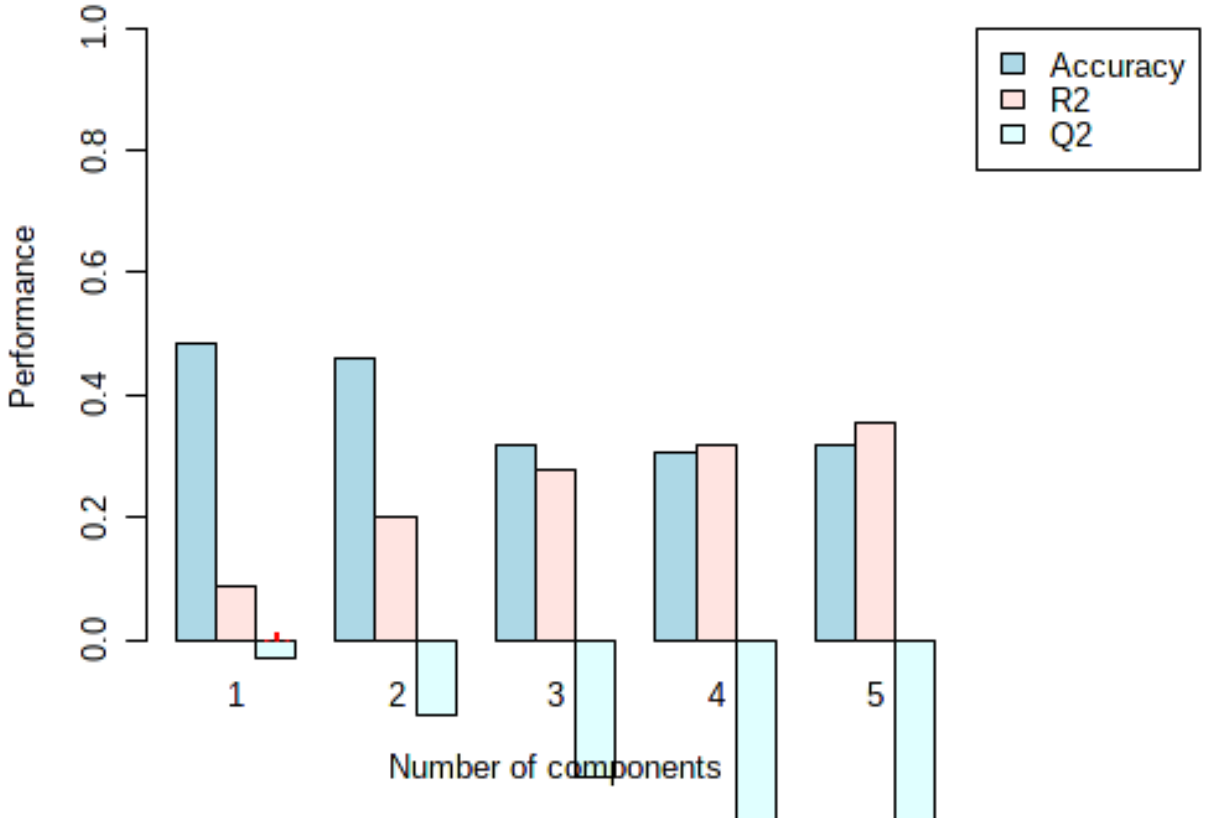

Figure 20. Significance related to value per hundred weight identified by Components Test:

PLS-DA classification using different number of components. There is not significance.

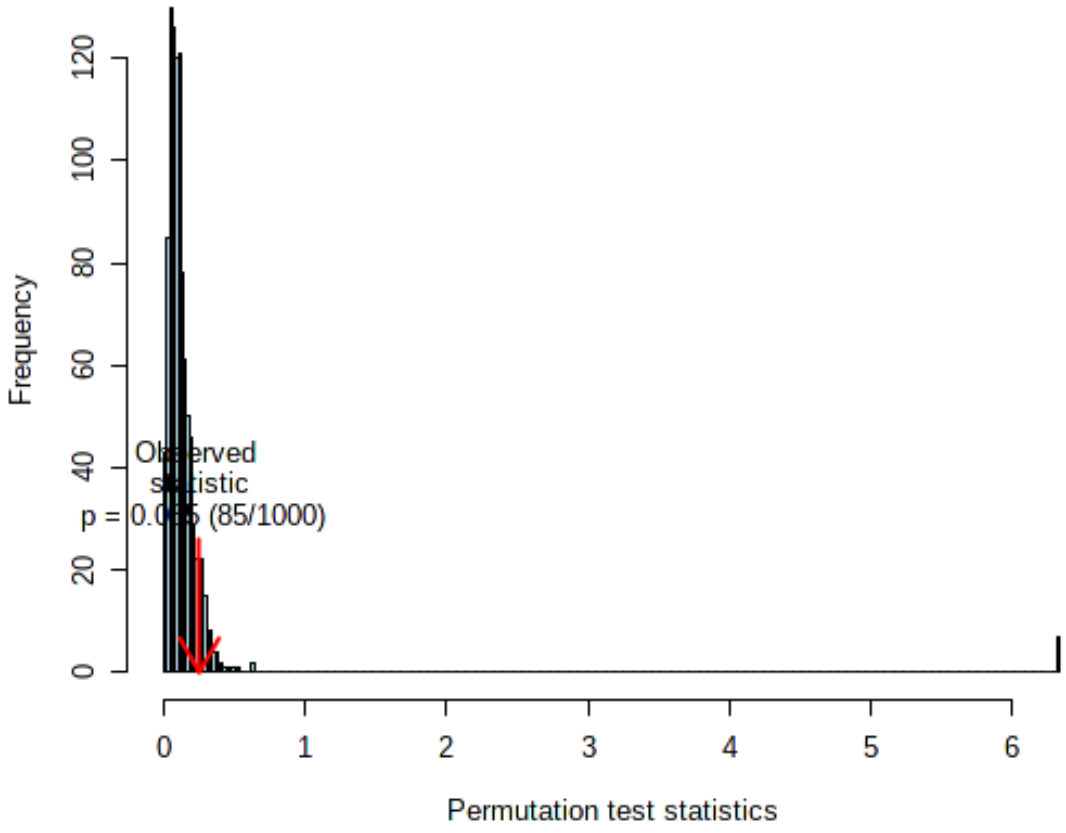

Figure 21. Significance realted to value per hundred weight identifed by Permutaiton Test: PLS-DA model validation by permutation test based on speratation distance. The P-value based on permutation is P = 0.085 (85/1000). This suggests there is a trend for a difference between the groups.

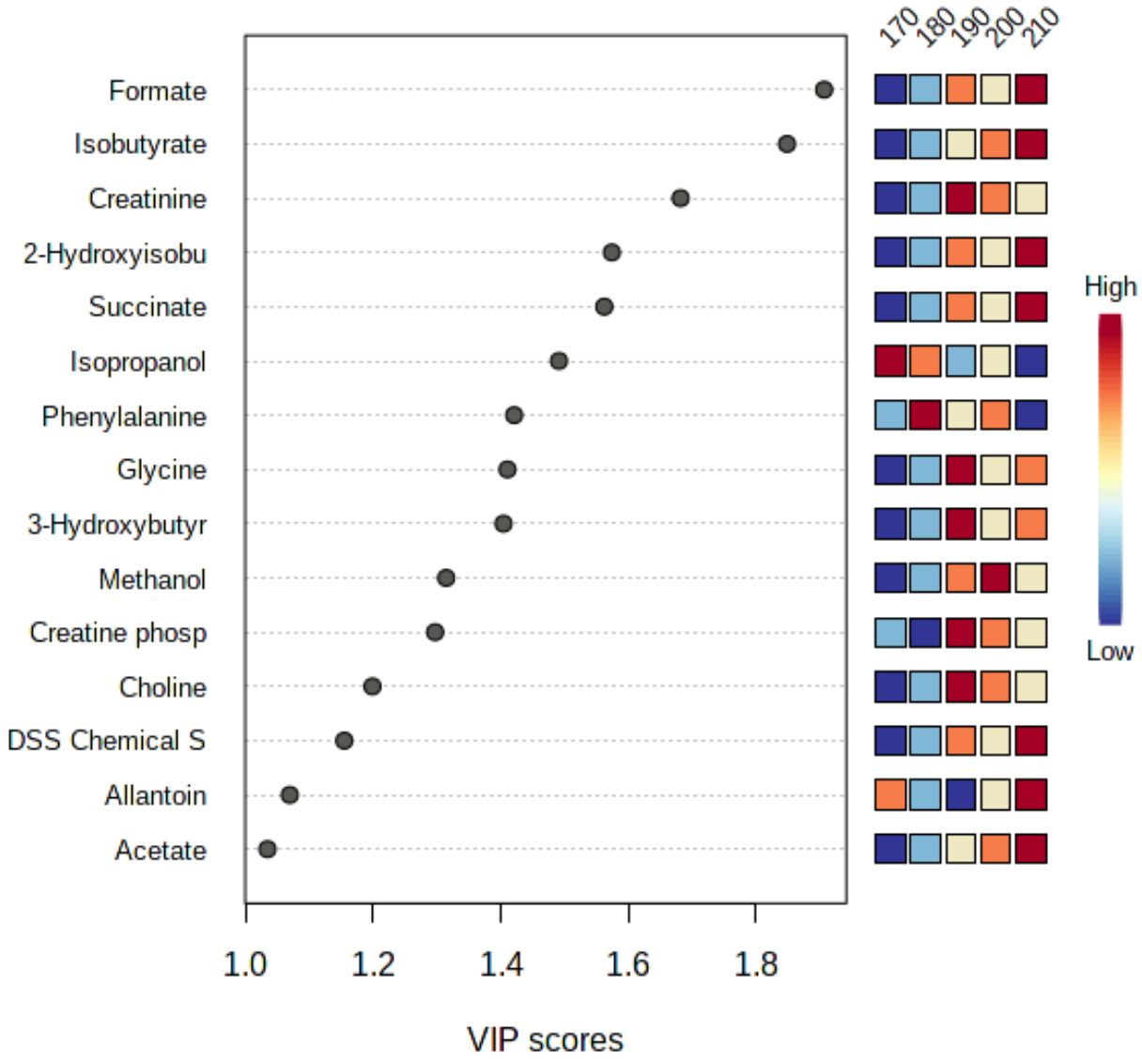

Figure 22. Significant Metabolites related to value per hundred weight identified by VIP Scores: Important featues identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

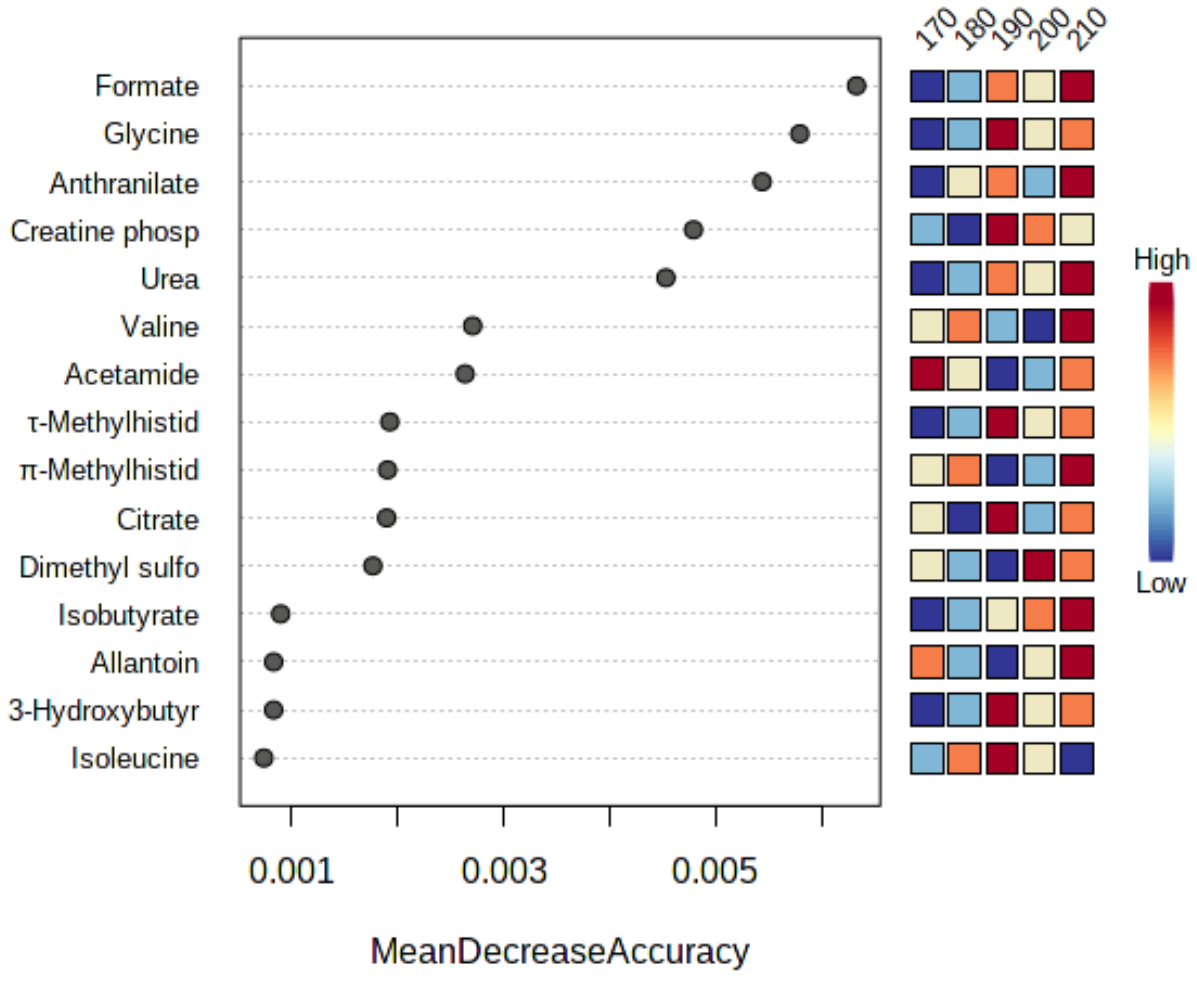

Figure 23. Significnat Metabolites related to value per hundred wieght identified by Mean Accuracy: Significant features identifed by Random Forest. The features are ranked by the mean decreased in classification accurancy when they are permuted.

Hot Carcass Weight

Hot carcass weights were assigned to three groups light, medium, and heavy. The groups were light from 278 kg to 340 kg, medium 341 kg to 401 kg and heavy carcasses were 402 kg to 464 kg. The data normalization for hot carcass weight is shown in Figure 24. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was first used to identify features that discriminate between hot carcass weight classifications. Figure 25 shows the important features identified by ANOVA analysis. PCA and PLSDA shown in Figure 26 and Figure 27 respectively show slight separation between the hot carcass weight groups. Figure 27 shows the PLSDA which is the supervised analysis and tries to maximize the differences between classifications. It indicates that there is no model for classification based on metabolite features for HCW. Figure 28 shows that the PLSDA model is not able to differentiate between HCW classes. The PLSDA validation with permutation shown in Figure 29 generated a *P*-value based on permutation of $P = 0.456$ (456/1000). This shows that the model cannot predict HCW any better than random chance. Figure 30 shows an ordered list of the most important features in discriminating between hot carcass weight classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are urea, t-methylhistidine, choline, and 2-hydroxyisobutyrate. Choline had the highest concentration in the heavy group and the lowest in the medium group. The data shown here suggests that heavier animals are utilizing less choline than the medium or light groups for fat metabolism. No patterns were evident in which features were highest or lowest concentrations by HCW classification. Figure 31 shows a similar analysis using a Random Forest approach. The single most important feature identified using this method was 2-hydroxyisobutrate.

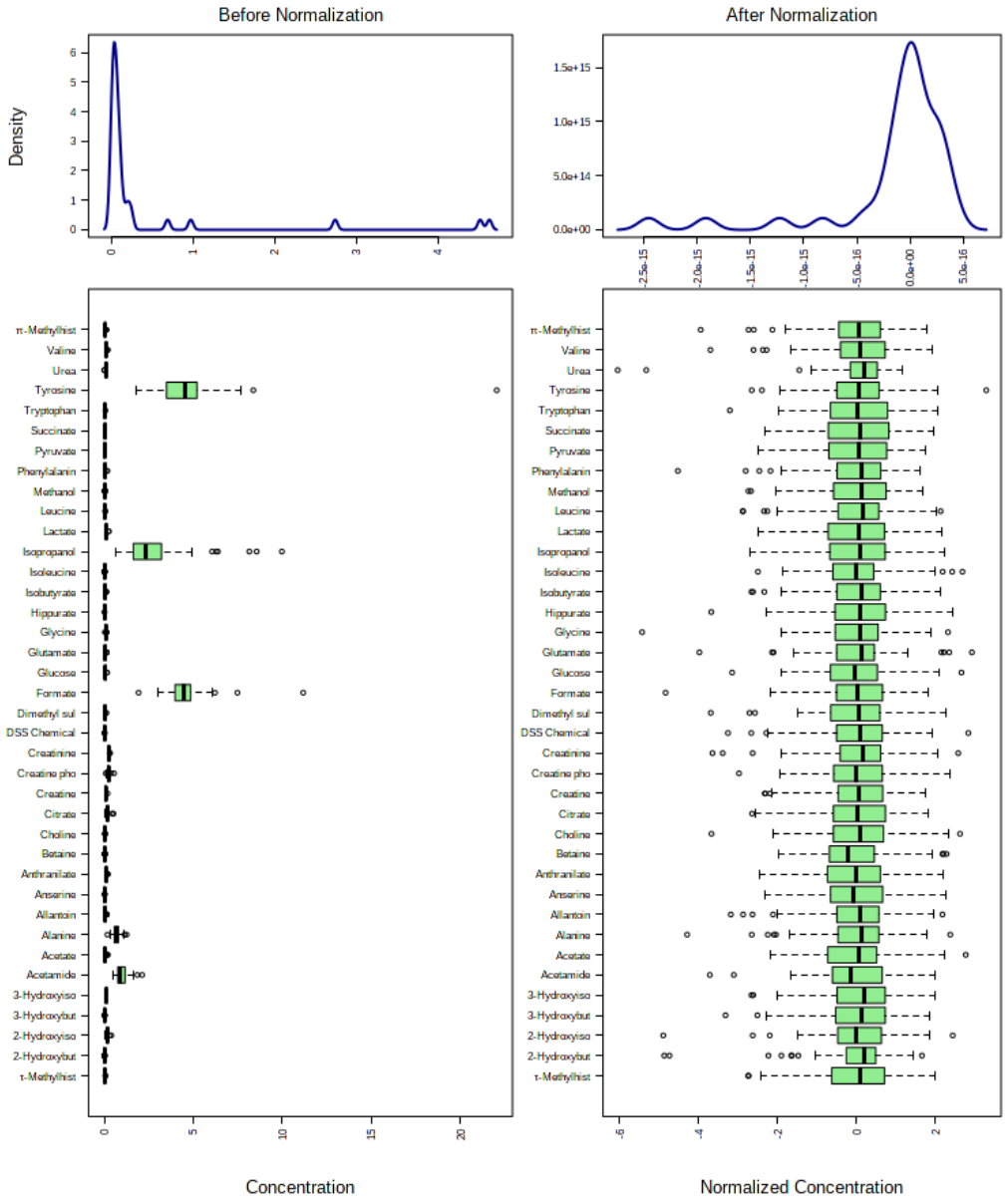

Figure 24. Hot Carcass Weight Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

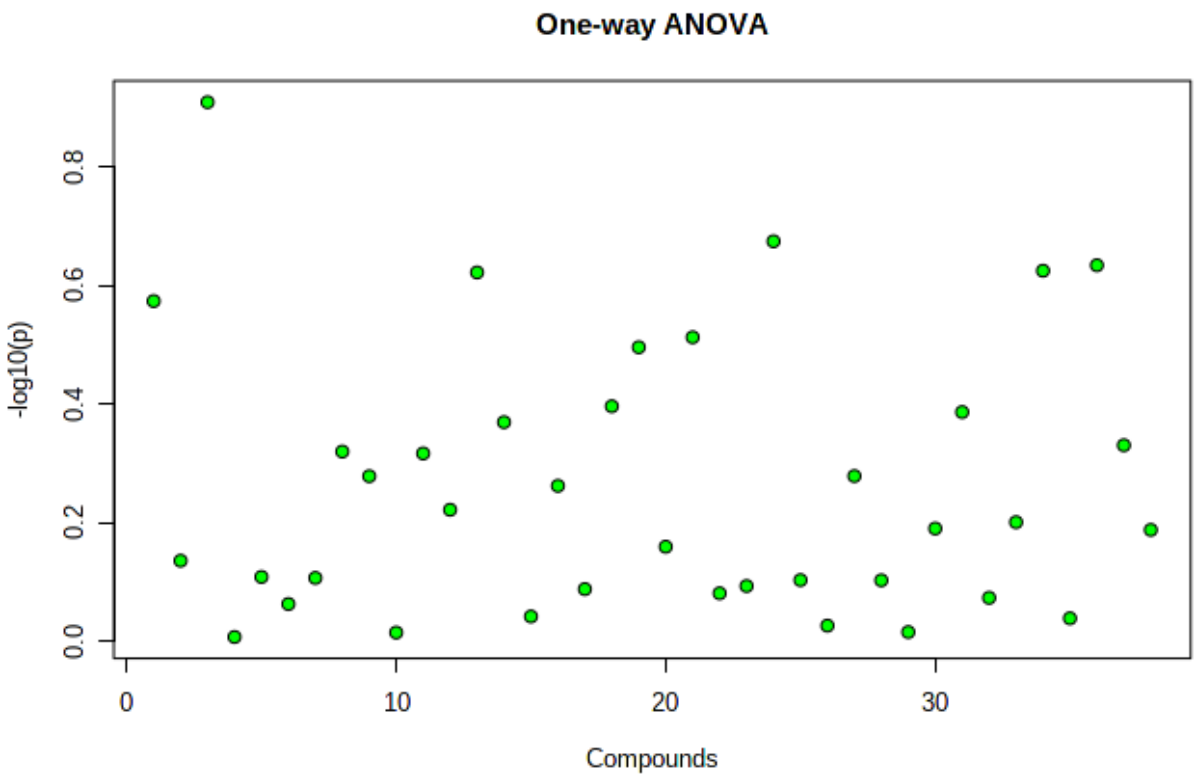

Figure 25. Significant features related to Hot Carcass Weight identified by Analysis of Variance: Important metabolites identified by ANOVA for Hot Carcass Weight categories, p-value threshold 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10 which is the adjustment made to find the level of significance of the metabolites in this list of 39.

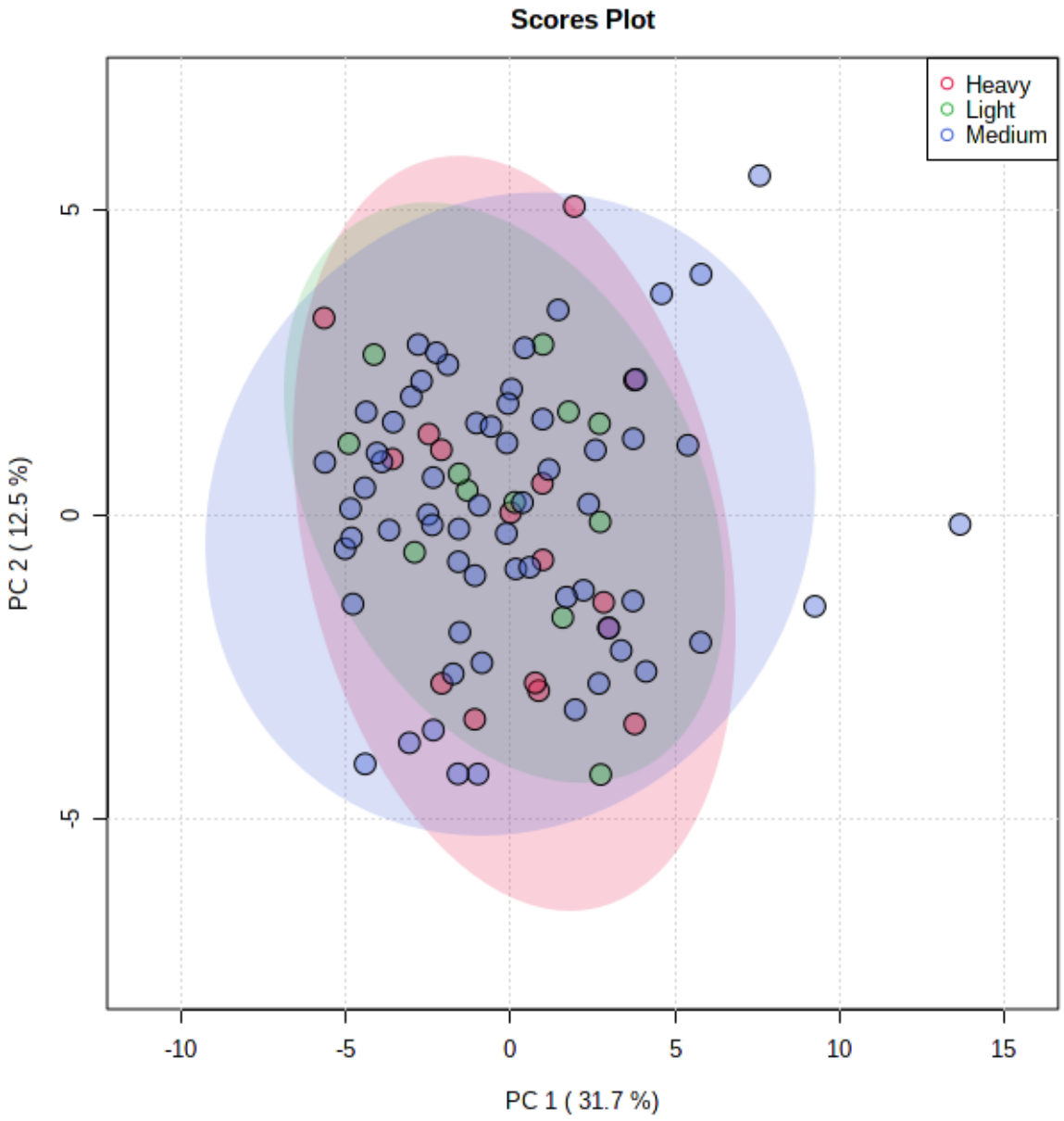

Figure 26. Significant features related to Hot Carcass Weight identified by PCA: Scores plot between the selected principal components or group classifications for Hot Carcass Weight. The variation explained by the first and second principal component is in parentheses. Little separation between HCW classes is shown.

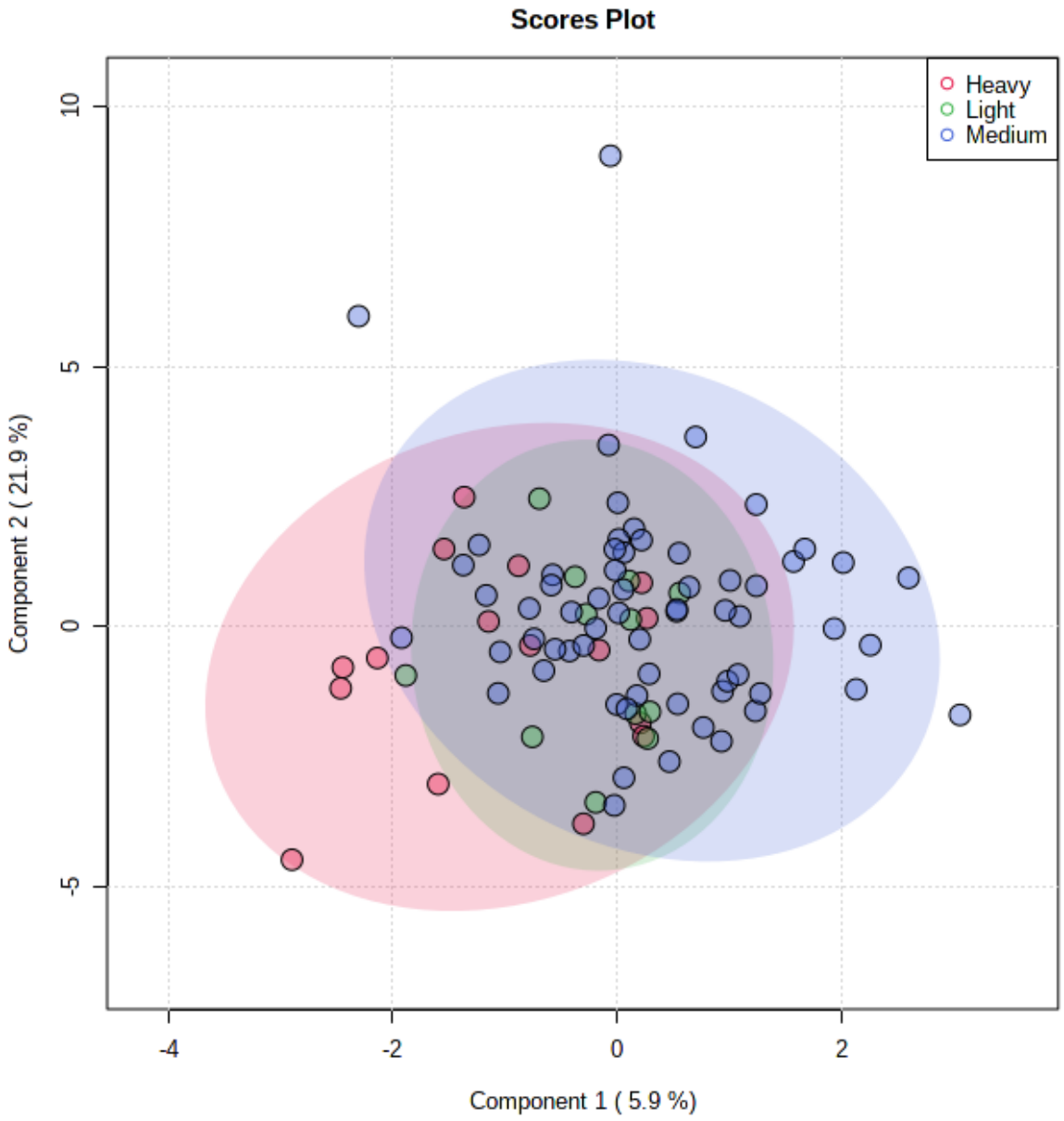

Figure 27. Significant features related to Hot Carcass Weight identified by PLS-DA: Scores plot for hot carcass weight between the selected principal components. The variance explained by the two principal components are shown in parentheses. Slight separation between the groups is shown.

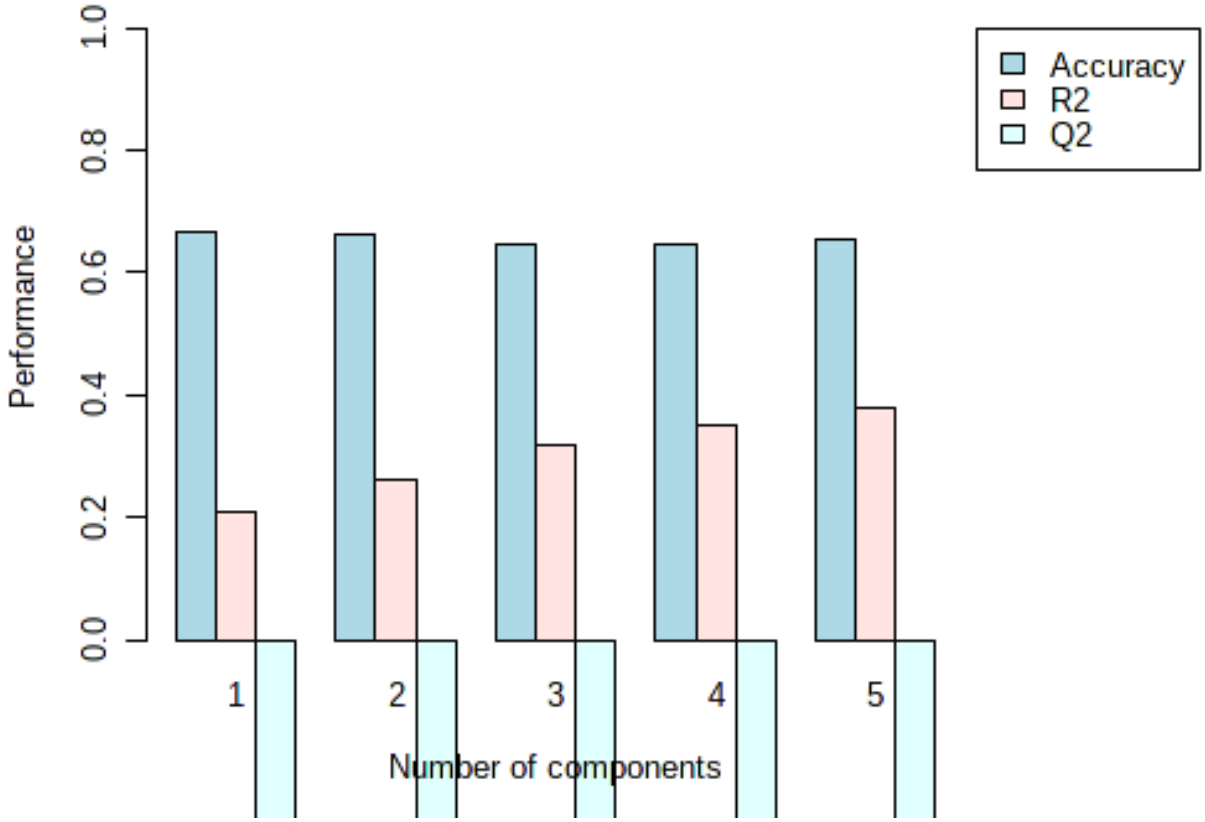

Figure 28. Significance related to Hot Carcass Weight identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present. As there is no star and all Q2 values are negative there is no significance in the separation in the groups.

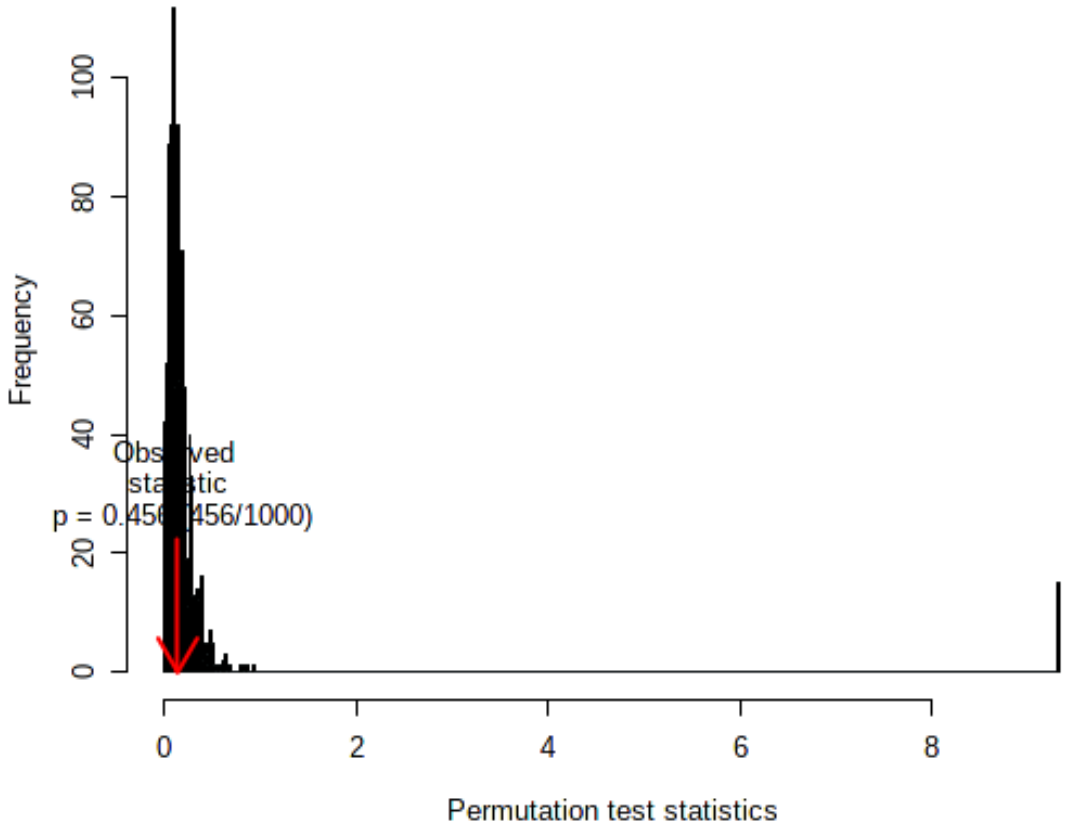

Figure 29. Significance related to Hot Carcass Weight identified by Permutation Test: PLS-DA model validation by permutation test based on separation distance. The p-value based on permutation is p = 0.456 (456/1000). There is no significant difference between HCW classes. The spike on the right hand indicates little variation in this trait.

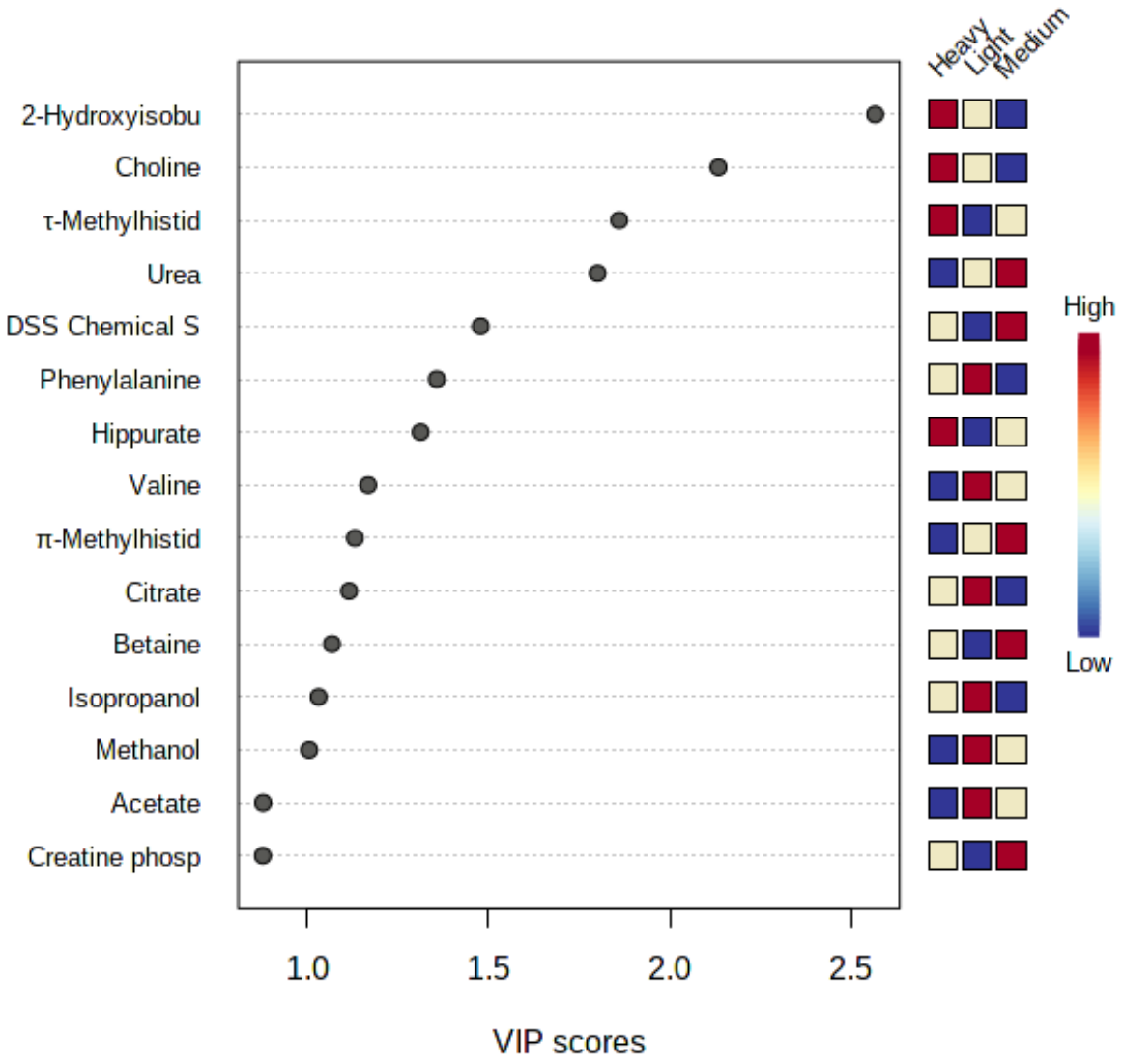

Figure 30. Significant Metabolites related to Hot Carcass Weight identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each classification.

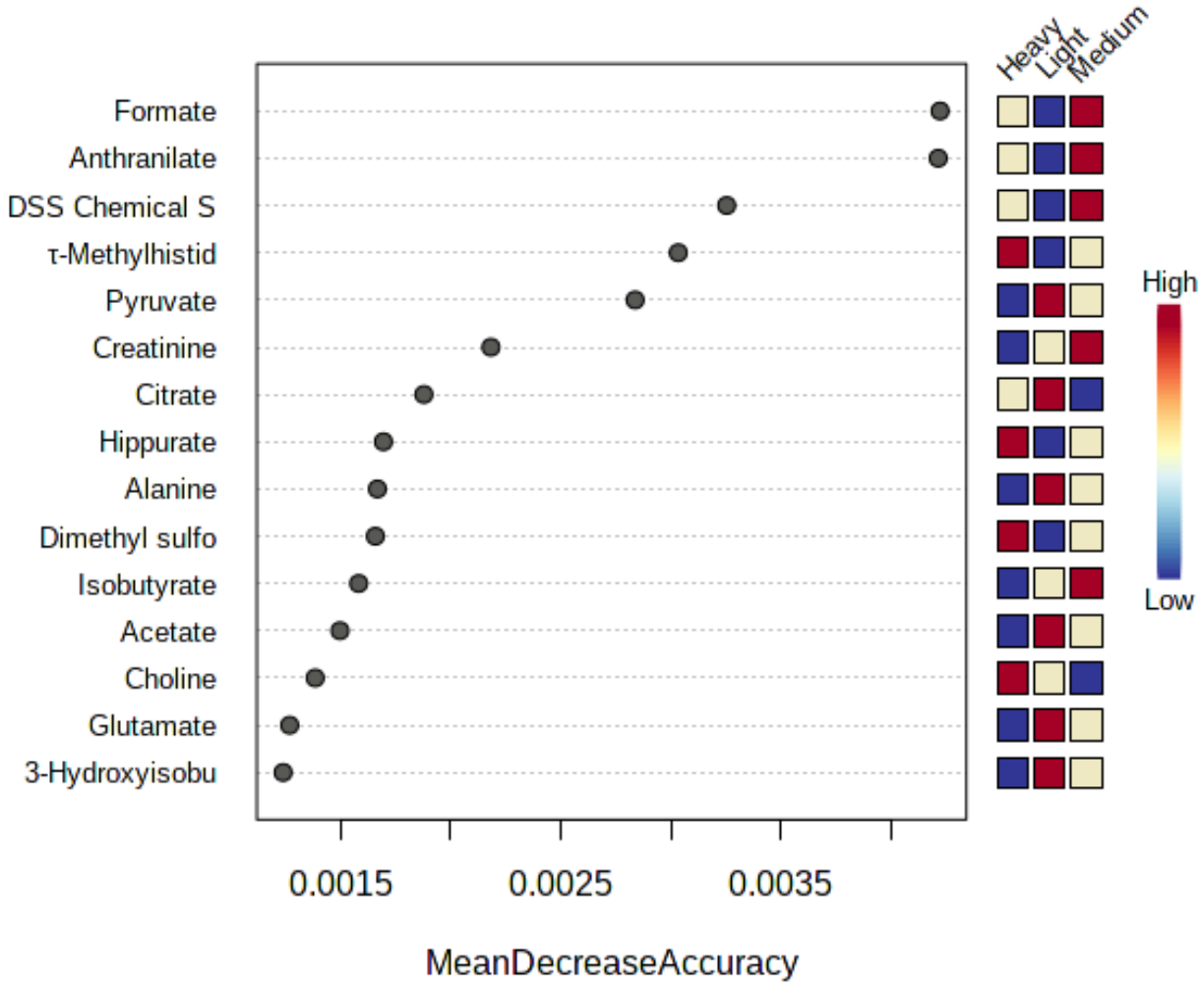

Figure 31. Significant Metabolites related to Hot Carcass Weight identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy.

Quality Grade classified into different levels of Select, Choice or Prime

Quality grades were classified as high Select, low Choice (-), Choice (0) and high Choice (+) and low Prime (-). These classifications are based on marbling score ranges: Select is 350-399, low Choice is 400-499 , average Choice is 500-599, high Choice is 600-699 and low Prime is 700-799. The data normalization for quality grade class is shown in Figure 32. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was used to identify features that discriminate between quality grade classes. Figure 33 shows the important features identified by ANOVA analysis. The post-hoc comparison column shows the comparisons between different levels that are significant given the *P* value threshold. PCA and PLSDA shown in Figure 34 and Figure 35 respectively and did not show separation between quality grade classification groups. The PLSDA validation with permutation shown in Figure 37 generated a *P*-value based on permutation of $P = 0.068$ (68/1000). This shows that there is a trend for a difference between the data set and the model. Figure 36 looks at the number of components that would allow for the best classifications between quality grade classes. It indicates that there is not a viable classification based on metabolite features possible for quality grade classification. Figure 38 shows an ordered list of the most important features in discriminating between quality grade classification from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are isopropanol, glucose, formate, acetamide, and isobutyrate. No patterns were evident in which features were highest or lowest concentrations by quality grade classification. Figure 39 shows a similar analysis using a Random Forest approach. The single most important feature identified using this method was citrate concentration.

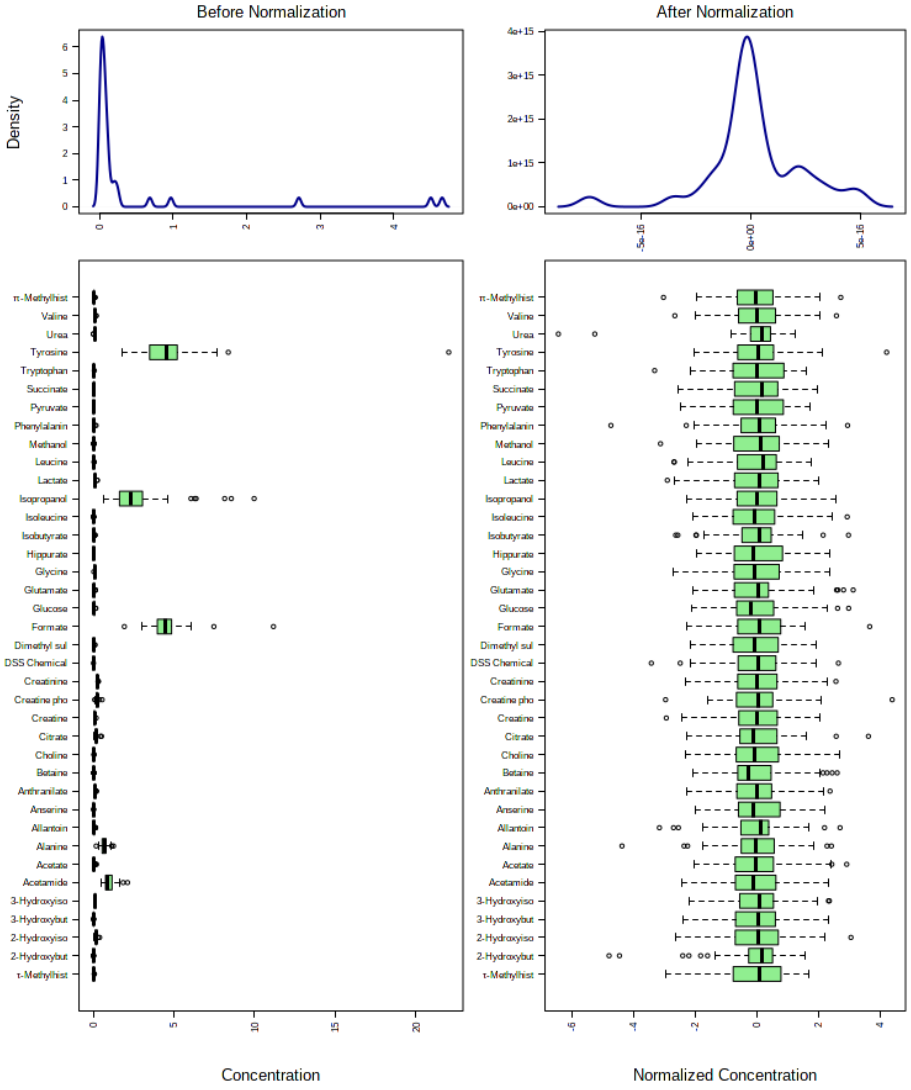

Figure 32. Quality Grade Class Data Before and After Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

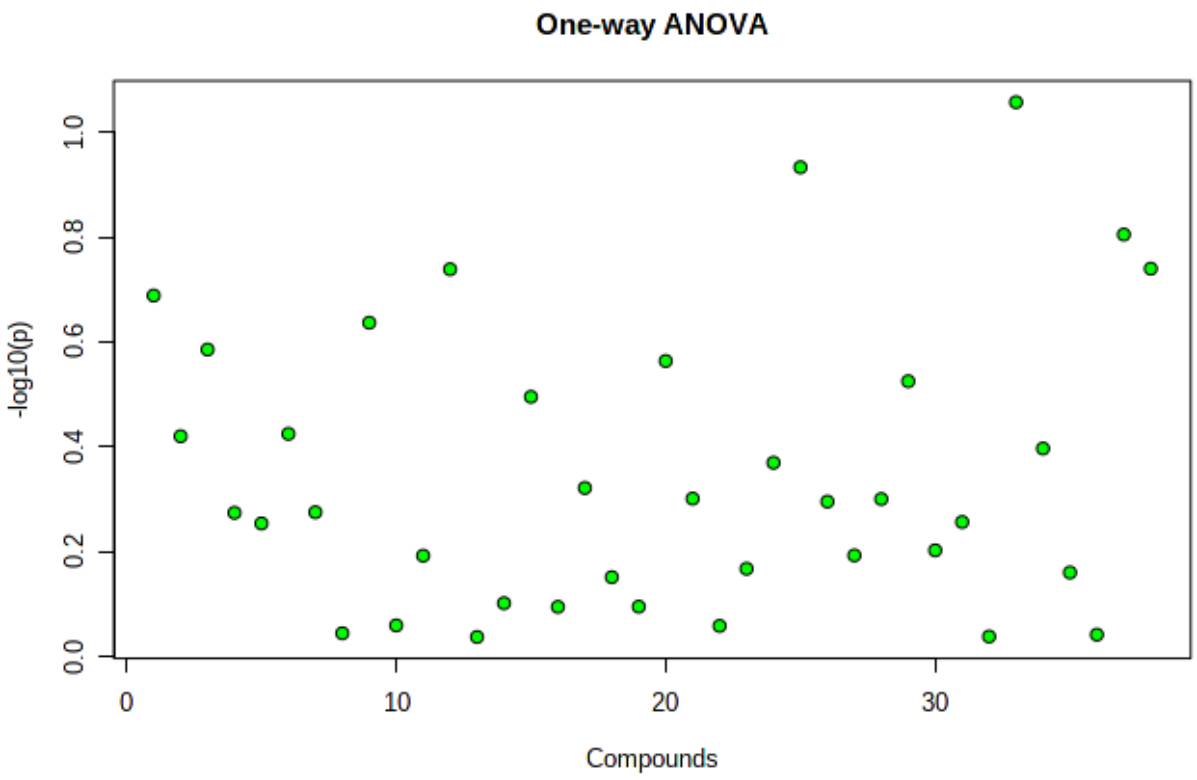

Figure 33. Significant features related to Quality Grade (-) (+) identified by Analysis of Variance: Important metabolites identified by ANOVA for quality grade classification categories, p-value threshold 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10 which is the adjustment made determine level of significance of the metabolites in this list of 39.

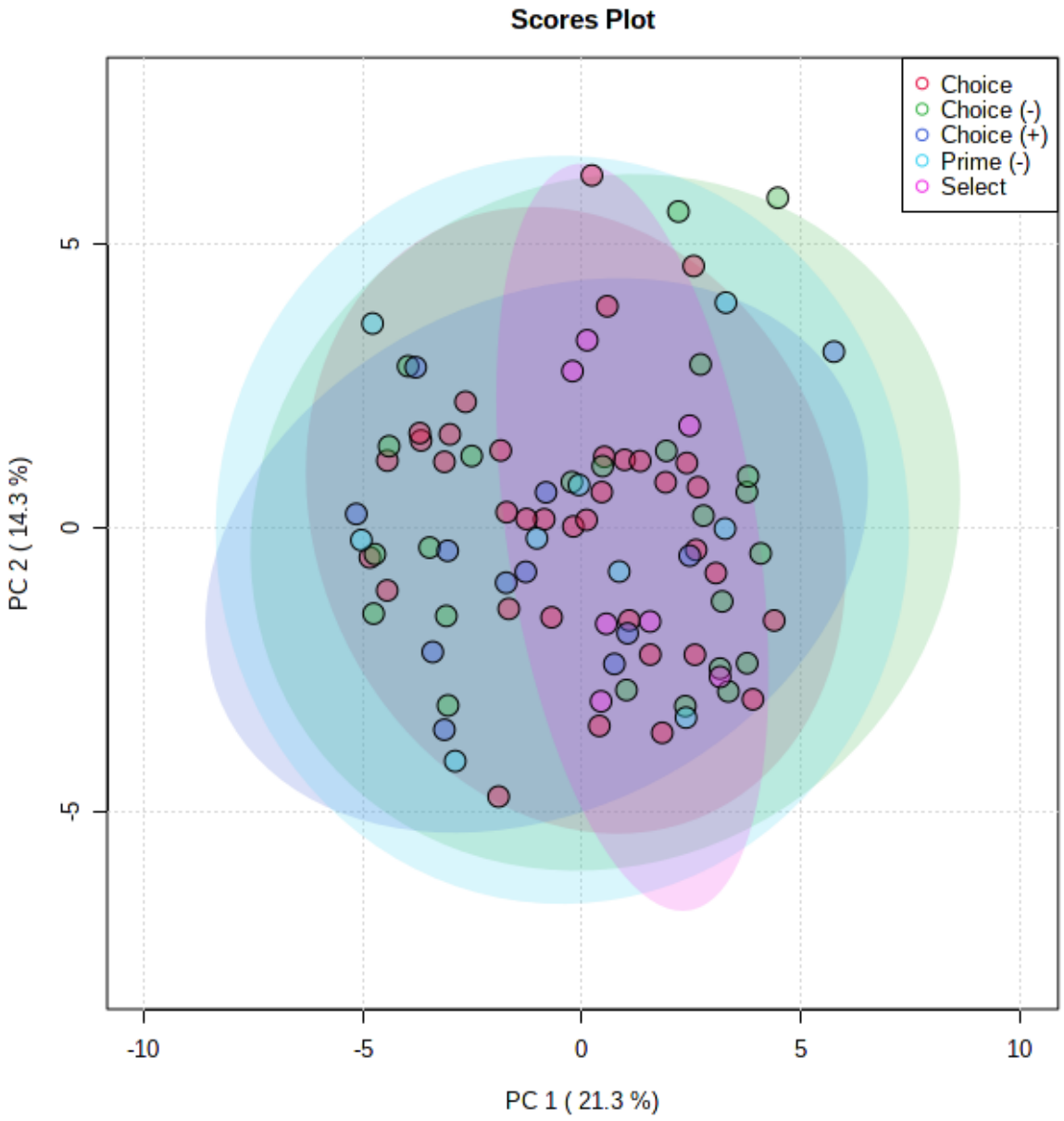

Figure 34. Significant features related to Quality Grade (-) (+) identified by PCA: Scores plot between the selected principal components or group classifications for quality grade. The variation explained by the first and second principal component is in parentheses.

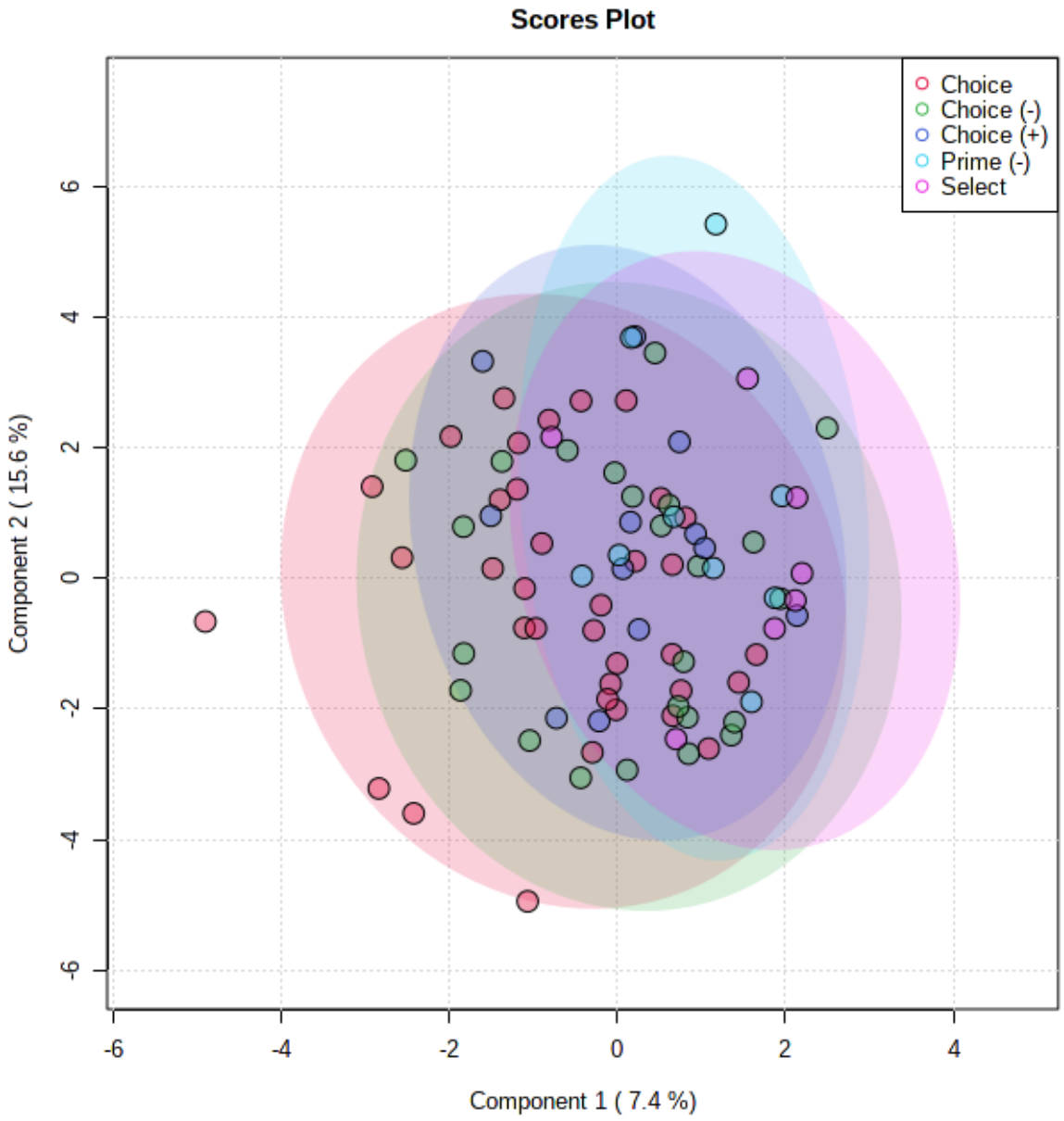

Figure 35. Significant features related to Quality Grade (-) (+) identified by PLS-DA: Scores plot for quality grade classes by selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is slight separation between the groups.

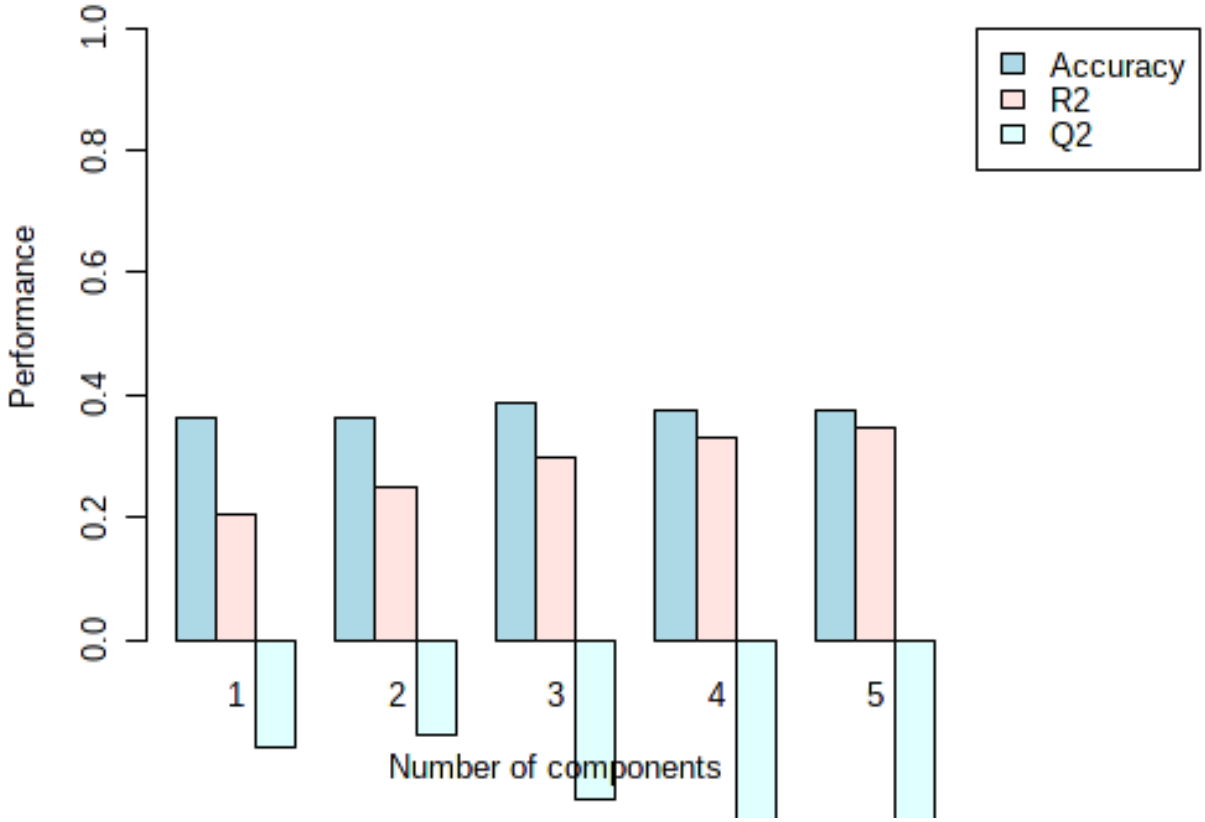

Figure 36. Significance related to Quality Grade Classification identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present, the negative Q2 shows no significance.

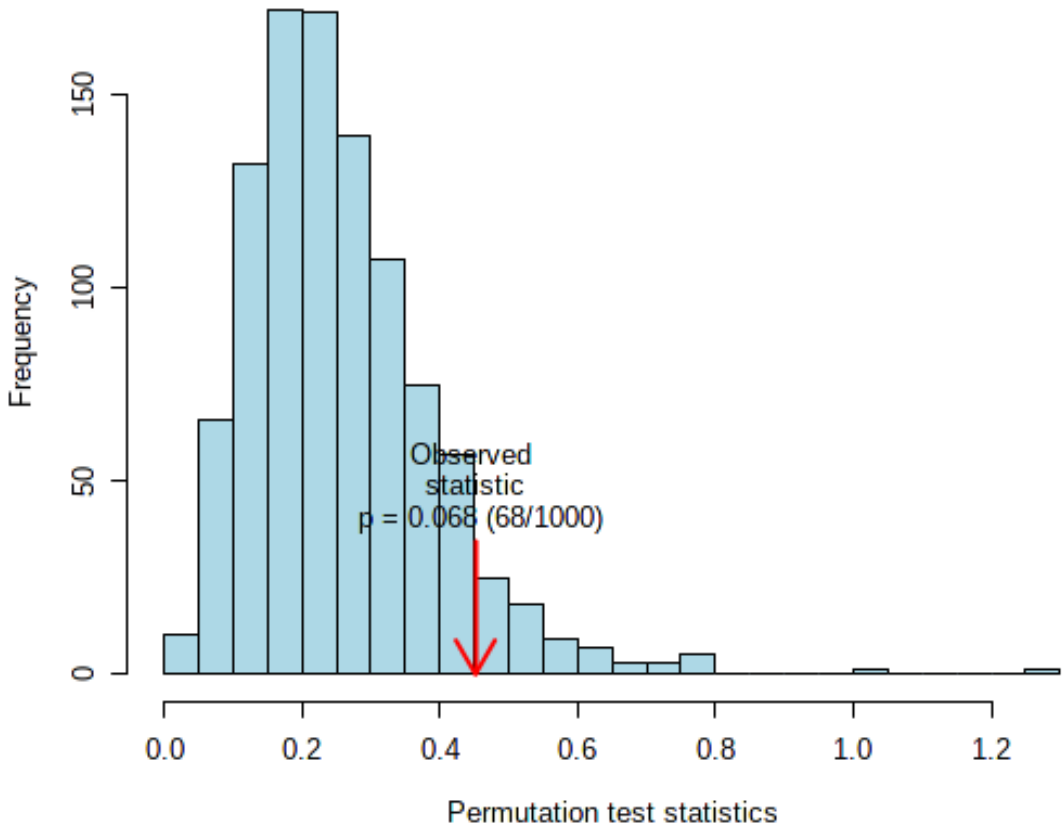

Figure 37 Significance related to Quality Grade Classification identified by Permutation Test: PLS-DA model validates by permutation test separation distance. The *P* – value based on permutation is *P* = 0.068 (68/1000) The *P* – value shows that there is a trend for a difference between the classifications in the model.

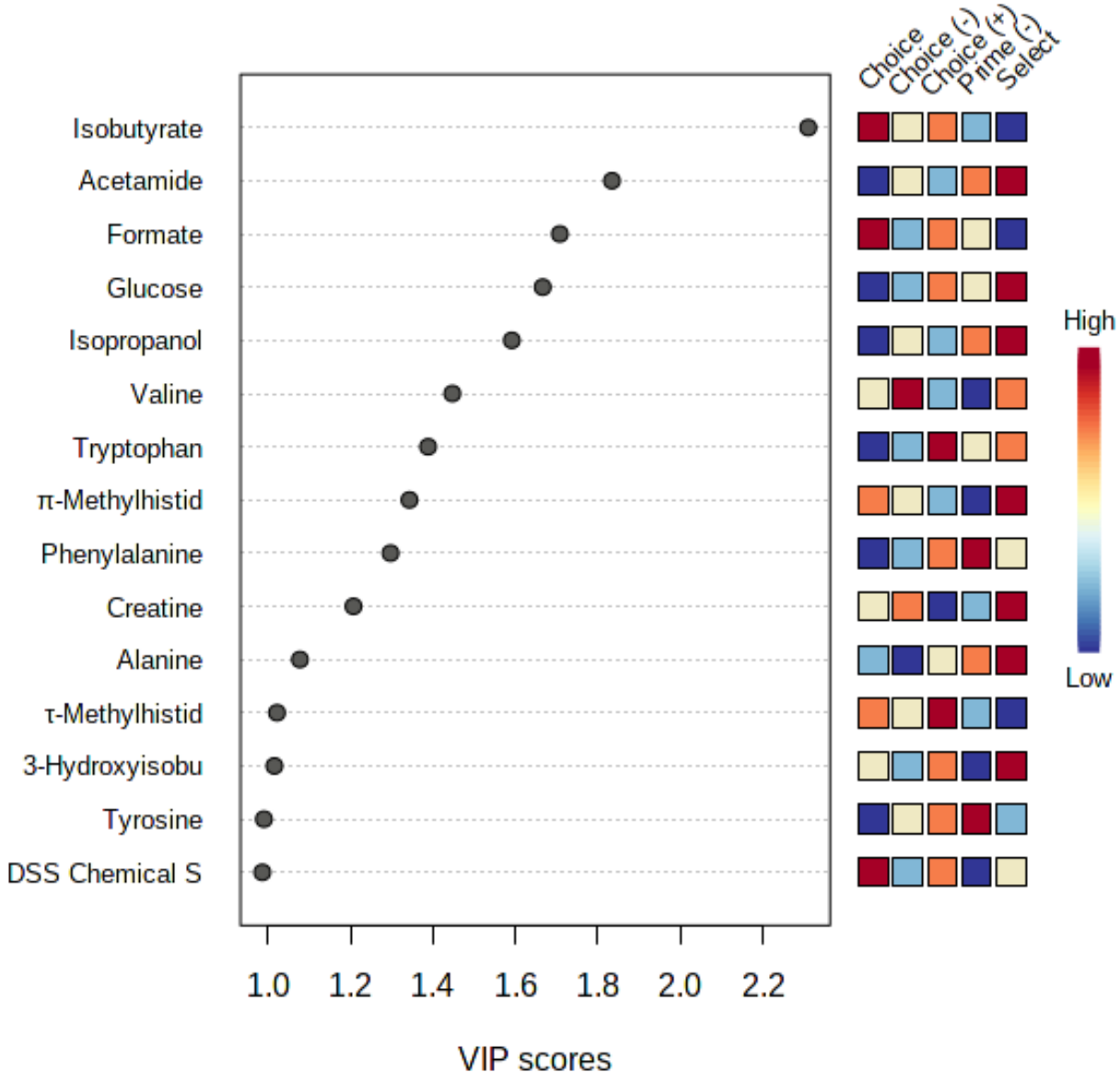

Figure 38. Significant Metabolites related to Quality Grade Classification identified by VIP Scores: Important features were identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each classification of quality grade.

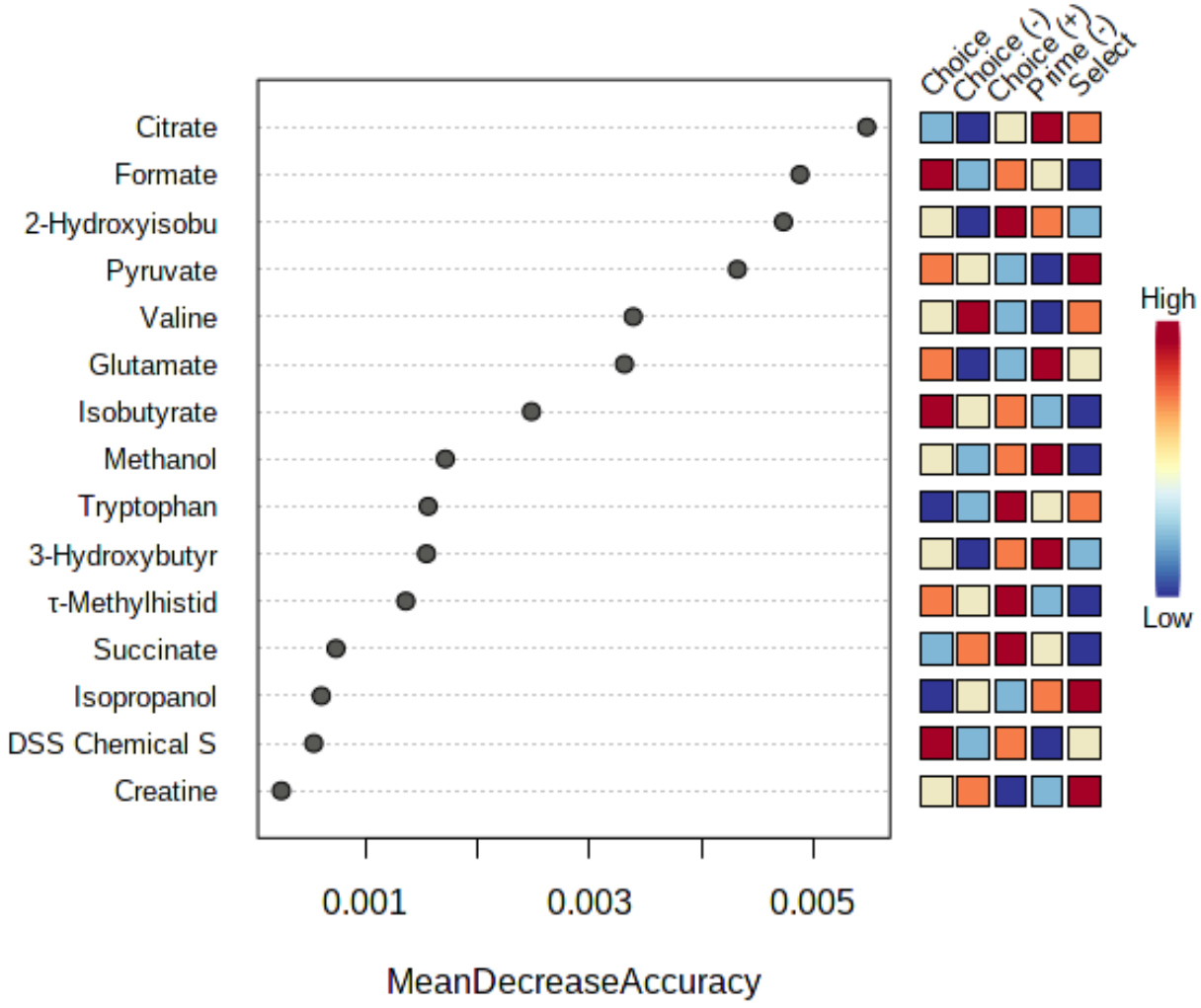

Figure 39. Significant Metabolites related to Quality Grade Classification identified by Mean Accuracy: Significant features were identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Rib Eye Area (cm^2)

Rib Eye Area (REA) was broken in five categories to give even number of samples in each category (70, 80, 90, 100 and 110 cm^2). The data normalization for REA is shown in Figure 40. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was used to identify features that discriminate between REA classes. Figure 41 shows the important features identified by ANOVA analysis, which found no significant features. PCA and PLSDA shown in Figure 42 and Figure 43 respectively did not show separation between REA classes. The PLSDA validation with the permutation shown in Figure 45 generated a *P*-value based on permutation of $P = 0.838$ (838/1000). This shows there no significant evidence of a difference between the classes in the model. Figure 44 looks at the number of components that would allow the best classifications. It indicates that there are no viable classifications based on metabolite features possible for REA. Figure 46 shows an ordered list of the most important features in discriminating between REA classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are isopropanol, betaine, succinate, anthranilate, and urea. No patterns were evident in which features had highest or lowest concentrations by REA classification. Figure 47 shows a similar analysis using a Random Forest approach. The two most important features identified using this method were betaine and anthranilate.

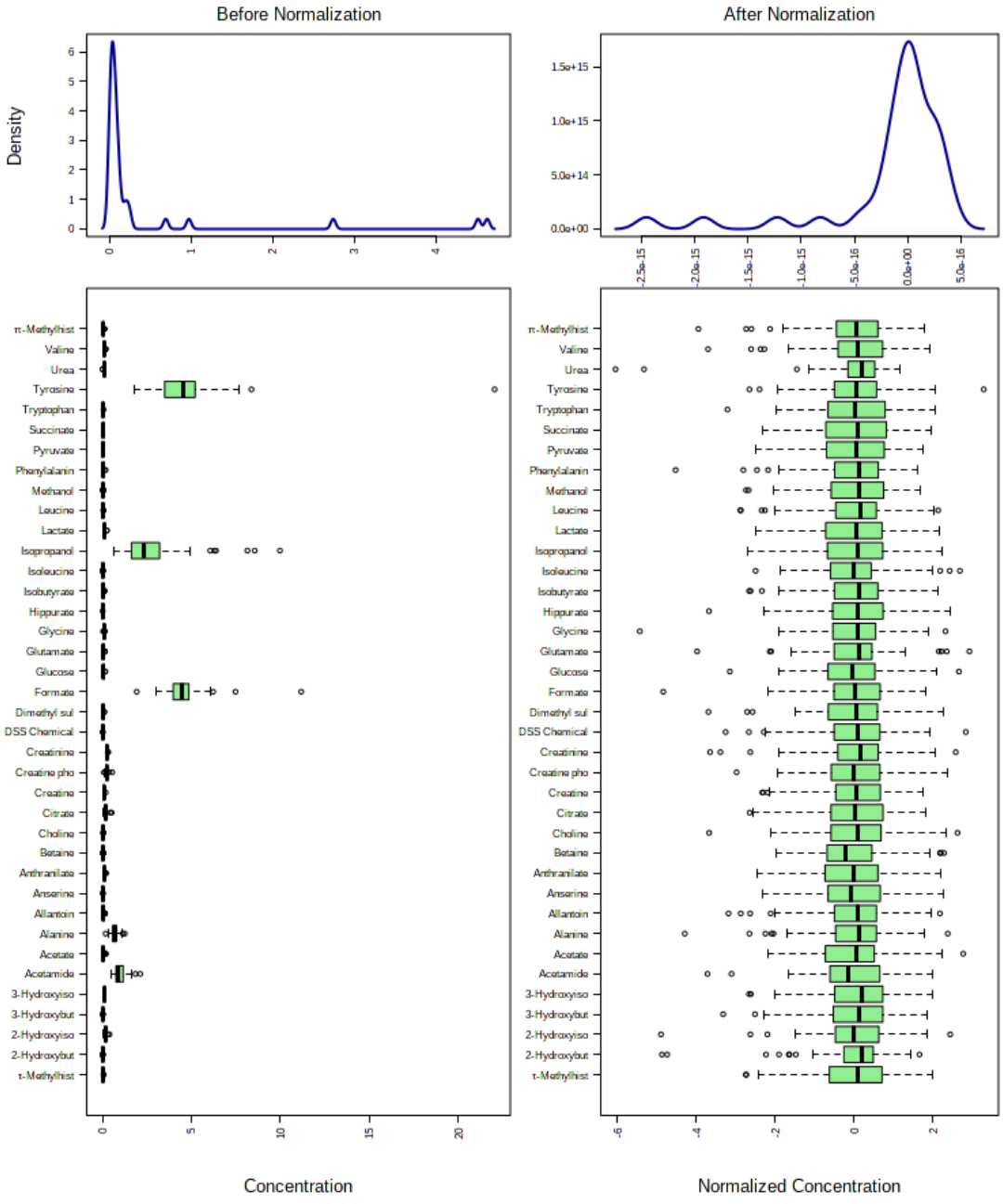

Figure 40. Rib Eye Area (cm^2) Date Before and After Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

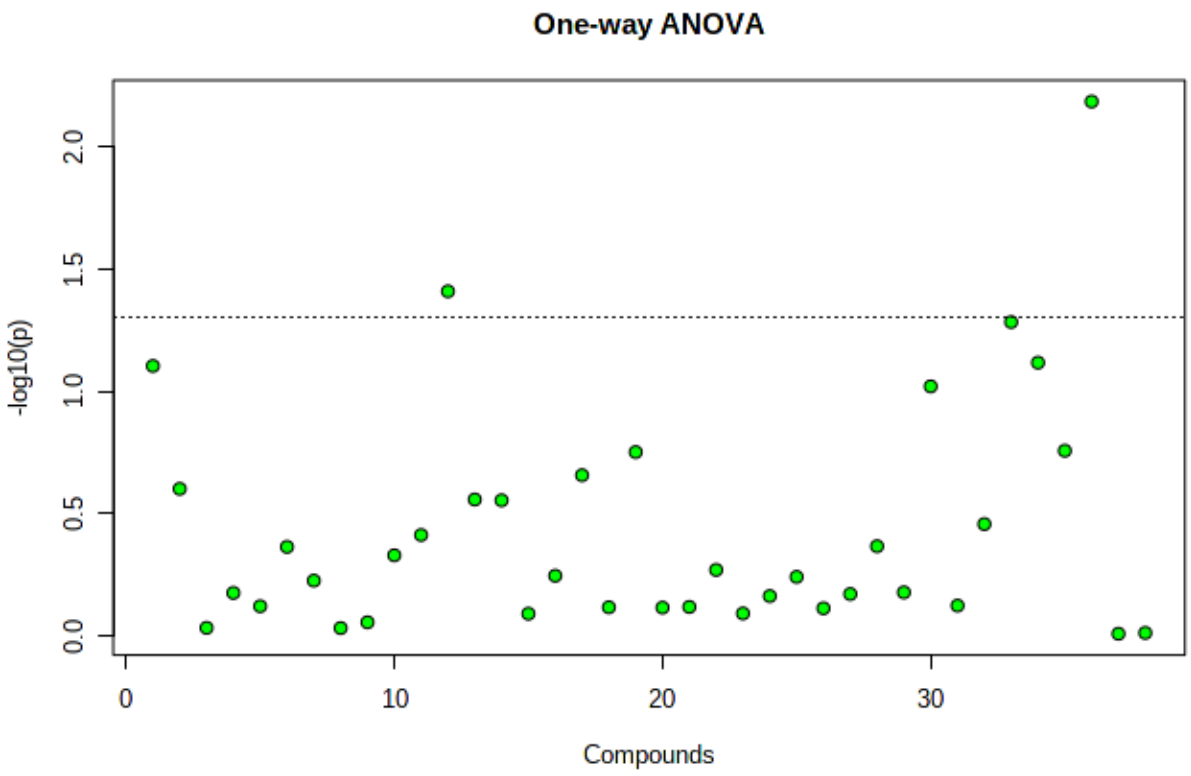

Figure 41. Significant features related to Rib Eye Area (cm^2) identified by Analysis of Variance: Important metabolites identified by ANOVA for rib eye area categories, *P* -value threshold 0.05. On the x-axis are the number of metabolites examined, the y-axis is the –log10 of the *P*-value which shows level of significance.

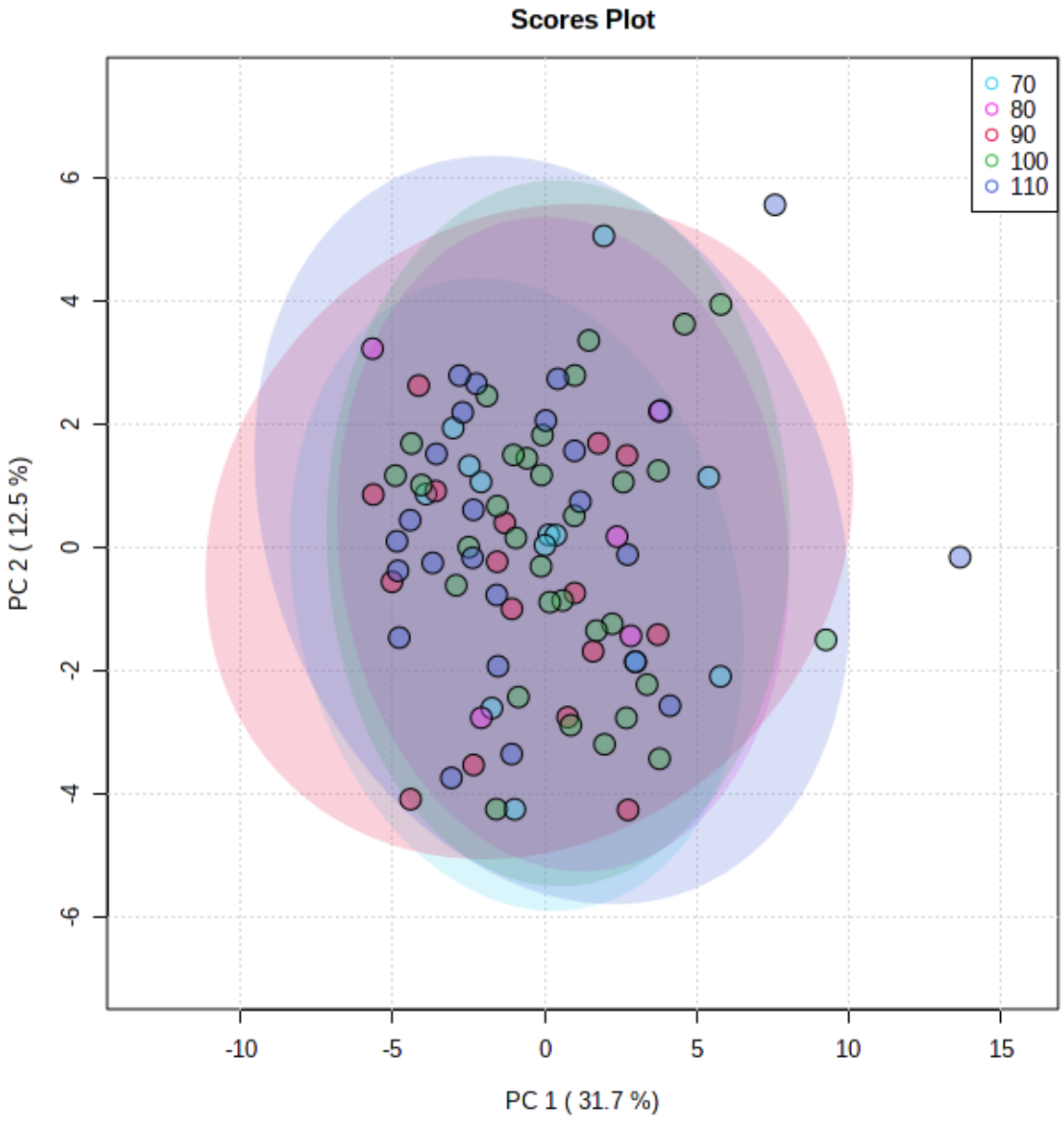

Figure 42. Significant features related to Rib Eye Area (cm^2) identified by PCA: Scores plot between the selected principal components or groups classifications for rib eye area. The variation explained by the first and second principal component is in parentheses.

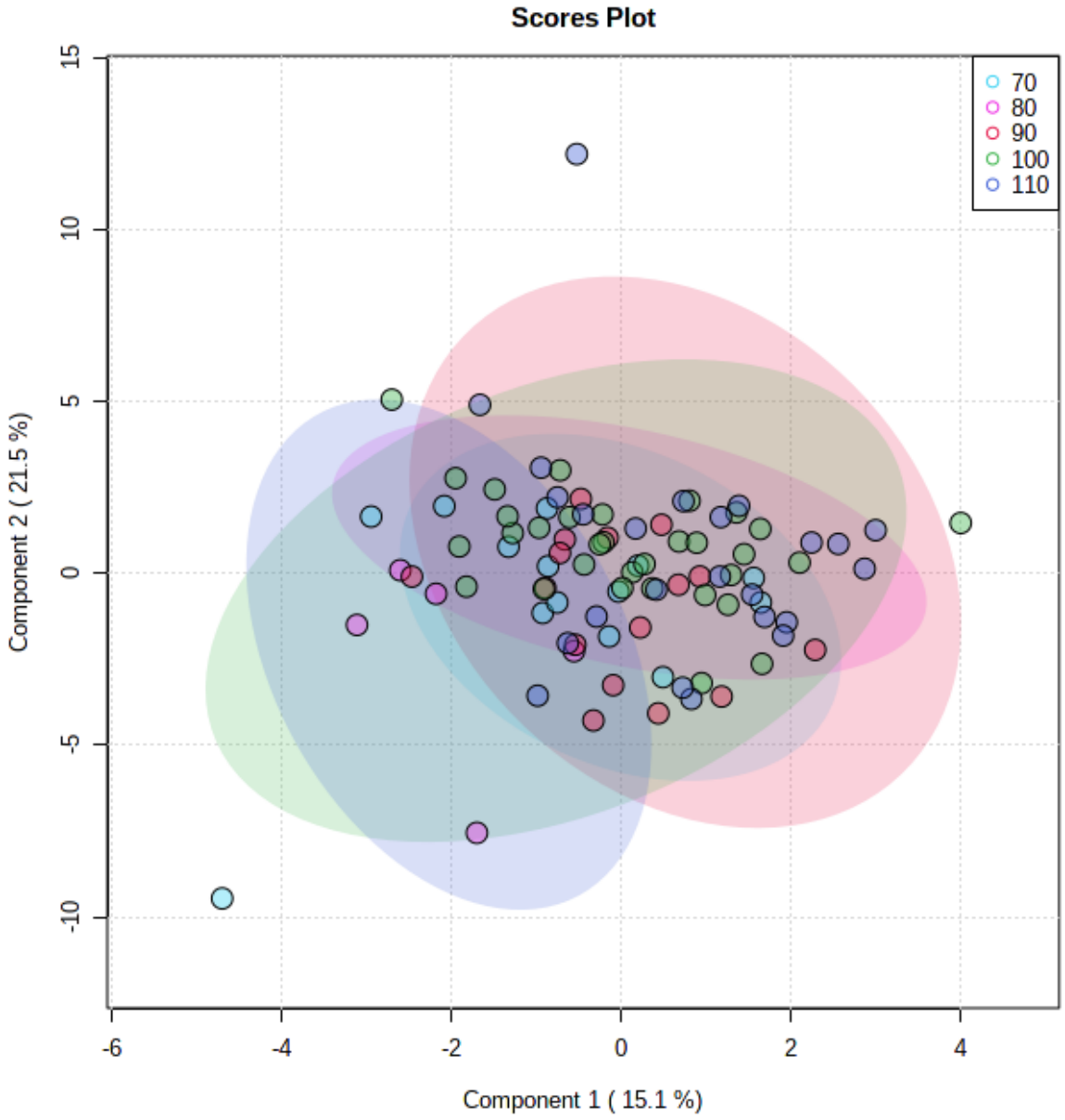

Figure 43. Significant features related to Rib Eye Area (cm^2) identified by PLS-DA: Scores plot for REA between principal components. The variance explained by the two principal components are shown in parentheses. In this image you can see slight separation between the REA classifications, but it is not significant.

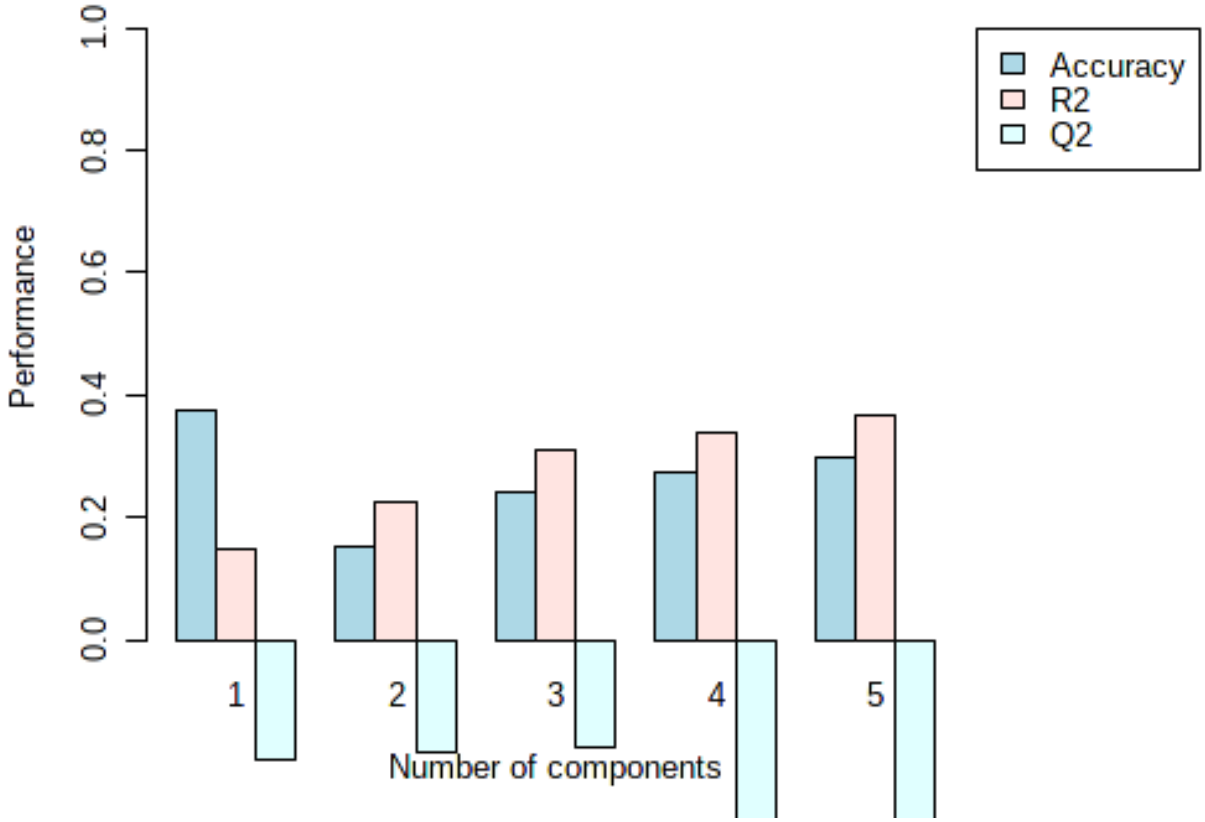

Figure 44. Significance related to REA (cm^2) identified by Components Test: PLS-DA classification using different numbers of components. The red star indicates the best classifier when present and the negative Q2 indicates a lack of significance.

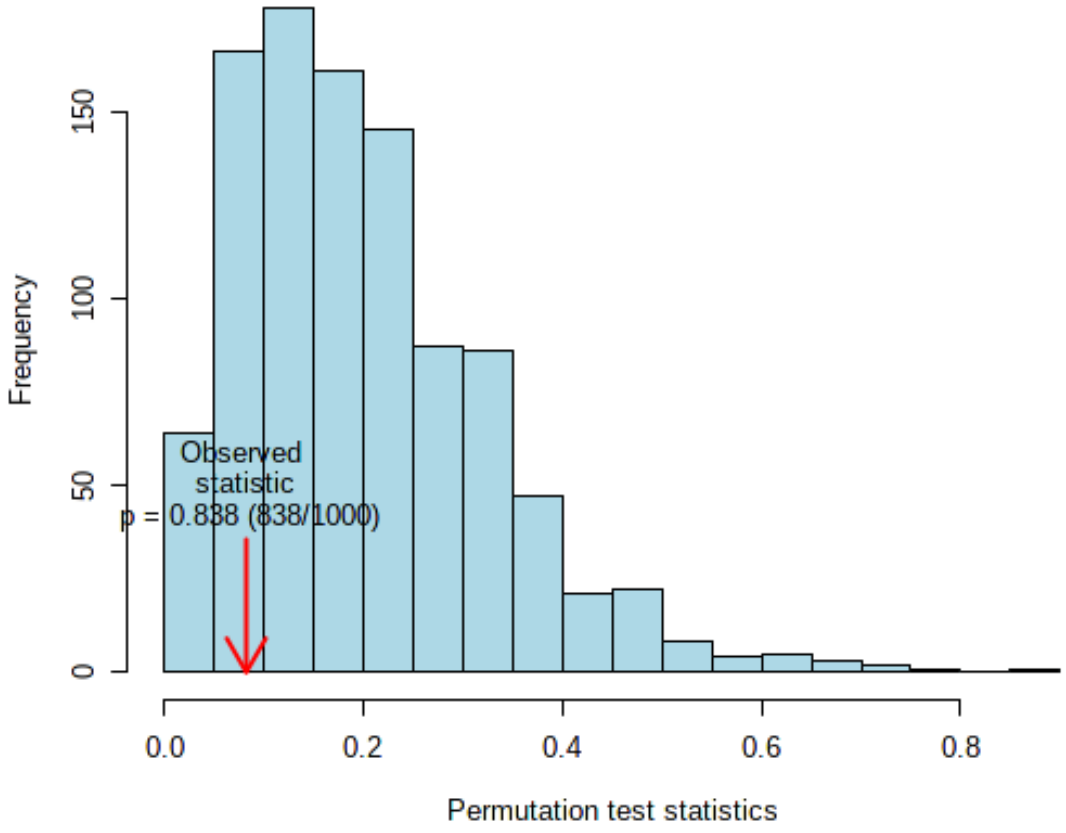

Figure 45. Significance related to REA (cm^2) identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P* value based on permutation is *P* = 0.838 (838/1000). The *P* value shows that there no significant evidence of a difference between classes.

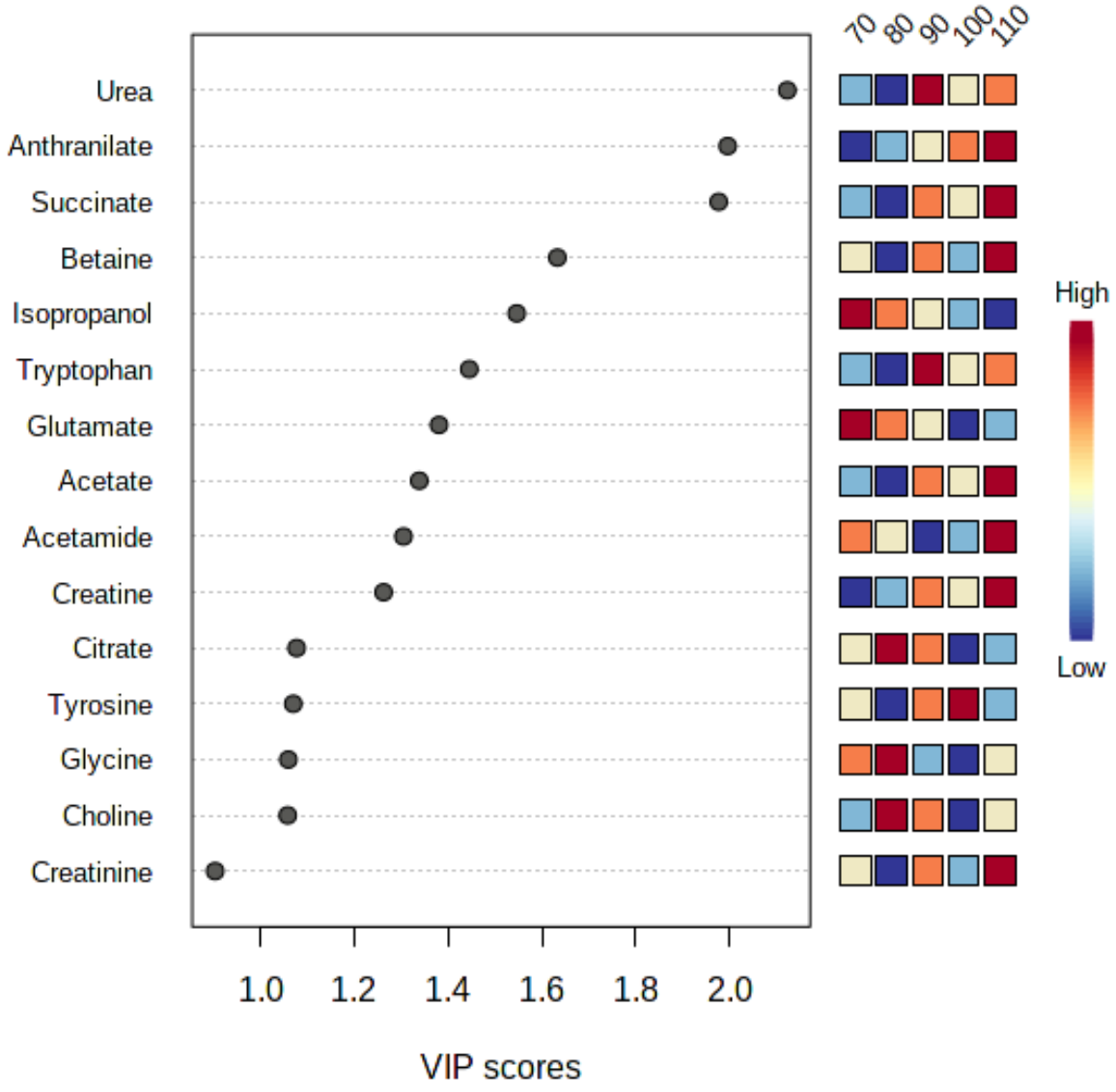

Figure 46. Significant Metabolites related to REA (cm^2) identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each classification.

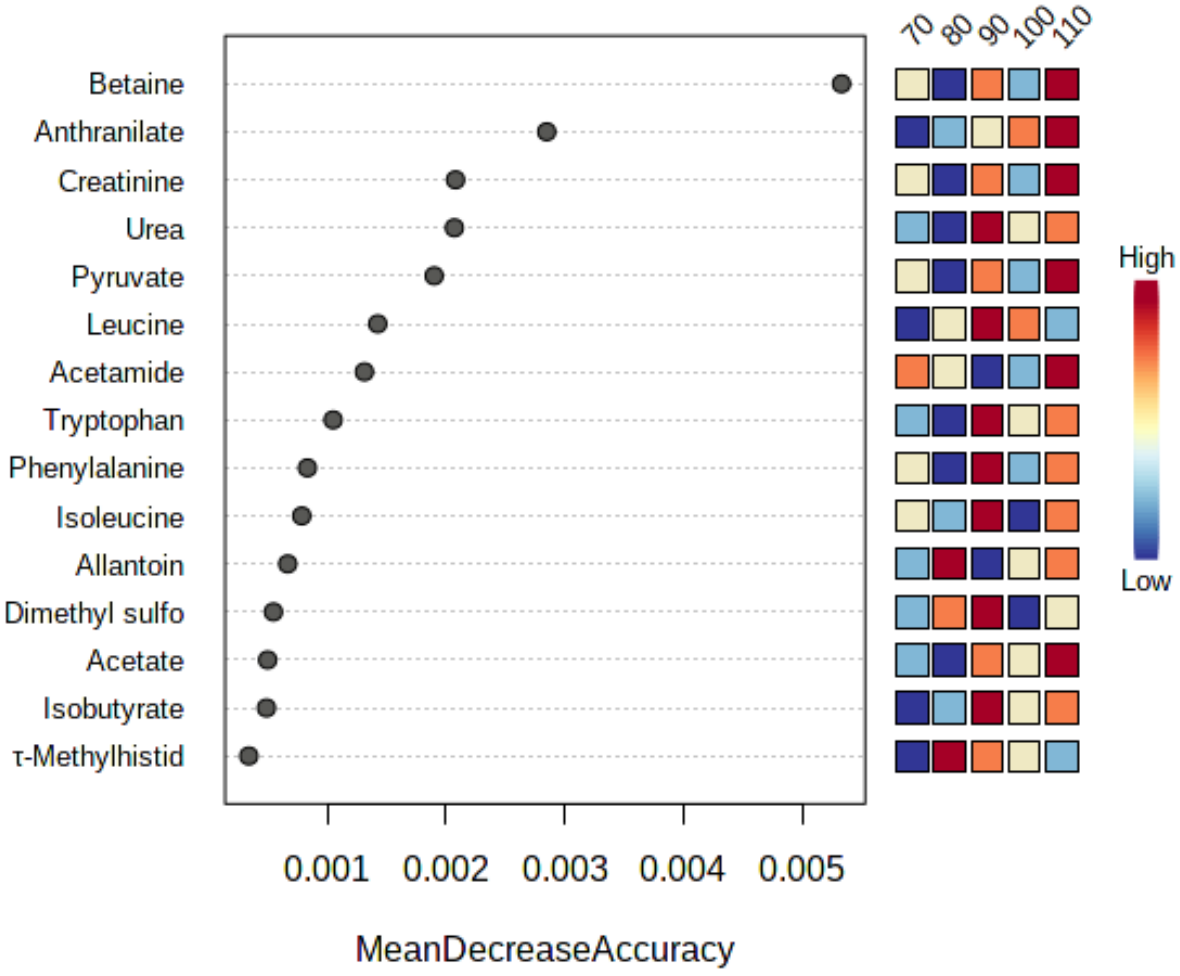

Figure 47. Significant Metabolites related to REA (cm^2) identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Exit Velocity (m/s)

Exit velocity was measured as the seconds to cross a specified distance once they left the chute. This value was then converted to meters per second. Animals were classified as fast if their exit velocity was higher than one standard deviation from the mean, medium if their exit velocity as within a standard deviation of the mean and slow if their exit velocity was lower than one standard deviation from the mean. The data normalization for exit velocity is shown in Figure 48. A one-way Analysis of Variance (ANOVA) was first used to identify features that discriminate between exit velocity classes. Figure 49 shows the important features identified by ANOVA analysis. Table 2 shows the details of these features. The post-hoc comparison column shows the comparisons between different classifications that are significant at the *P*-value threshold. The compounds identified are methanol, isopropanol, lactate, isobutyrate, and pyruvate ($P < 0.01$). The PCA and PLSDA are shown in Figure 50 and Figure 51 respectively, and show little separation between exit velocity classification groups. In the PLS-DA the slow group shows slight separation from the medium and fast classifications. The PLSDA validation with permutation shown in Figure 53 generated a p-value based on permutation of $P = 0.295$ (295/1000). This shows that the model does not differentiate exit velocity classes. Figure 52 looks at the number of components that would allow the best classifications. Figure 54 shows an ordered list of the most important features in discriminating between exit velocity classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are lactate, creatinine, 3-hydroxybutyrate, methanol, isobutyrate, and isopropanol. No patterns were evident in feature concentration for exit velocity classification. Figure 55 shows a similar analysis using a Random Forest approach. The three

most important features identified using this method were methanol, π-methylhistidine, and isopropanol.

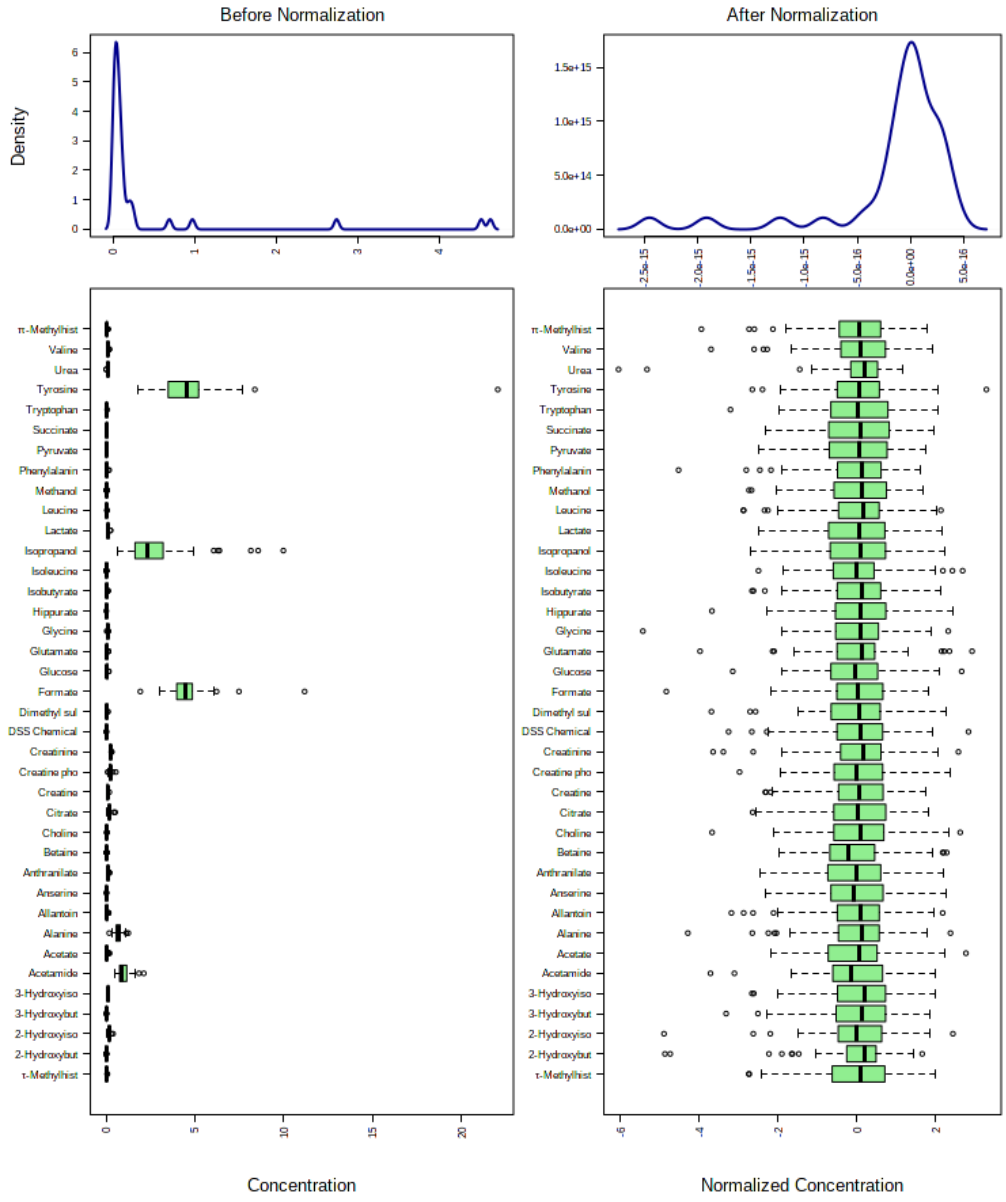

Figure 48. Exit Velocity (m/s) Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

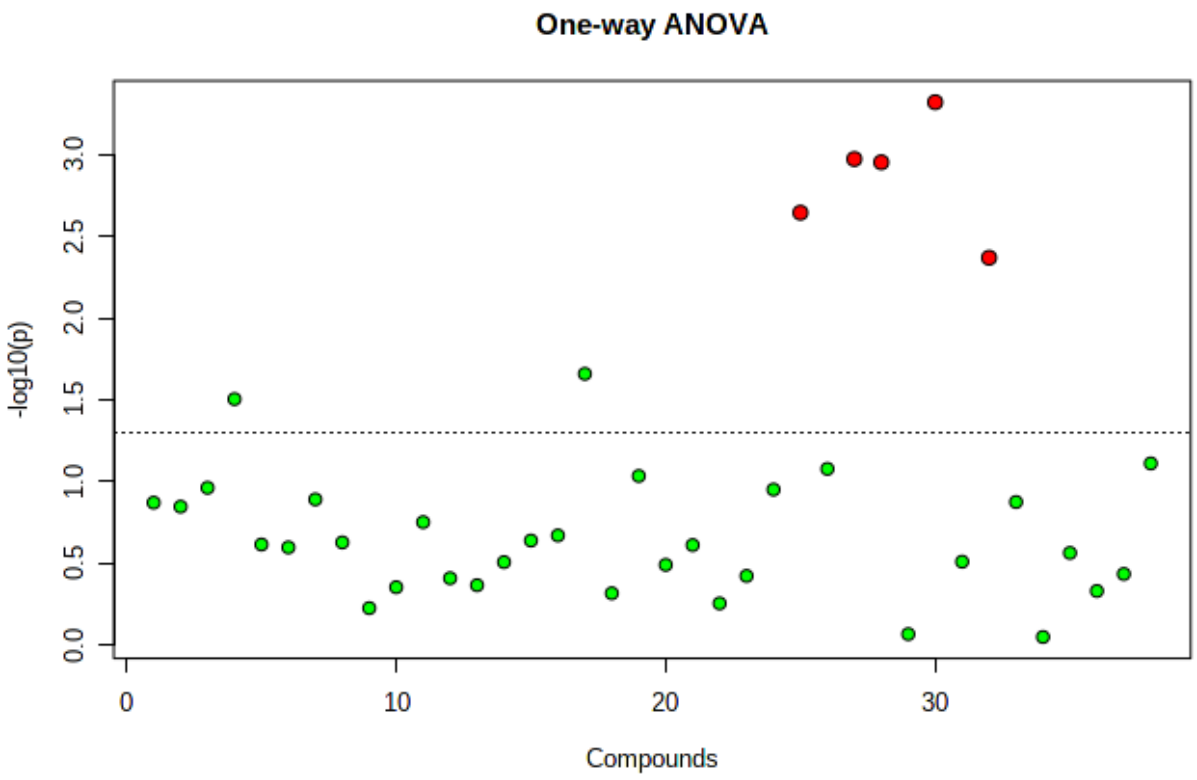

Figure 49. Significant Features related to Exit Velocity (m/s) identified by Analysis of Variance: Important metabolites identified by ANOVA for exit velocity (m/s) categories, p-value threshold 0.05. On the x-axis are the number of metabolites, the y-axis is the –log10 of the *P*-value which indicates significance of the metabolites. The reason for some metabolites shown as green even though they are at or above the level of significance is because they were corrected using the false discovery rate, which made them non-significant.

Table 2. Significant features related to Exit Velocity (m/s) identified by One-Way ANOVA and post-hoc analysis: Important Features identified are Methanol, Isopropanol, Lactate, Isobutyrate, and Pyruvate.

Compound	**F.value**	***P*value**	**$Log^{10}(P)$**	**FDR**	**Fisher's LSD**
Methanol	8.3518	0.0005	3.3211	0.0141	Fast - Slow; Medium – Slow
Isopropanol	7.4076	0.0011	2.9733	0.0141	Slow - Fast; Slow – Medium
Lactate	7.3543	0.0011	2.9535	0.0141	Fast - Slow; Medium – Slow
Isobutyrate	6.5316	0.0023	2.6449	0.0215	Fast - Slow; Medium – Slow
Pyruvate	5.8078	0.0043	2.3692	0.0325	Fast - Slow; Medium – Slow

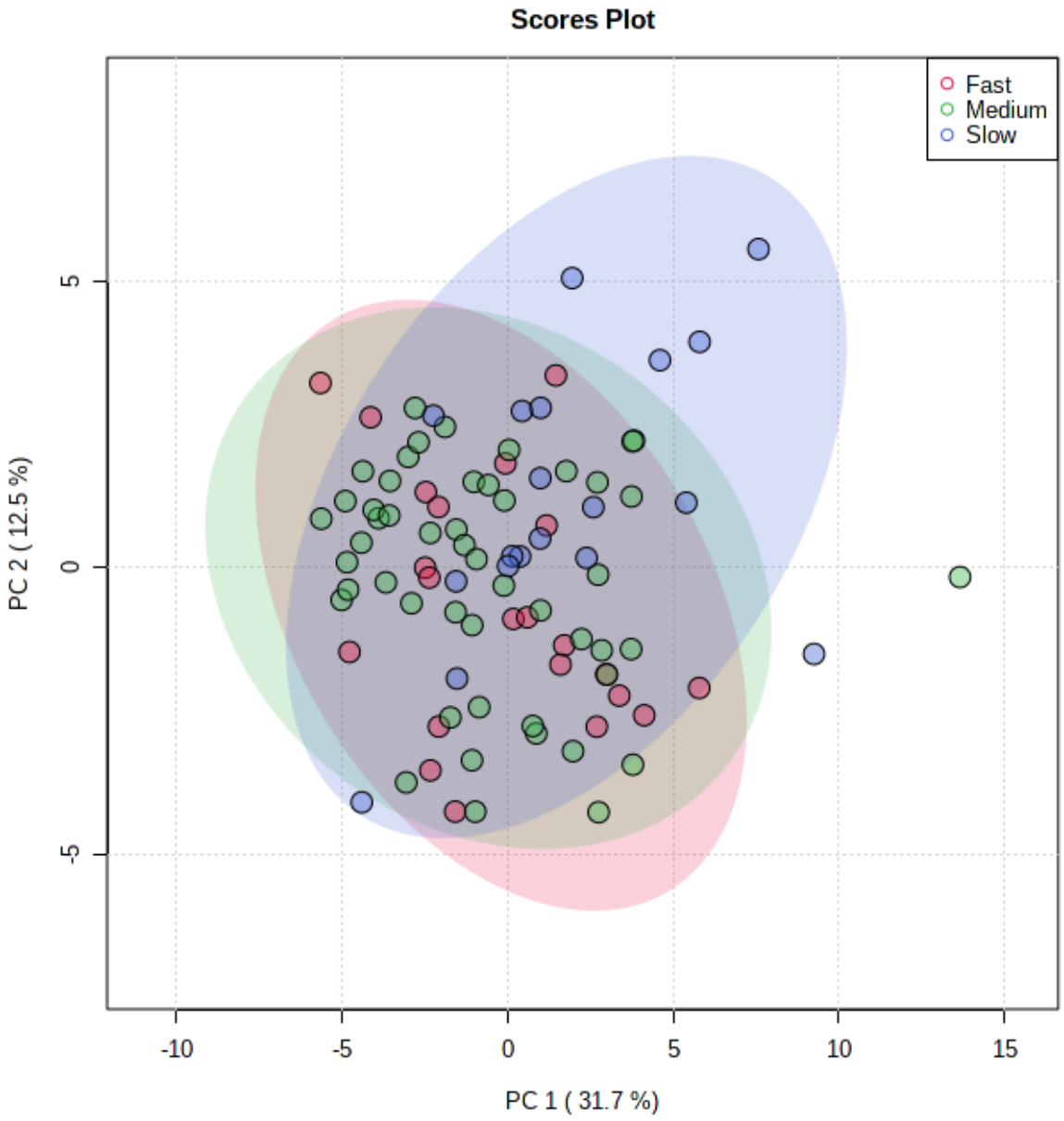

Figure 50. Significant features related to Exit Velocity (m/s) identified by PCA: Scores plot of the selected principal components between fast, medium and slow exit velocities (m/s). The variation explained by the first and second principal component is in parentheses.

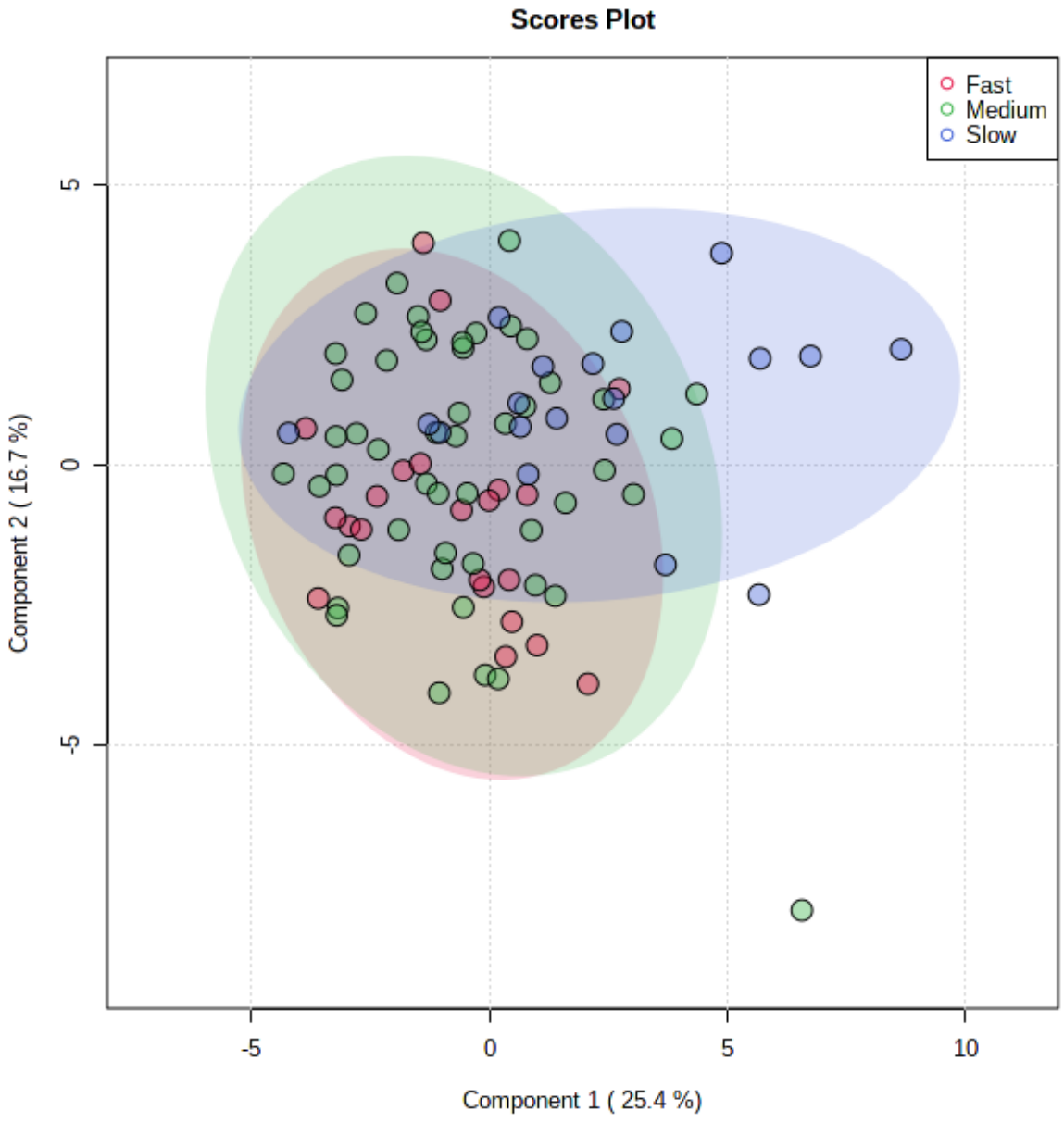

Figure 51. Significant features related to Exit Velocity (m/s) identified by PLS-DA: Scores plot for exit velocity (m/s) between the selected principal components, fast, medium, and slow. The variance explained by the two principal components are shown in parentheses. In this image you can see slight separation between the groups.

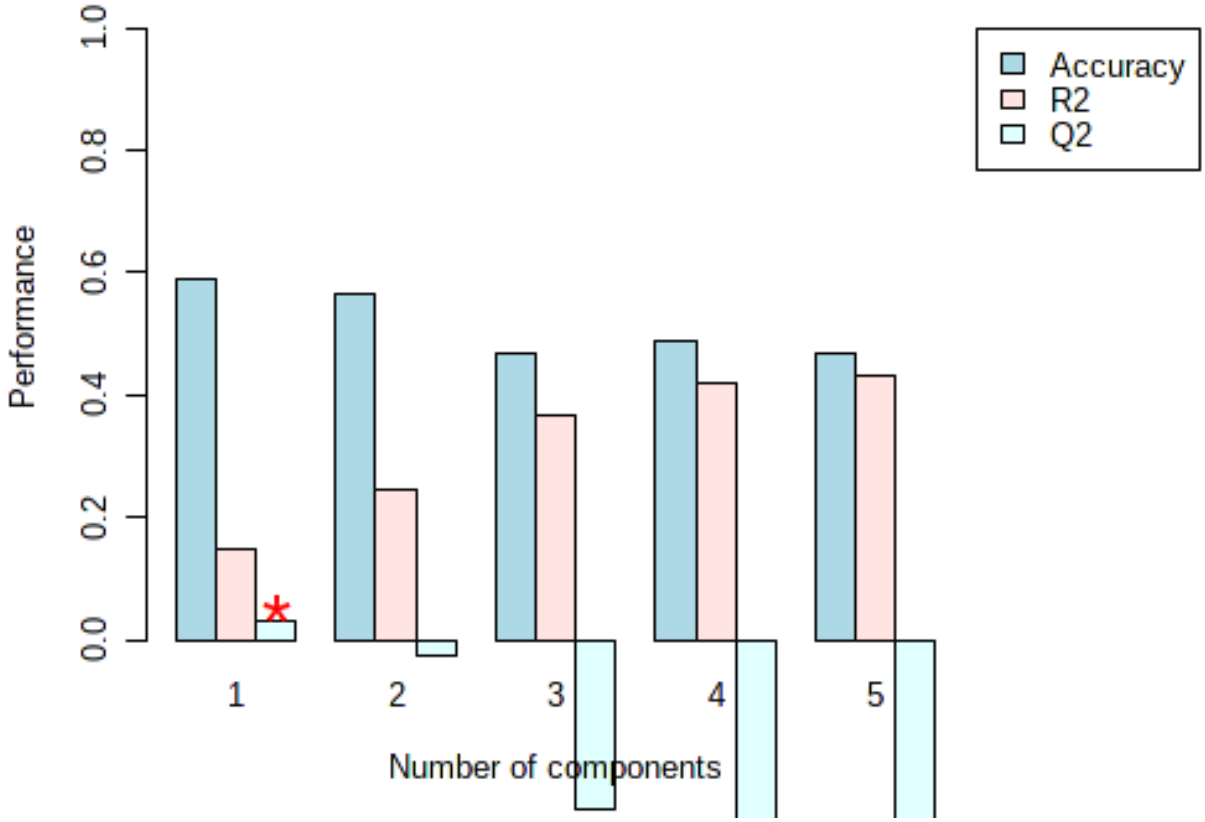

Figure 52. Significance related to Exit Velocity (m/s) identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier. The negative Q2 indicates little significance between exit velocity classes.

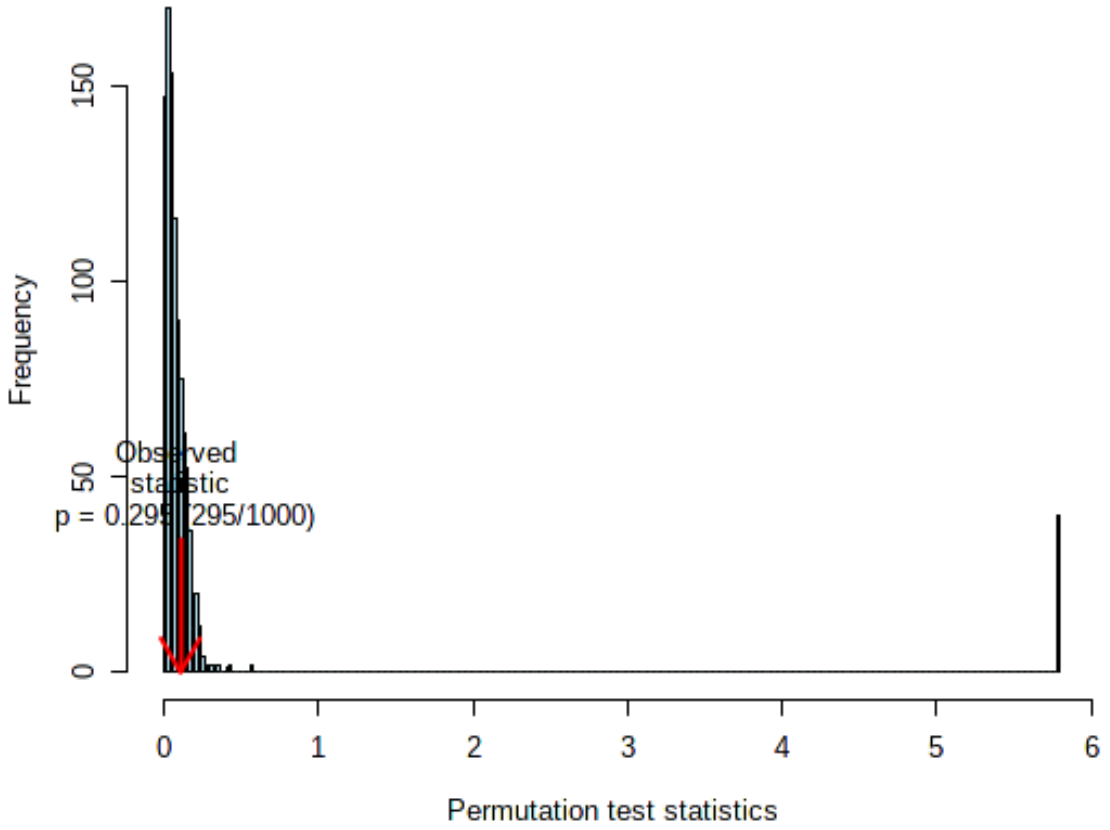

Figure 53. Significance related to Exit Velocity (m/s) identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is *P* = 0.244 (244/1000), meaning there no distinguishing between exit velocity classes in this model. The reason for the spike on the right hand low variation in this trait.

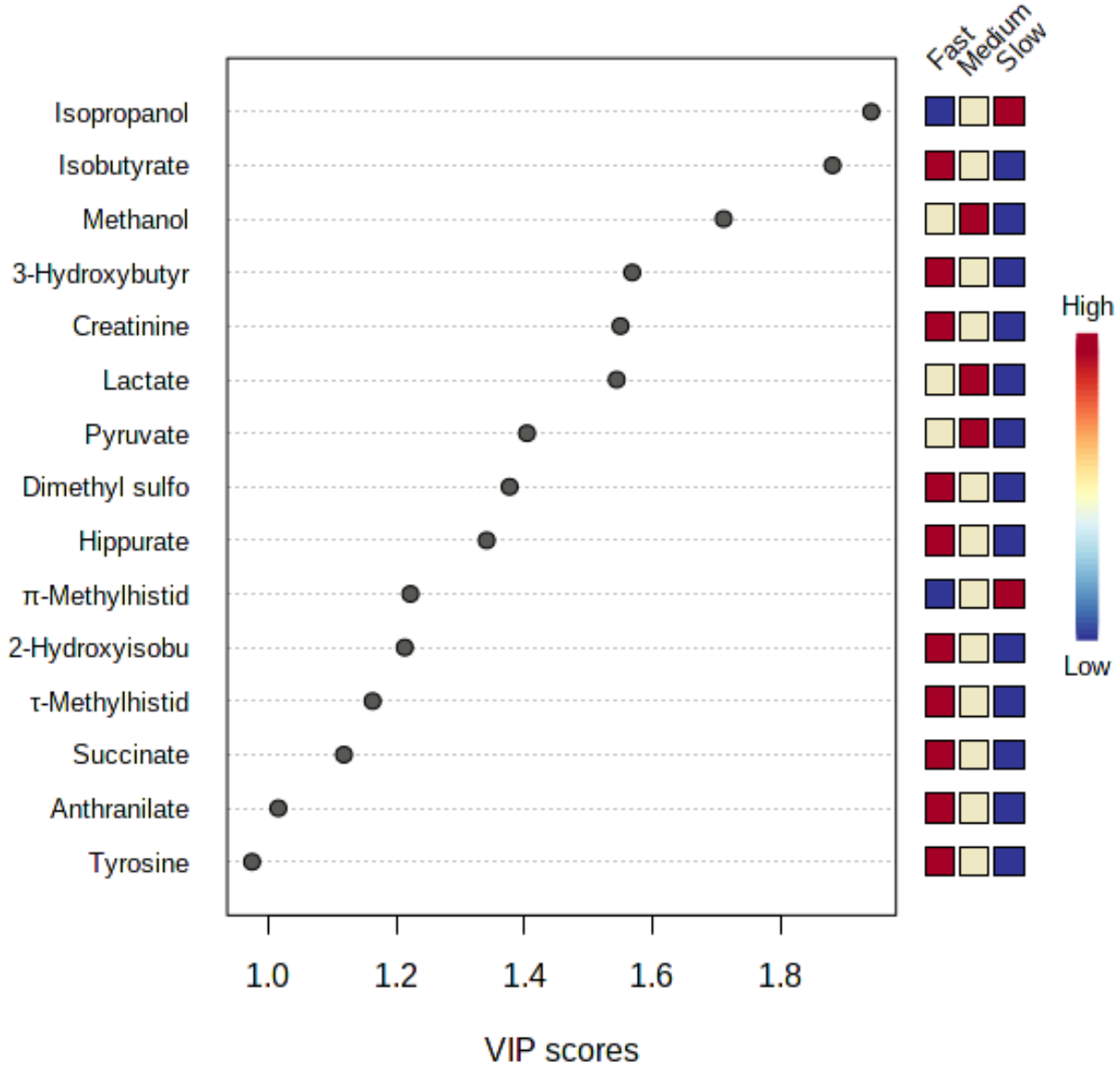

Figure 54. Significant Metabolites related to Exit Velocity (m/s) identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

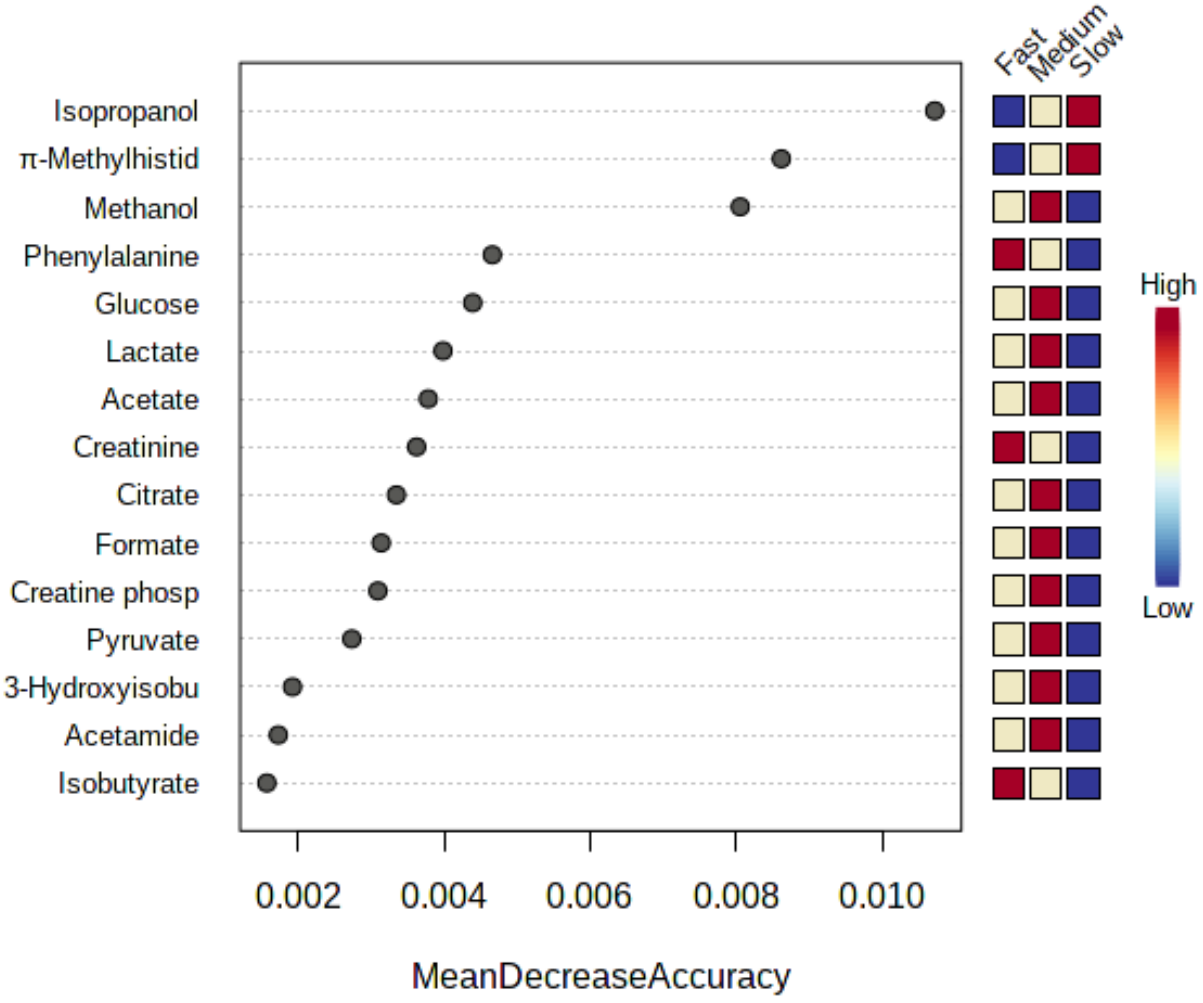

Figure 55. Significant Metabolites related to Exit Velocity (m/s) identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in exit velocity classification.

Temperament Scores (Chute Scores)

One of the traits observed in this study is a measurement of temperament (chute scores). Temperament of the animal based on chute score was assigned by a single observer. The data normalization for temperament scores is shown in Figure 56. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was first used discriminate between temperament scores. Figure 57 shows the important features identified by ANOVA analysis. Table 3 shows the details of these features. The post-hoc comparison column shows the comparisons between different levels that are significant given the *P*-value threshold. All seven significant features identified for temperament scores were significant at $P < 0.01$. PCA and PLSDA are shown in Figure 58 and 59 respectively and show little separation between chute score classification groups. The PLSDA validation with permutation shown in Figure 61 generated a *P*-value based on permutation of $P = 0.556$ (556/1000), therefore, the model cannot differentiate classification of chute score accurately. Figure 60 shows the components that would allow the best classifications. Figure 62 shows an ordered list of the most important features in discriminating between chute score classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important for this analysis. The features above this threshold are succinate, dimethyl sulfone, 3-hydroxybutyrate, hippurate, and methanol. Similar patterns in metabolite concentrations were seen in high chute score animals having the lowest concentrations. However, the medium chute score animals had the highest concentrations in many of the observed metabolites with high VIP scores. Figure 63 shows a similar analysis using a Random Forest approach. The two most important features identified using this method were urea and dimethyl sulfoxide.

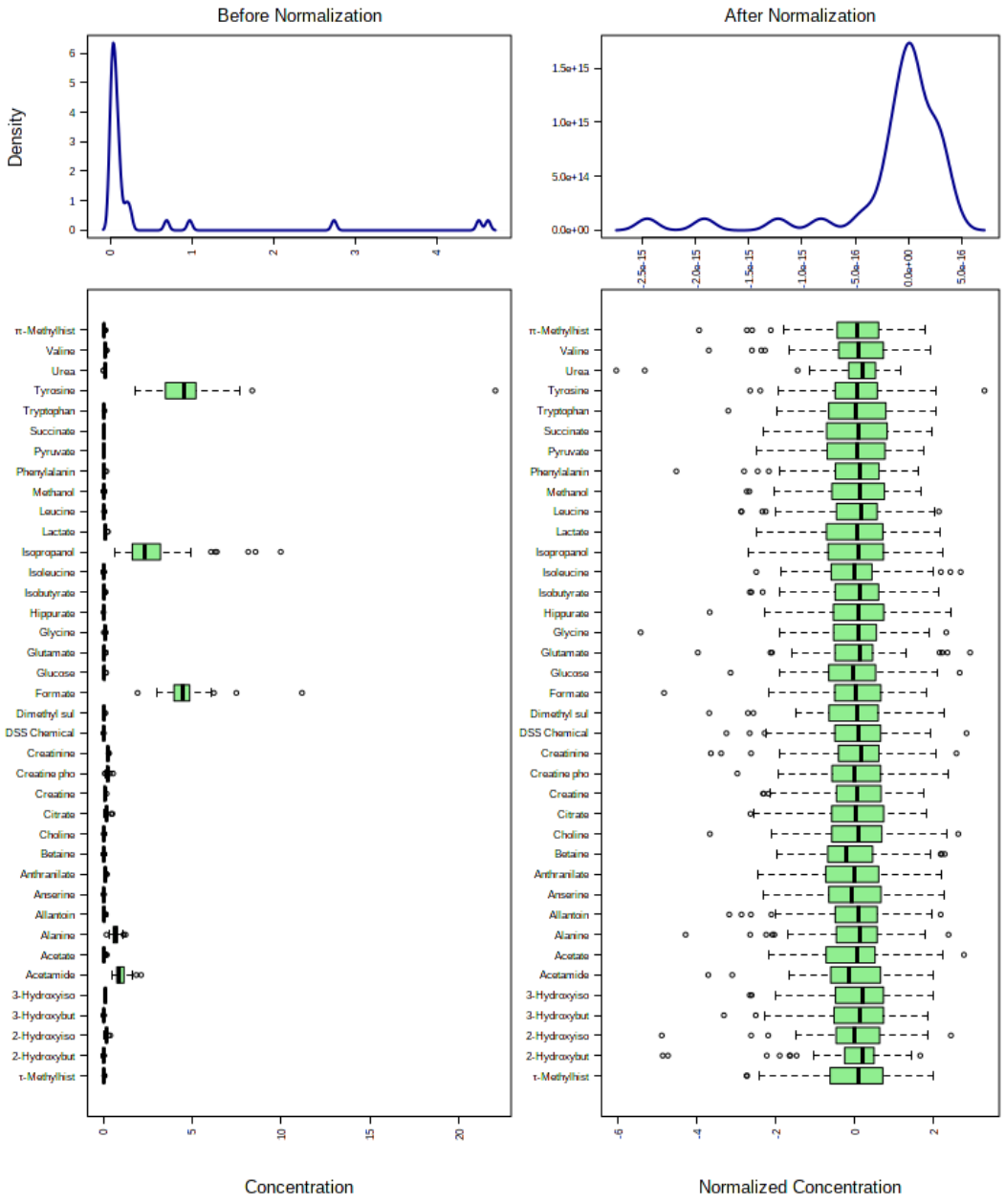

Figure 56. Temperament Scores (Chute Scores) Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

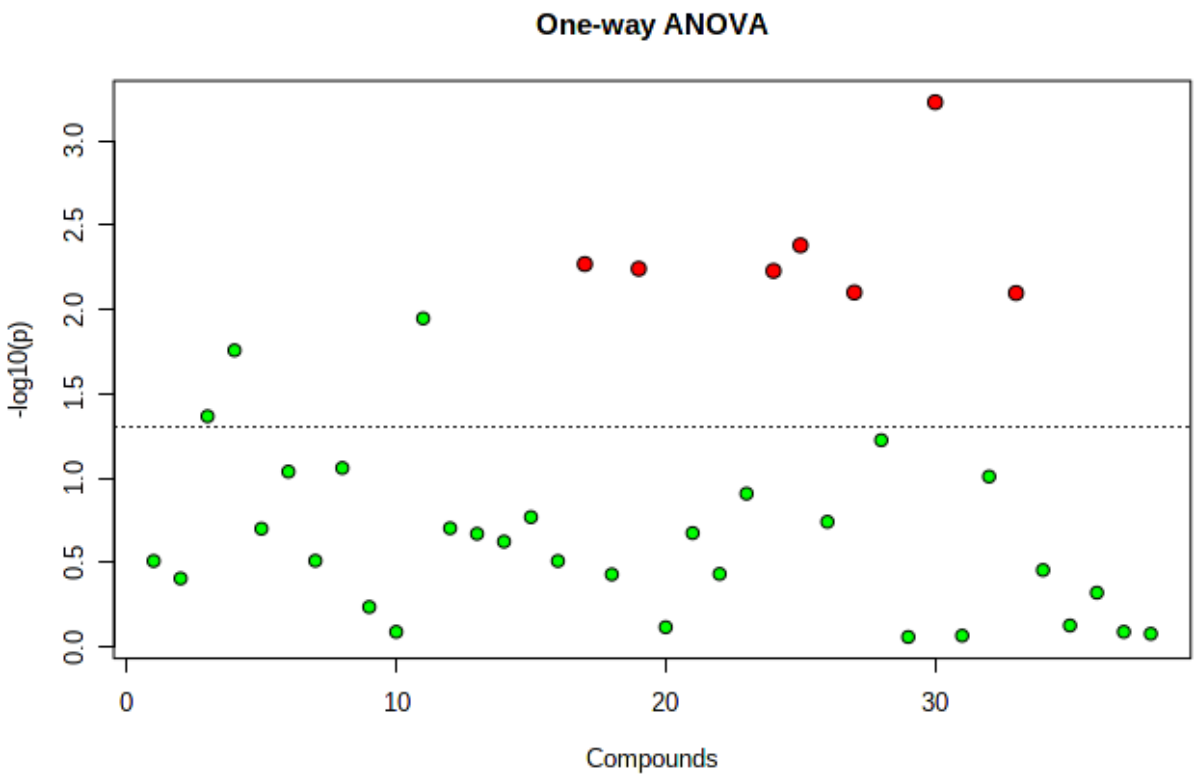

Figure 57. Significant features related to Temperament Scores (Chute Scores) Identified by Analysis of Variance: Important metabolites identified by ANOVA for temperament scores categories, p-value threshold 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10(p) which is the adjustment made to find the level of significance of the metabolites. The reason for some metabolites shown as green even though they are at or above the level of significance is due to FDR correction.

Table 3. Significant features related to Temperament Scores (Chute Score) identified by One-Way ANOVA and post-hoc analysis: The important metabolites identified are methanol, Isobutyrate, creatine, dimethyl-sulfone, Hippurate, isopropanol, and succinate.

Compound	**F.value**	***P*-value**	**-LOG^{10}(*P*)**	**FDR**	**Fisher's LSD**
Methanol	8.095	0.0005	3.227	0.0225	Low - High; Medium - High
Isobutyrate	5.833	0.0042	2.379	0.0435	Low - High; Medium – High
Creatinine	5.545	0.0054	2.268	0.0435	Low – High
Dimethyl sulfone	5.473	0.0058	2.240	0.0435	Low - High; Medium – High
Hippurate	5.440	0.0059	2.228	0.0435	Low - High; Medium - High
Isopropanol	5.110	0.0080	2.100	0.0435	High -Low
Succinate	5.101	0.0080	2.096	0.0435	Low - High; Medium – High

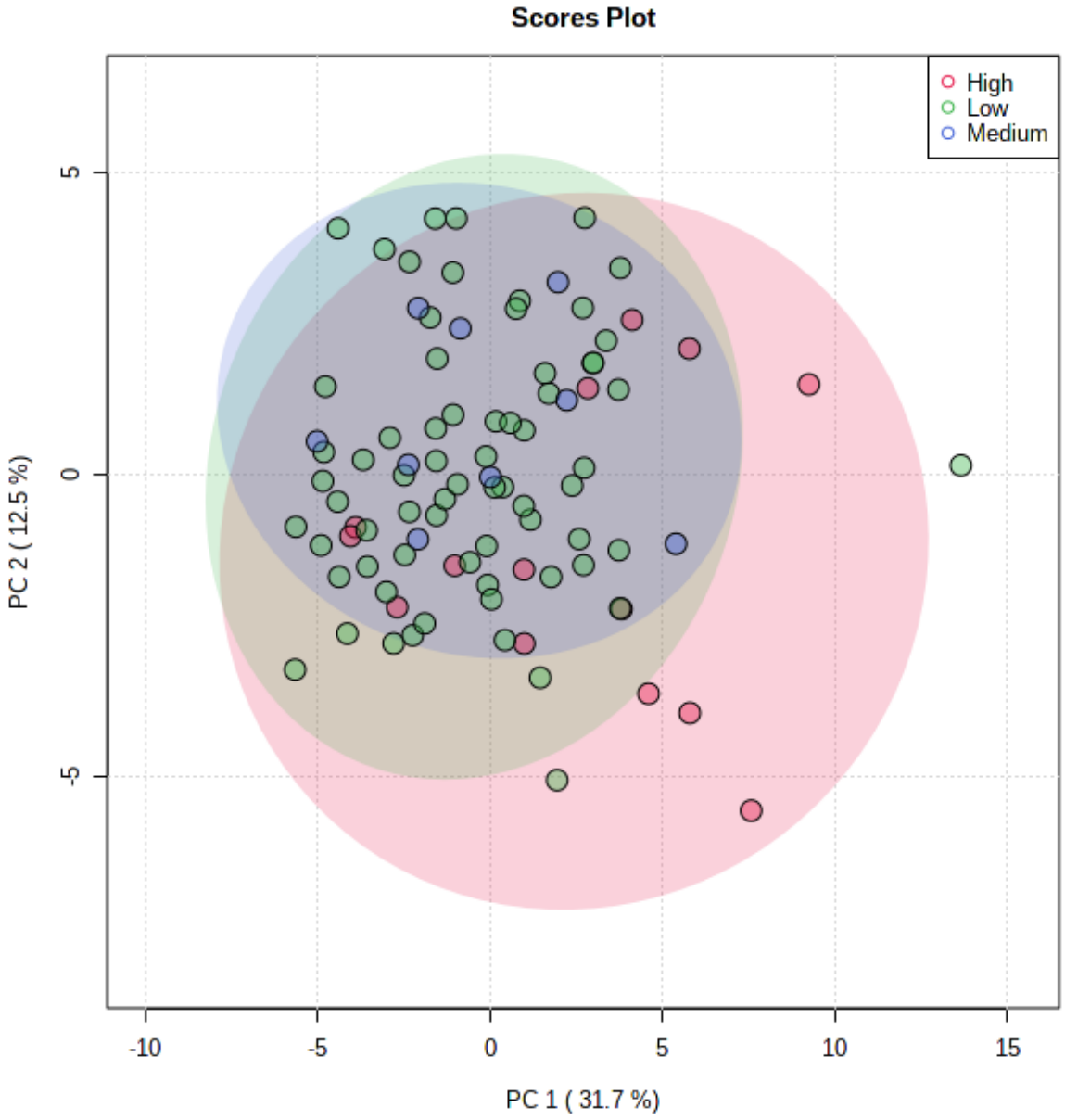

Figure 58. Significant features related to Temperament Score (Chute Score) identified by PCA: Scores plot between the selected principal components or group classifications for chute scores. The variation explained by the first and second principal component is in parentheses.

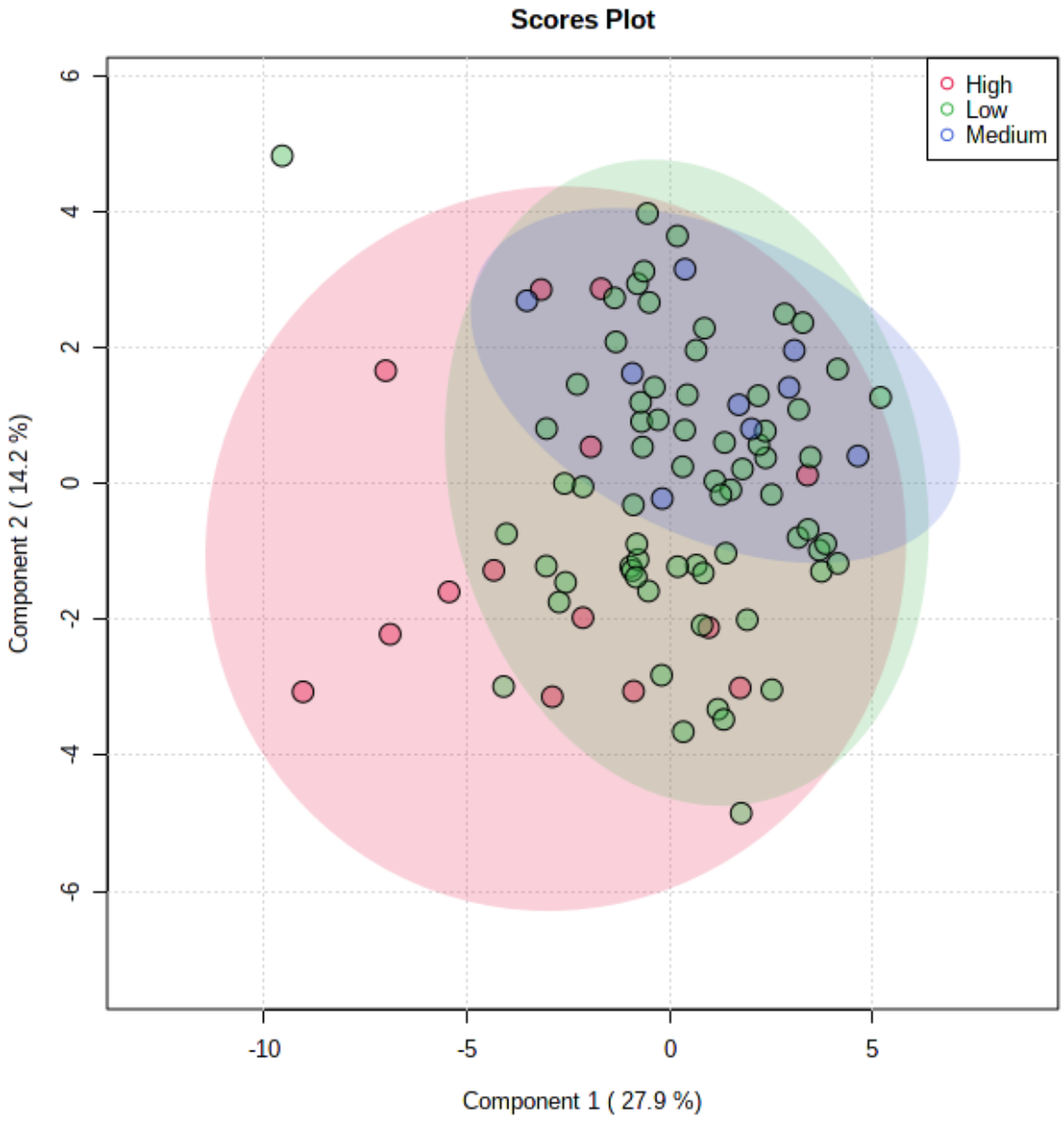

Figure 59. Significant features related to Temperament Score (Chute Score) identified by PLS-DA: Scores plot for temperament scores between the selected principal components. The variance explained by the two principal components are shown in parentheses. In the image above you can see slight separation between temperament groups.

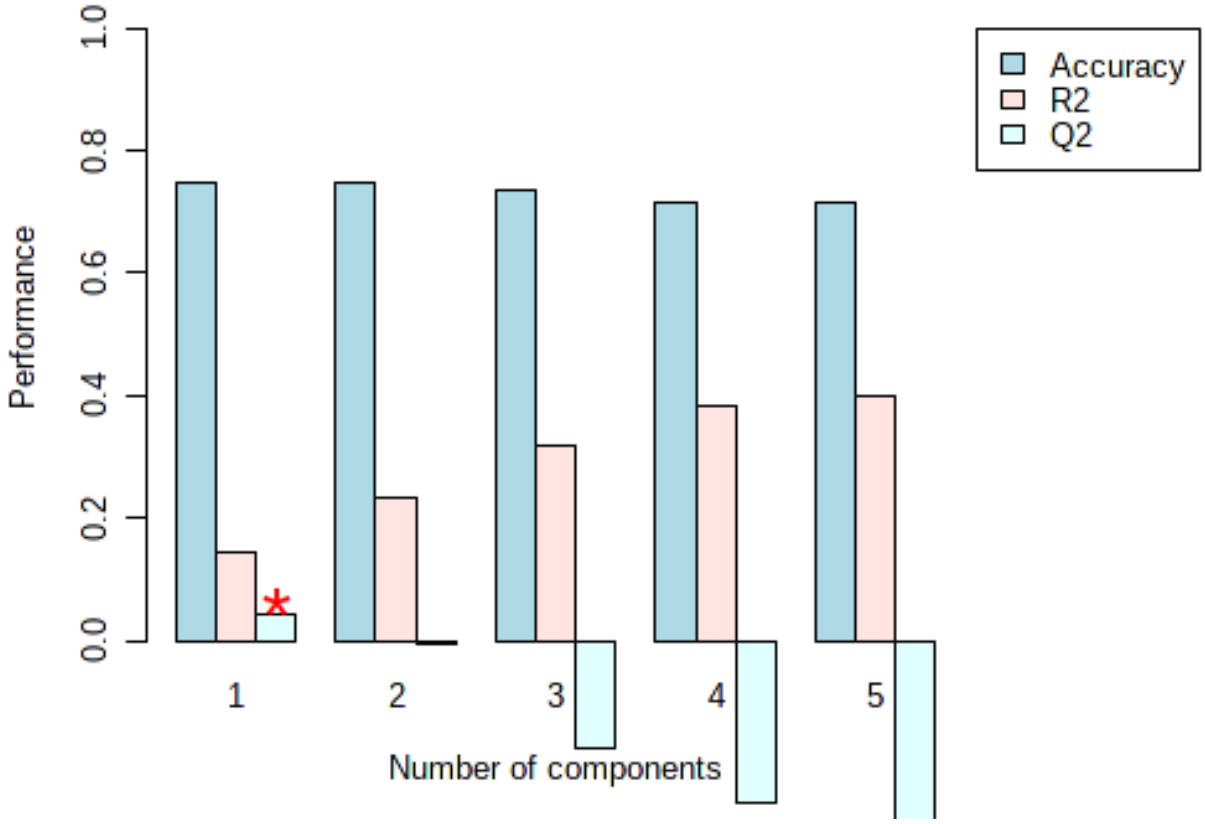

Figure 60. Significance related to Temperament Score (chute scores) identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier, the negative Q2 indicates a lack of significance.

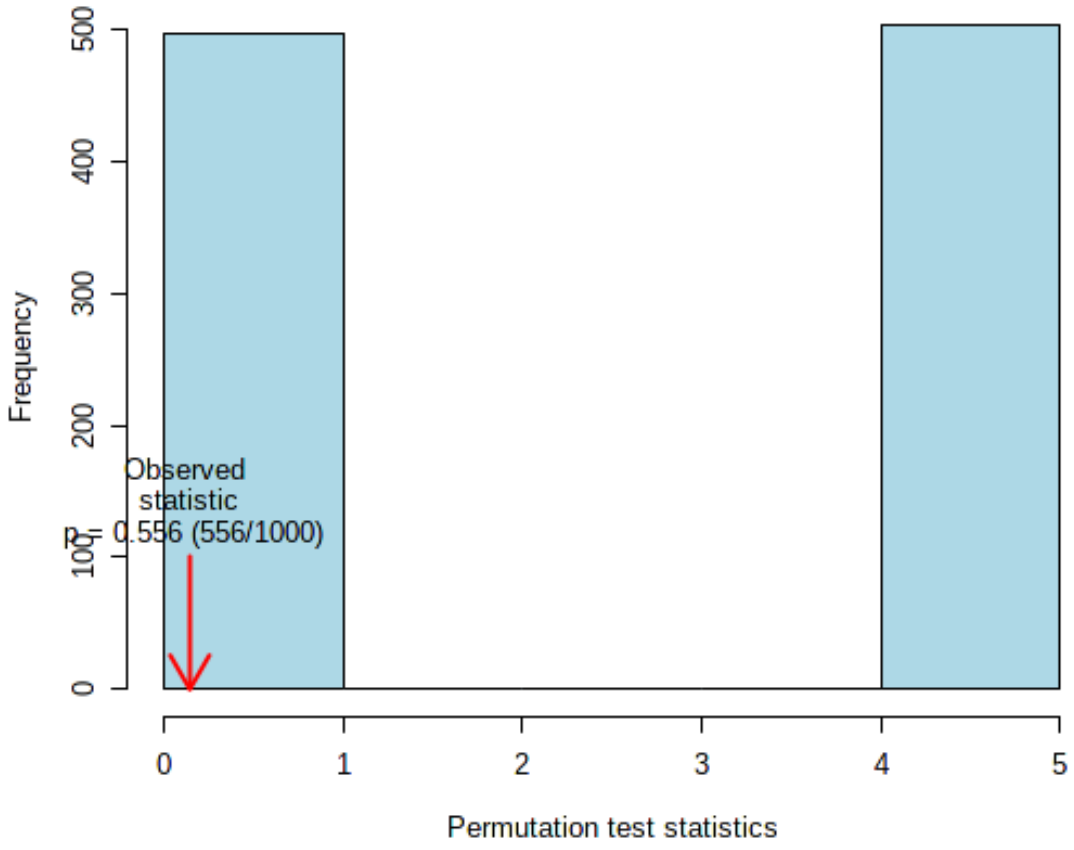

Figure 61. Significance related Temperament Score (Chute score) identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is $P = 0.556$ (566/1000). The *P*-value shows that the model cannot predict differences based on chute score classification.

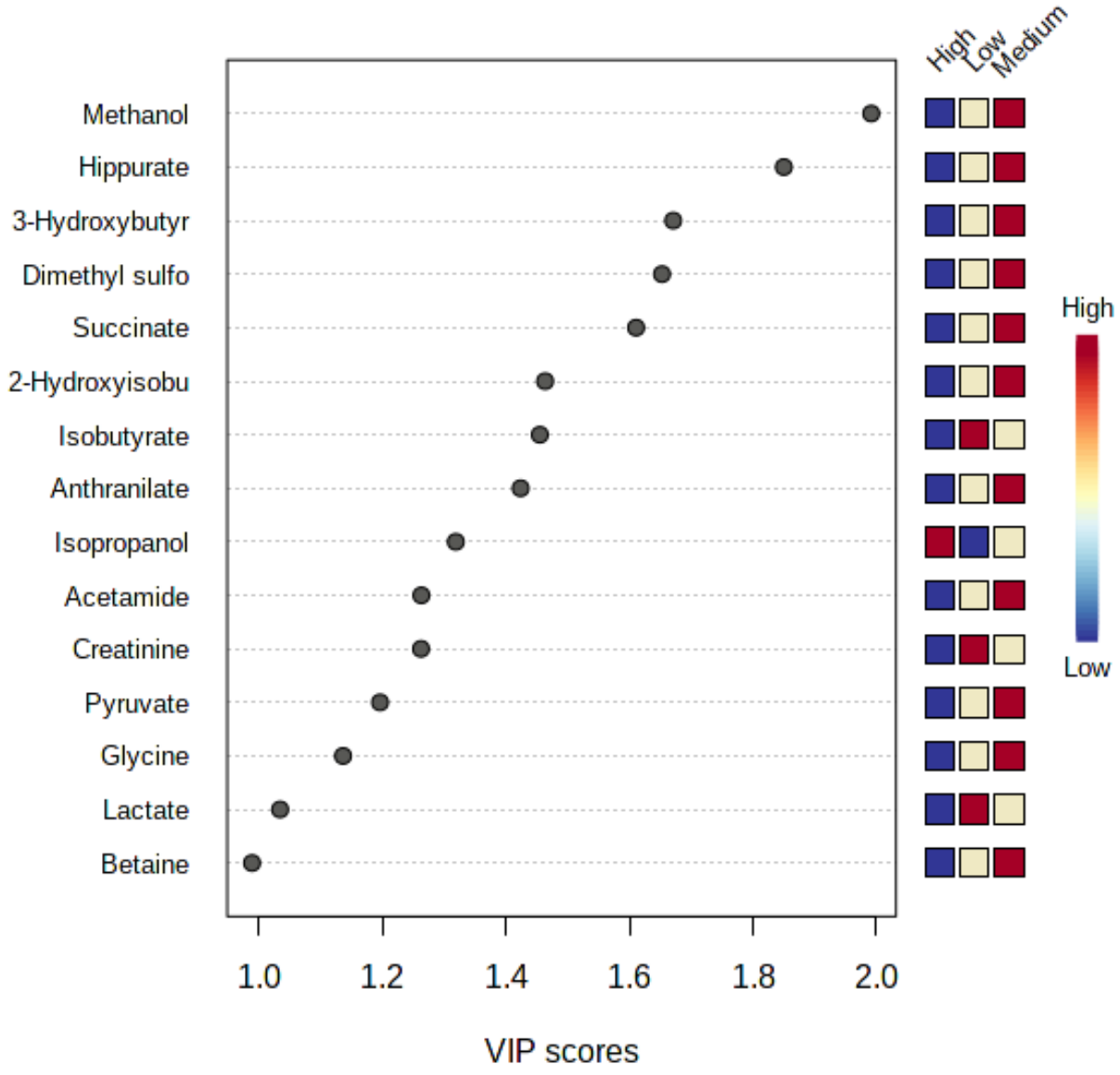

Figure 62. Significant Metabolites related to Temperament Score (Chute Score) identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each chute score class..

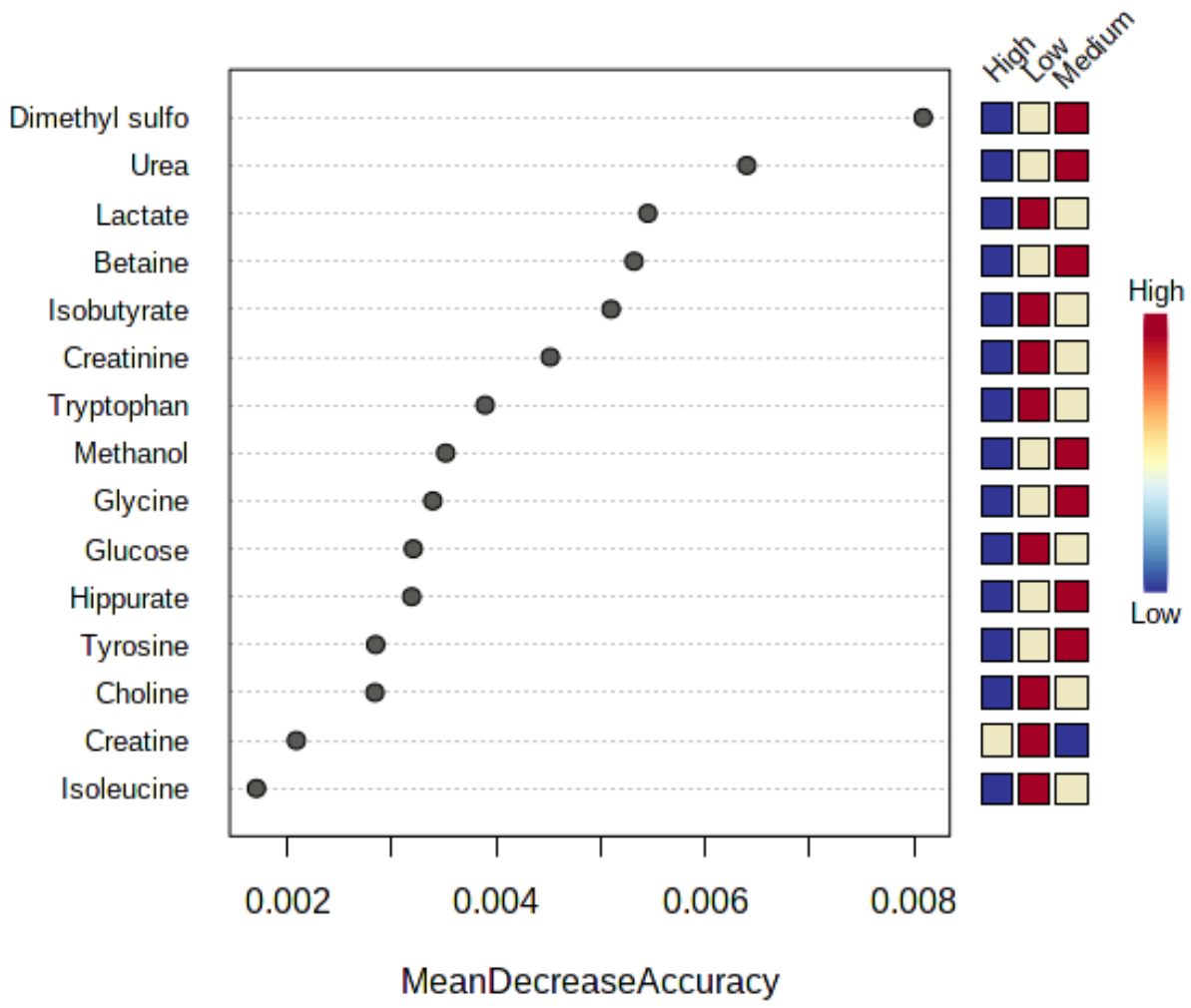

Figure 63. Significant Metabolites related to Temperament Score (chute score) identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy.

Discussion

The ability of an NMR metabolite profile to predict feedlot performance traits was examined. One of the traits that differed in metabolite concentrations was back fat thickness or subcutaneous fat. This was unexpected as serum metabolites were measured at entry into the feedlot and back fat thickness was measured at harvest. The ANOVA for back fat showed that there were two metabolites, urea and 2-hydroxyisobutyrate, that were significantly different $P < 0.05$. Urea is an important metabolite that is used in protein deposition and utilization of energy. The difference in urea concentration at entry into the feedlot could reflect differences in energy utilization or in the efficiency of urea recycling which could spare energy for storage. This would be reflected in increased fat deposition at harvest. The second metabolite, 2 hydroxyisobutyrate could reflect alterations in rumen microbial fermentation or production of microbial protein which could also impact efficiency during the feedlot period. However, the observations for this metabolite were not consistent with fatness levels at harvest and may not be biologically relevant. In contrast, Consolo et al., 2020 found glutamine, betaine, creatinine, and isoleucine were important metabolites associated with muscle protein and fat deposition amongst rapidly maturing and slow maturing cattle. Further, the PLS-DA (Figure 11) was unable to differentiate between back fat classifications. The VIP scores for backfat classifications identified Hippurate, Creatinine, Tyrosine, and 2-Hydroxyisobutrate as metabolites contributing to differences between classes. Both Hippurate and creatinine are related to amino acid supply and metabolism and could reflect more efficient protein uptake or deposition. Additionally, Consolo et al., 2020 also identified creatinine in association with protein and fat deposition in feedlot cattle. There is potential application for this finding if metabolites can be identified early in the feeding period

related to energy utilization and fat deposition as this could lead to precision management to limit inputs and increase consistency in feedlot cattle endpoints.

A trend for a difference between classifications was seen with $CWT, or the carcass value per hundred pounds of carcass based on value reported to the feedlot producer and is heavily influenced by quality grade. A one-way Analysis of Variance (ANOVA) was performed, but there were no significant metabolites identified as different between the carcass value classificaitons at the P <0.05 threshold. The PLS-DA (Figure 18) model show slight separation between $CWT classification groups. The slight separation is highlighted by the permutation test. The PLS-DA validation with permutation shown in Figure 20 generated a P-value based on permutation of P = 0.085 (85/1000). This shows that there is a trend for the ability of the model to differentiate between the classes of $CWT. Furthermore, looking at the VIP scores for $CWT there are metabolites listed above a 1.5 threshold that contribute to differentiating the classes. Metabolites identified include succinate, 2-hydroxyisobutyrate, creatinine, isobutyrate, and formate. These metabolites are primarily involved in energy metabolism or single carbon metabolism and could be related to fat deposition and storage which could be reflected in fat deposition driving quality grade and influencing carcass value.

The third trait in which we illustrated differences was hot carcass weight. The PLS-DA (Figure 26) model shows slight separations between hot carcass weight groups between the medium and heavy groups specifically. The P-value of P = 4.56 indicates that there is no significant evidence that the model can predict HCW. However, the VIP scores identified four metabolites contributed to predicting HCW. The two most significant VIP for HCW were urea and choline. As mentioned above, urea is related to energy metabolism. Choline was also

identified by Consolo et al., 2020 who reported that choline concentrations were different between high growth and high precocity, with a higher concentration of choline in the high growth animals. The authors speculated that this was due to alterations in the level of fatty acids used for energy in the high precocity group. We observed our heavy animals had the highest concentration of choline while the medium had the lowest concentration similar to the findings of Consolo et al., 2020. This may indicate that animals in the heavy or high growth class use choline for fat metabolism.

Once again, a one-way analysis of variance (ANOVA) was first used to compare quality grade classes. The PLS-DA (Figure 35) shows slight separation between quality grade classes. The PLSDA validation with permutation (Figure 37) generated a $P = 0.068$ (68/1000). This demonstrates a trend for a difference between quality grade classes. The VIP score analysis (Figure 38) identified isopropanol, glucose, formate, acetamide, and isobutryate as metabolites differentiating between the classes. There were no clear patterns seen in the metabolite concentrations. Metabolites were similar to what was seen with back fat thickness and quality grade but was unexpected as metabolites were measured upon entry into the feedlot and carcass characteristics were measured at harvest.

The PLS-DA (Figure 43) shows slight separation amongst the classifications of ribeye area (REA). The VIP score analysis identified isopropanol, betaine, succinate, anthranilate, and urea as the important metabolites, and there was a similar pattern in the concentrations of succinate, betaine, and anthranilate in relation to REA. The metabolite Betaine was found as a possible predictive metabolite for protein synthesis. Consolo et al., 2020 also found betaine to be higher in early maturing animals.

The metabolites identified in the ANOVA as significantly different for exit velocity were methanol, isopropanol, lactate, isobutyrate, and pyruvate. As exit velocity was used as a measure of temperament, these metabolites indicate variation in energy expenditure with a heavy push towards anaerobic energy production. Burfeind and Heuwieser (2012) found a high correlation between exit velocity, docility score, and blood lactate. Lactate concentrations increased with flighty temperament. There have been other studies done that strongly suggest that blood lactate could be an effective objective measure to assess temperament (Williams et al., 2019; Holmes et al., 1972; Chloupek et al., 2001) The PLS-DA (Figure 51) shows slight separation between exit velocity classification groups but significance wasn't observed. The VIP score analysis identified lactate, creatinine, 3-hydroxybutyrate, methanol, isobutyrate, and isopropanol. The identification of a similar set of metabolites utilizing multiple methods increases confidence in the importance of these metabolites in distinguishing exit velocity classes. The concentrations of lactate, methanol, and pyruvate show a similar pattern in relation to exit velocity classification. The expression of highest concentrations of pyruvate, lactate, and methanol were seen in the Medium group whereas the lowest concentration of these metabolites was observed in the slow group. A potential explanation for these observations is that the higher exit velocity or flightier animals have utilized all quickly available energy and have transitioned to anaerobic metabolism while the medium class still has readily available aerobic energy. Another option is that animals with higher exit velocity have already exported lactate from the cells while the medium animals are still responding to the stimuli.

Another measurement of animal temperament was chute score (scale of 1-5). A one-way analysis of variance (ANOVA) was used to compare classifications. The metabolites that were significant at $P < 0.05$ are methanol, isobutyrate, creatinine, dimethyl sulfone, hippurate, isopropanol, and succinate (Table 3). The concentrations of these metabolites suggest that animals are making energy for the flight or fight response to maintain homeostasis. Succinate is an intermediate of the TCA cycle and feeds the electron transport chain to manufacture ATP. additional energy metabolites show that chute score is a reflection of mobilization of energy stores in response to handling stress. The PLS-DA (Figure 59) shows a slight separation between the classifications of temperament; high, medium and low. The lack of significant separation may be low variation and overall mild to docile temperament in the batch of cattle evaluated. Even with very little representation from the high temperament group, there is possible separation between groups in this study. VIP score analysis of metabolites (Figure 62) identified succinate, dimethyl sulfone, 3-hydroxybutyrate, hippurate, and methanol. High chute score animals had the lowest concentration of all these metabolites with medium chute score having the highest concentration of these metabolites in a similar pattern to what we saw with exit velocity. As above, this may indicate that higher chute score animals have transitioned from aerobic to anaerobic metabolism and have cleared some intermediates . To better understand what is being observed in the metabolic profile in regards to chute score or more, broadly, animal temperament we will need a more variable group of cattle better representing the extremes of chute score.

CHAPTER FIVE

CONCLUSION

In this study our objective was to identify metabolite predictors for animal health. We also examined the relationship between metabolite profile at entry into the feedlot with feedlot performance and final carcass characteristics.

Animal Health

We did not find any predictive metabolites for health status as predicted by treatment records, cost of treatment or mortality.

Animal Temperament

Metabolites associate with energy metabolism were identified in different temperament classifications. However, an unexpected result was that medium classification in both exit velocity and chute score had higher metabolite concentrations than fast exit velocity or high chute scores. Further research to examine temporal changes in metabolite concentrations is needed to illustrate the dynamic nature of the response to stimuli.

Carcass Traits

A possible relationship between metabolite profile on entry into the feedlot and carcass characteristics evaluated at harvest was not expected in this study. More directed research towards early prediction of carcass characteristics could elucidate the potential mechanisms including protein and fat deposition that was seen here and in other published studies.

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APPENDICES

APPENDIX A:

TEMPERAMENT AND CARCASS TRAITS EXPLORED WITH NO SIGNIFICANT FINDINGS

Days on Feed NMR Data

Days on Feed (kg)

Days on feed was the difference between the entry date into the feedlot and the harvest days. There were three different harvest days for the steers sampled. Days on feed is a measurement of the days to reach a marbling endpoint. Chappell Feedlot routinely uses ultrasound to determine the days to grading Choice. This is how the feedlot operator determines which animals are sent to harvest. Data normalization for days on feed was normalized by sum using a row-wise procedure and then a $\log_{10}$ transformation (Appendix Figure 1). A one-way Analysis of Variance (ANOVA) was first used to identify metabolites that discriminate between days on feed classes. Appendix Figure 2 shows metabolites identified by ANOVA analysis; no significant differences were seen for metabolites when data was categorized into three different harvest days. PCA and PLSDA (Figure 3 and 4, respectively) did not show any significant separation between days on feed classification groups. The PLSDA validation with permutation (Figure 6) generated a p-value based on permutation of P = 0.905 (905/1000). This shows that the model cannot differentiate days of feed class. Figure 5 looks at the number of components that would allow the best classifications. It indicates that there is not a viable classification based on metabolites for days on feed. Figure 7 shows an ordered list of the most important metabolites in discriminating between days on feed classifications from the PLSDA analysis. Metabolites with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are acetate, methanol, and isobutyrate. No patterns were evident in which features were highest or lowest concentrations by days on feed classification. Figure 8 shows a similar analysis

using a Random Forest approach. The two most important features identified using this method were methanol and 2-hydroxyisobutyrate.

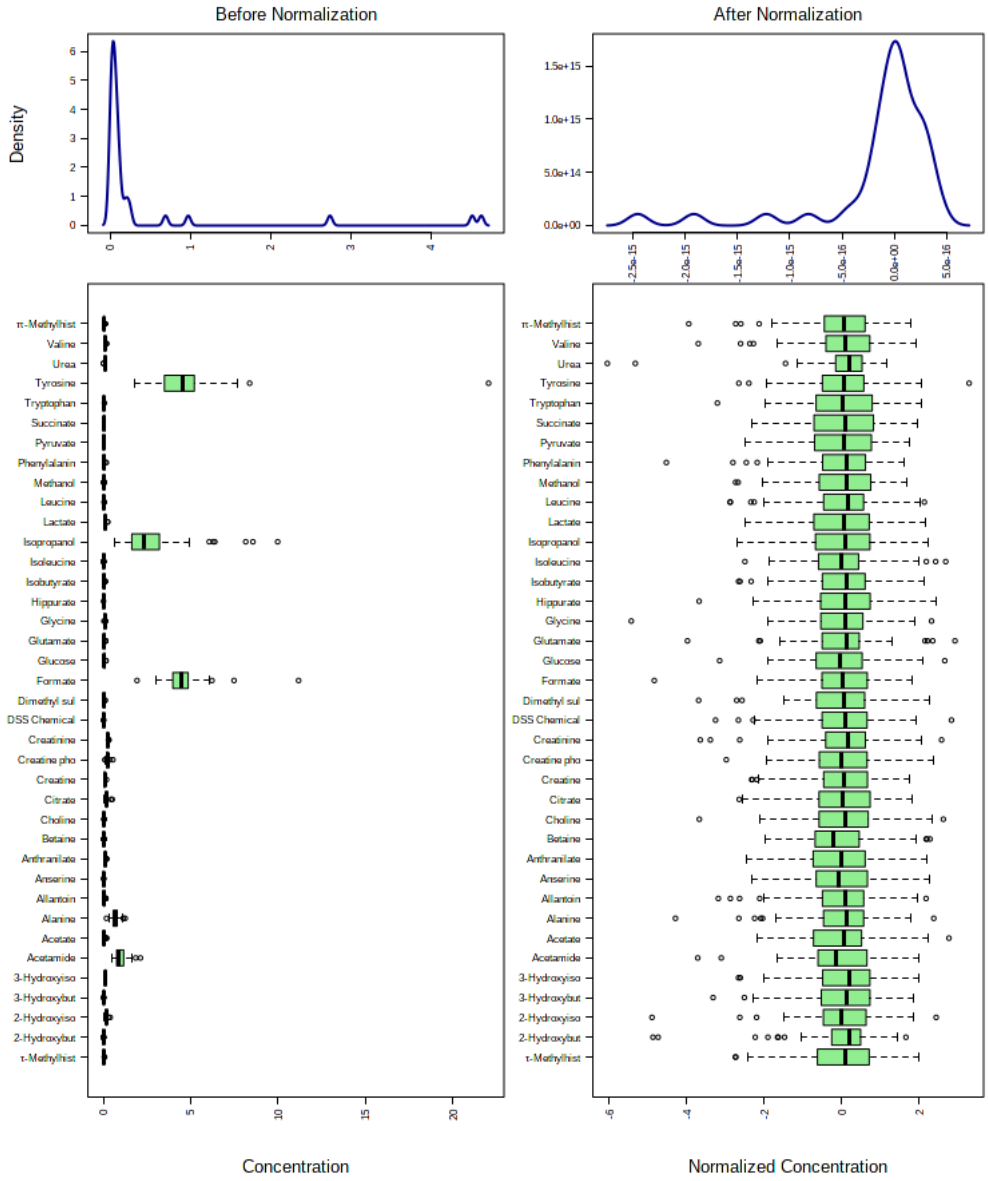

Figure 1. Days on Feed Data Before and After Normalization Procedures: Box plots and kernel density plots before and after normalization for days on feed data. The boxplots show at most 50 metabolites due to space limit. The density plots are based on all samples. Selected methods: Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

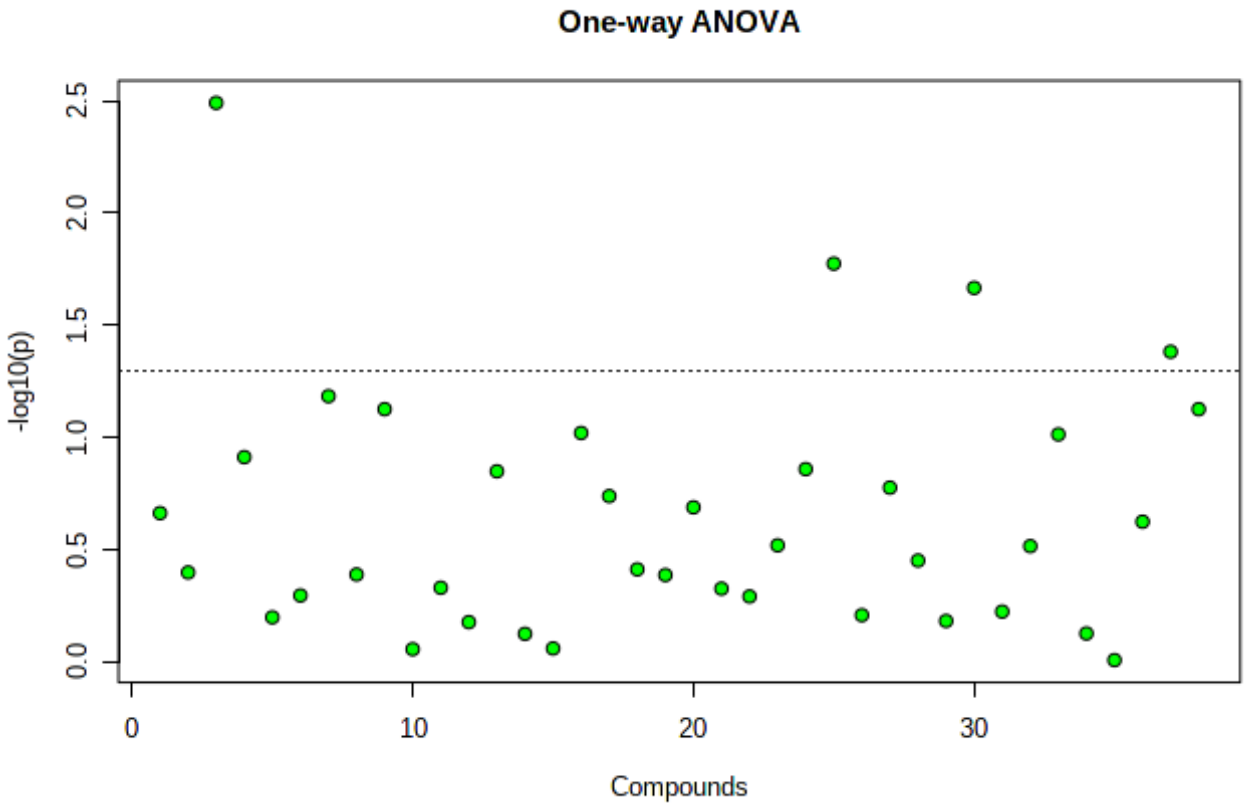

Figure 2. Significant features related to Days on Feed identified by Analysis of Variance: Important metabolites identified by ANOVA for days on feed categories, *P*-value threshold 0.05. There are no significant features found using the given threshold. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10(*P*). The reason for some metabolites shown as green even though they are at or above the level of significance is because of FDR adjustment.

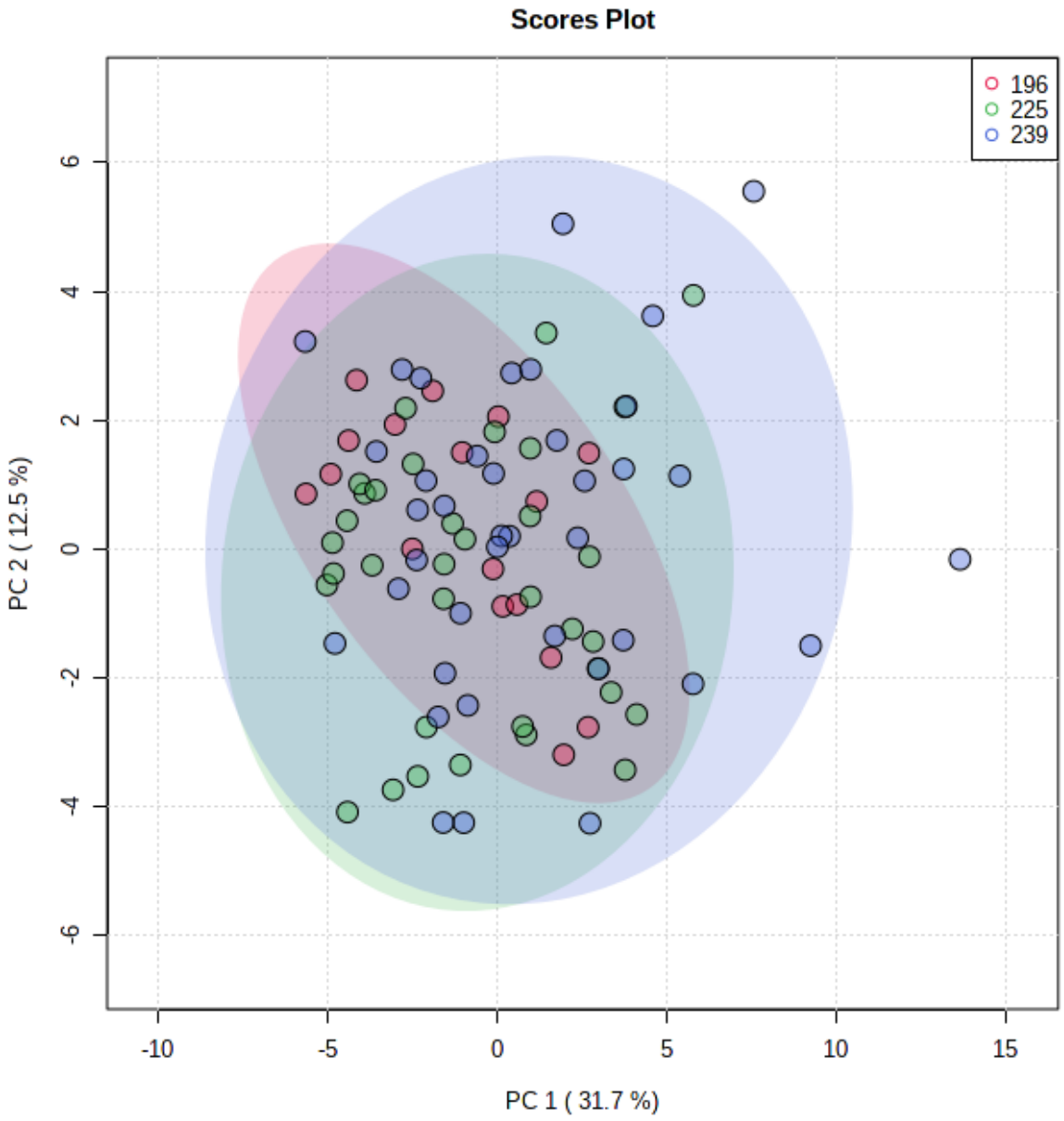

Figure 3. Significant features related to Days on Feed identified by PCA: Scores plot between the selected principal components or group classifications for days on feed. The variation explained by the first and second principal components is in parentheses. In this image there is no significant separation between the groups.

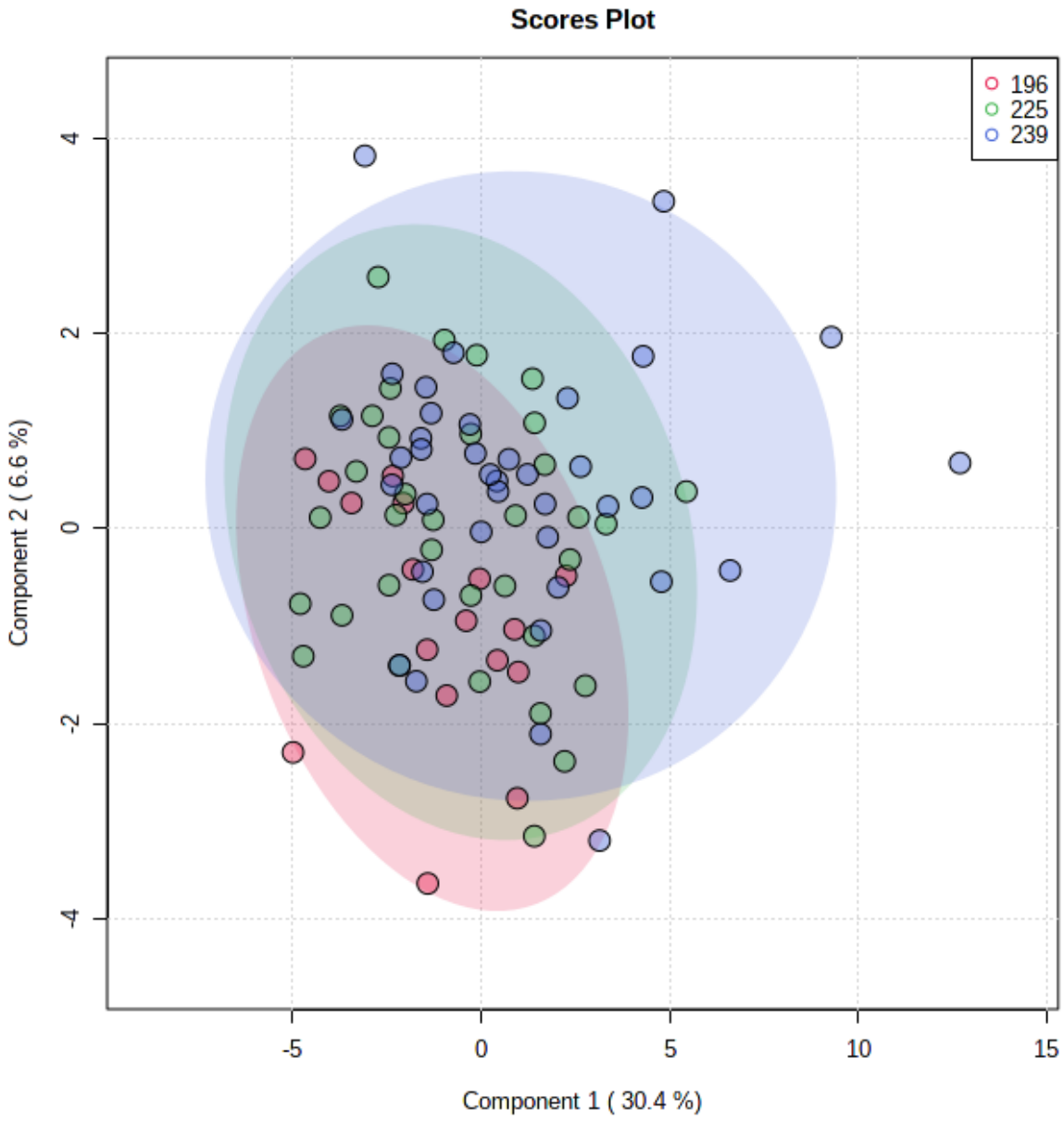

Figure 4. Significant features related to Days on Feed identified by PLS-DA: Partial Least Squares – Discriminant Analysis (PLS-DA)**.** Scores plot for days on feed between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is no significant separation between the groups.

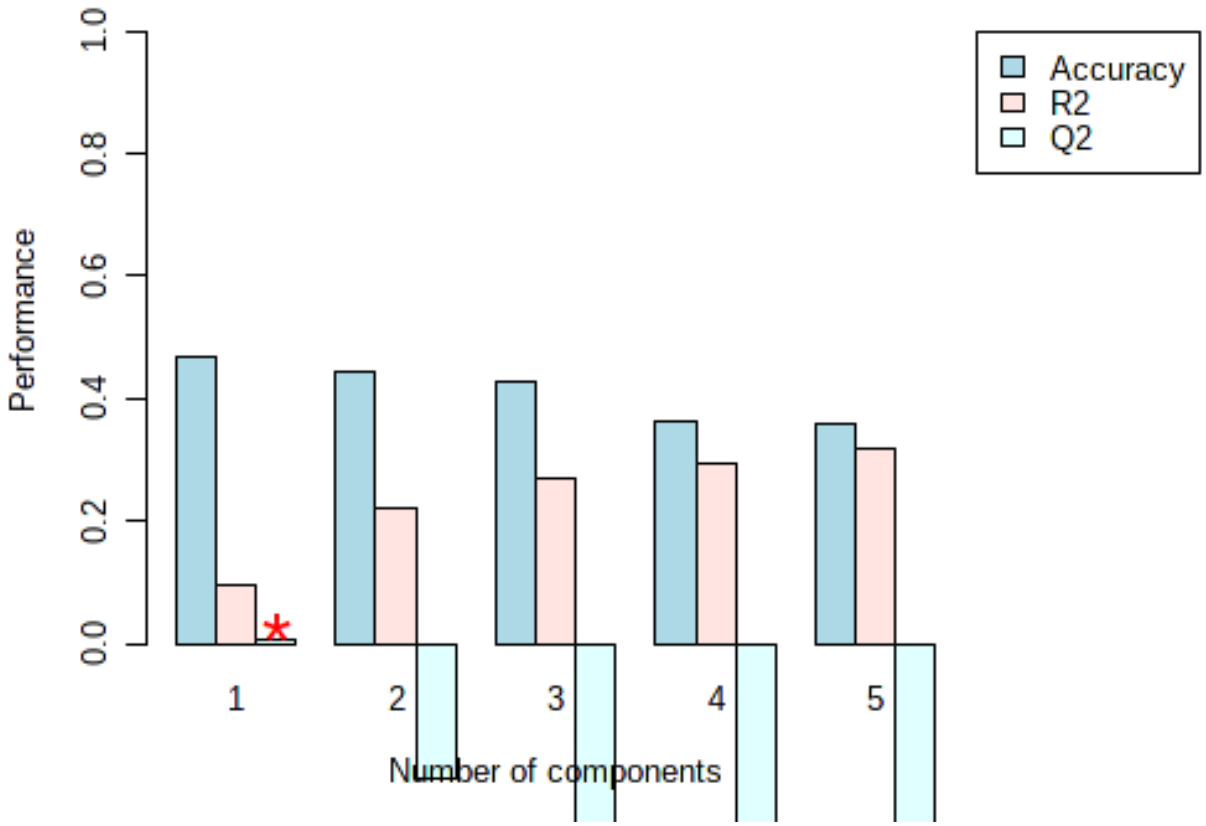

Figure 5. Significance related to Days on Feed identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier, the negative Q2 means there is no significance.

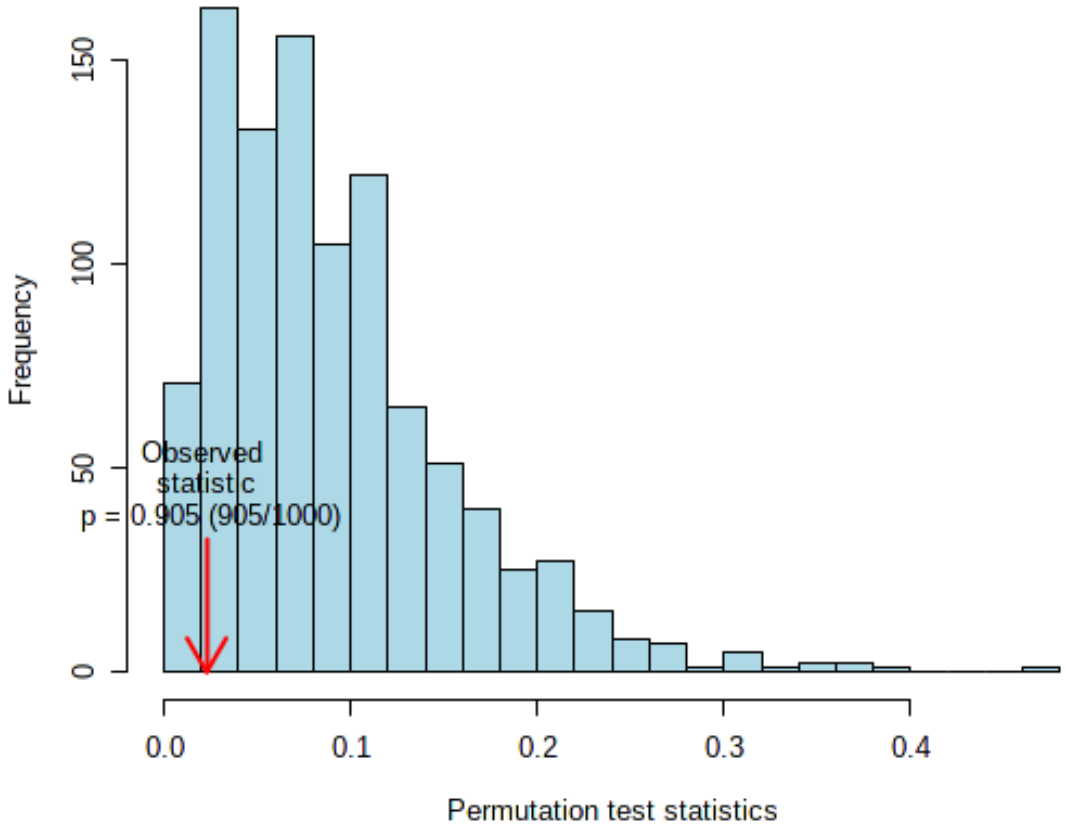

Figure 6. Significance related to Days on Feed identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance for days on feed. The *P*-value based on permutation is *P* = 0.905 (905/1000). The *P*-value shows that there is no difference between classes.

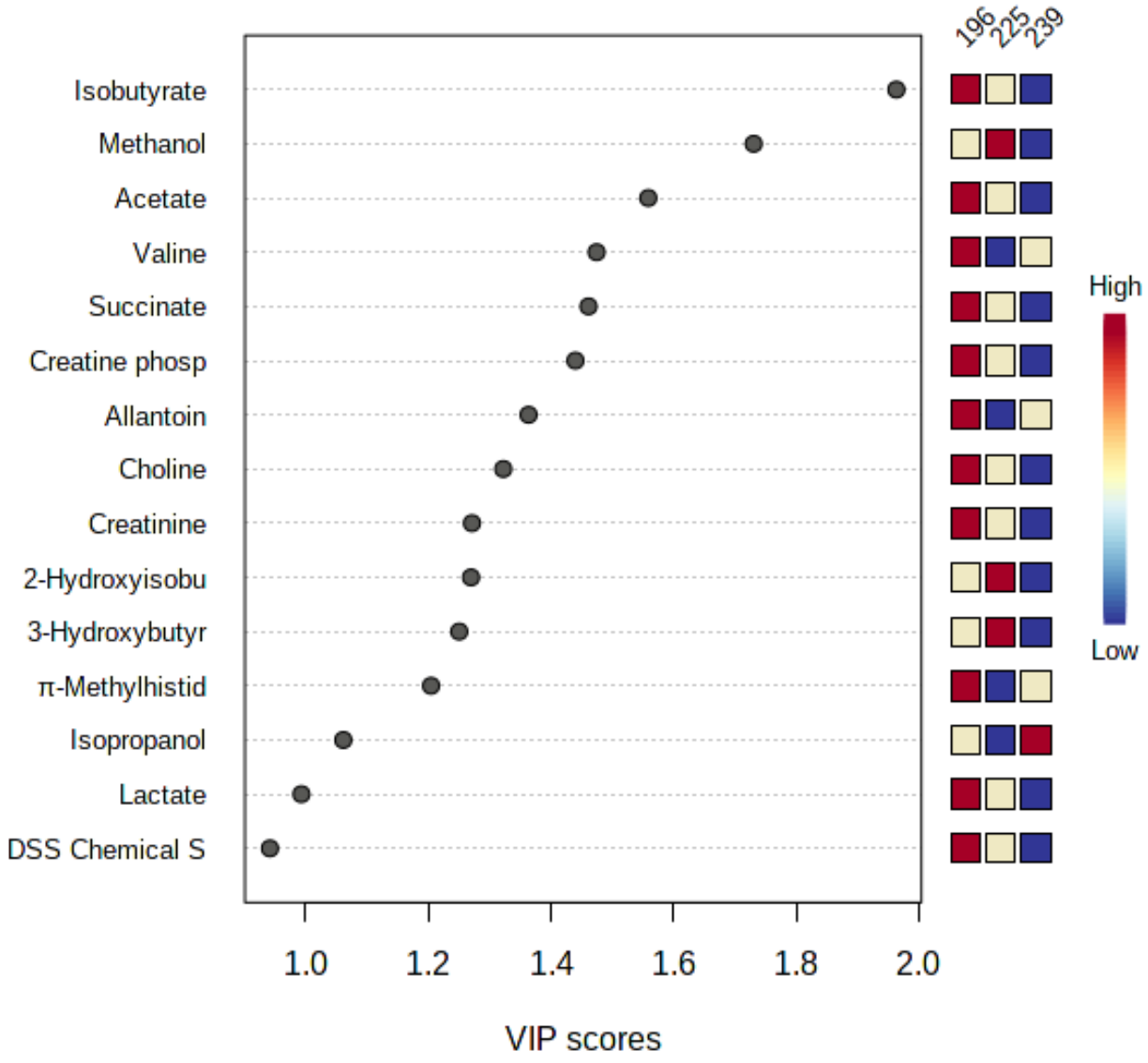

Figure 7. Significant Metabolites related to Days on Feed identified by VIP Scores: Important metabolites identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

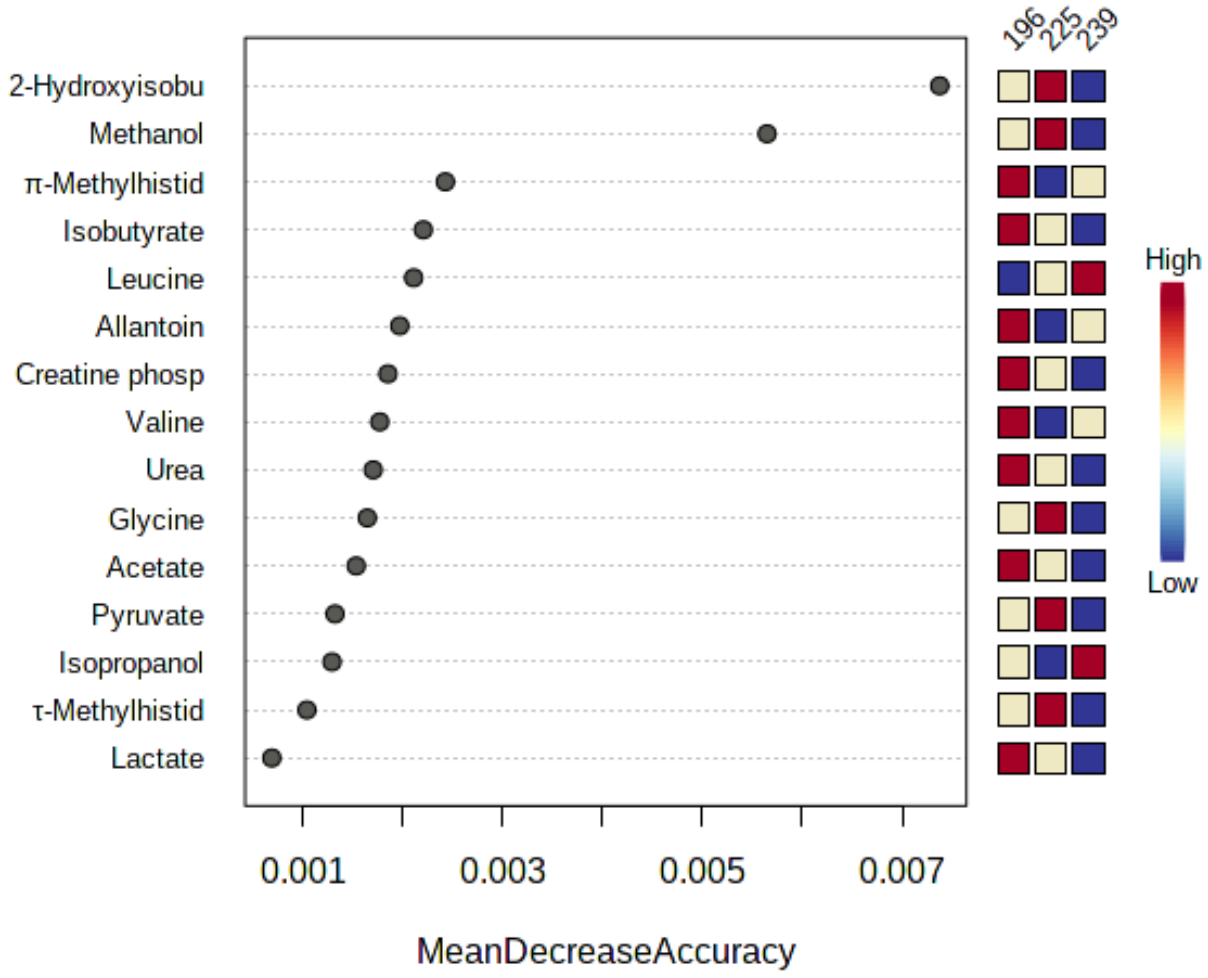

Figure 8. Significant Metabolites related to Days on Feed identified by Random Forest: Significant metabolites identified by Random Forest. The metabolites are ranked by the mean decrease in classification accuracy when they are permuted.

Average Daily Gain NMR Data

Average Daily Gain (ADG)

Average daily gain was calculated from the number of days on feed and the weight gained while on feed. This information was collected by the feedlot and provided by the operator. Actual entry weights were not obtained so the load weight was used to calculate the entry weight for average daily gain. ADG Data was normalized by sum using a row-wise procedure (Appendix Figure 9) and then $\log_{10}$ transformed. PCA and PLSDA (Appendix Figure 10 and 11, respectively) did not show separation between average daily gain groups. The PLSDA validation with permutation (Appendix Figure 11) generated a *P*-value based on permutation of $P = 0.755$ (755/1000). The model generated cannot predict average daily gain class. The number of components (Appendix Figure 13) that would allow the best classifications indicates that a viable model cannot be generated.. Appendix Figure 14 shows an ordered list of the most important features in discriminating between average daily gain classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are t-methylhistidine, urea, and choline. No patterns were evident in concentrations by average daily gain classification. Appendix Figure 15 shows a similar analysis using a Random Forest approach. The three most important metabolites identified using this method were 3-hydroxybutyrate, acetate, and choline. The metabolites identified in both analyses indicate that energy metabolism may be important in average daily gain classification.

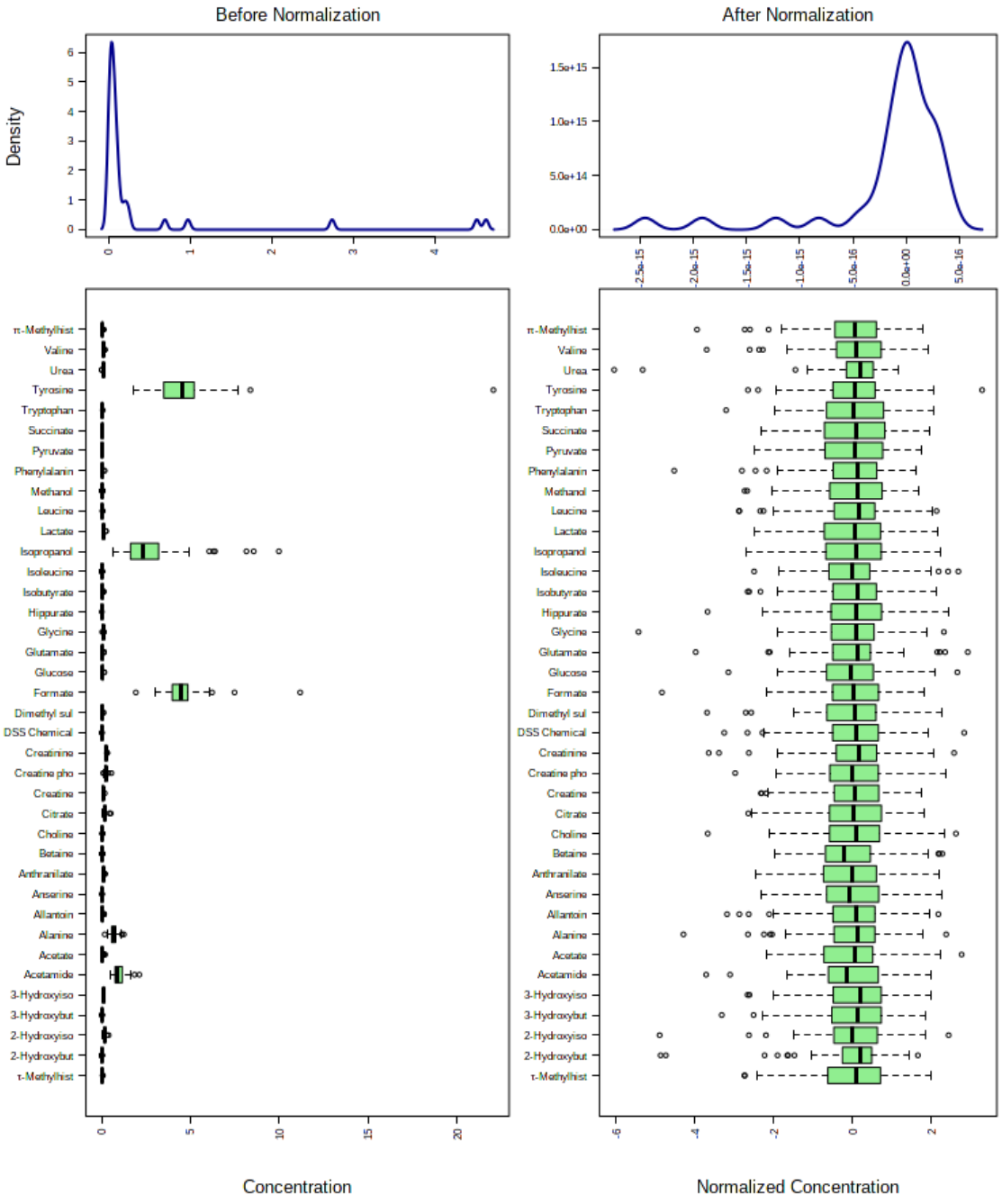

Figure 9. Average Daily Gain before and after Normalization Procedures: Box plots are kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

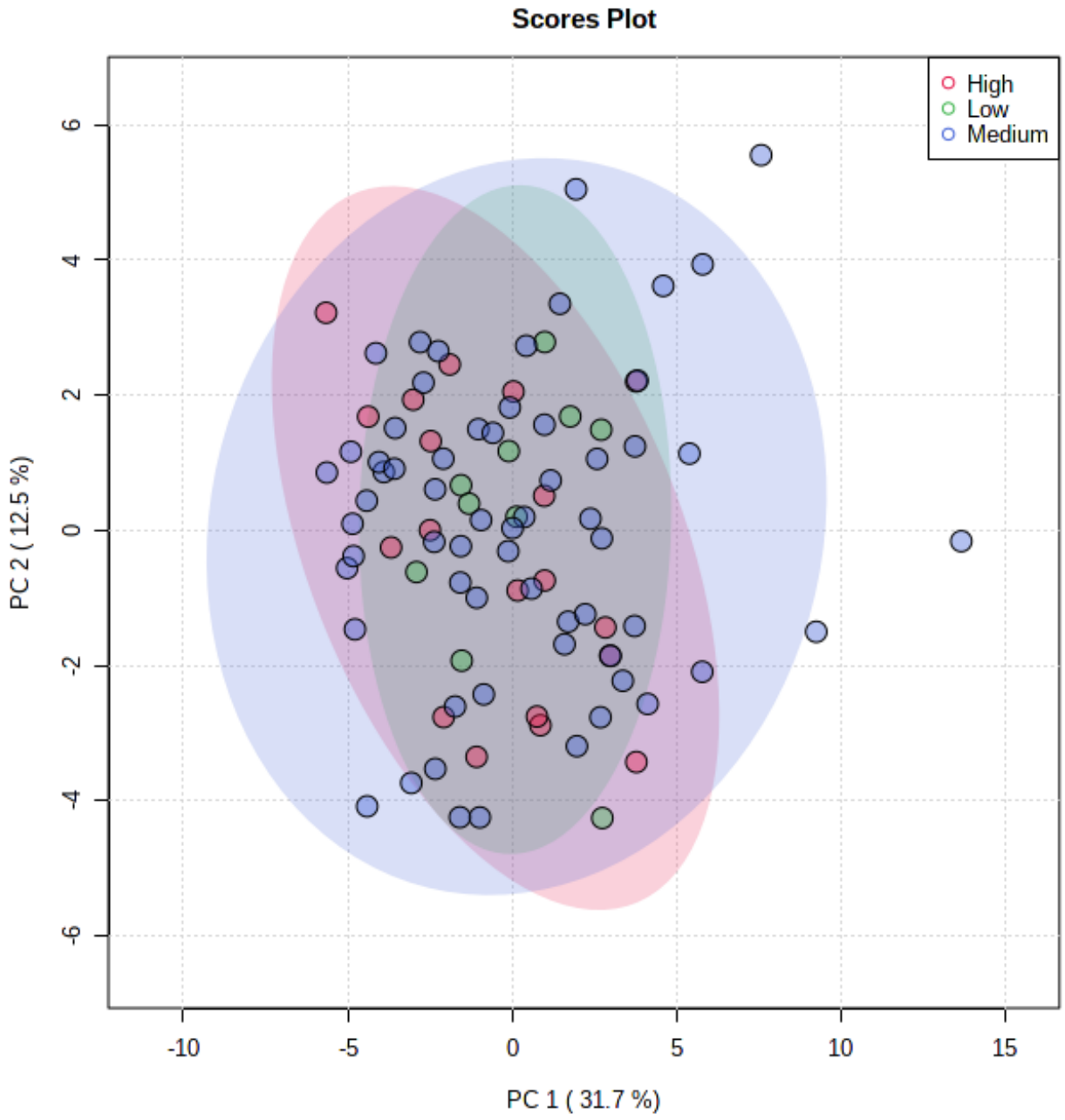

Figure 10. Significant features related to Average Daily Gain Identified by Analysis of Variance: Scores plot between the selected principal components or group classifications for average daily gain. The variation explained by the first and second principal component is in parentheses. In this image there is no significant separation between the groups.

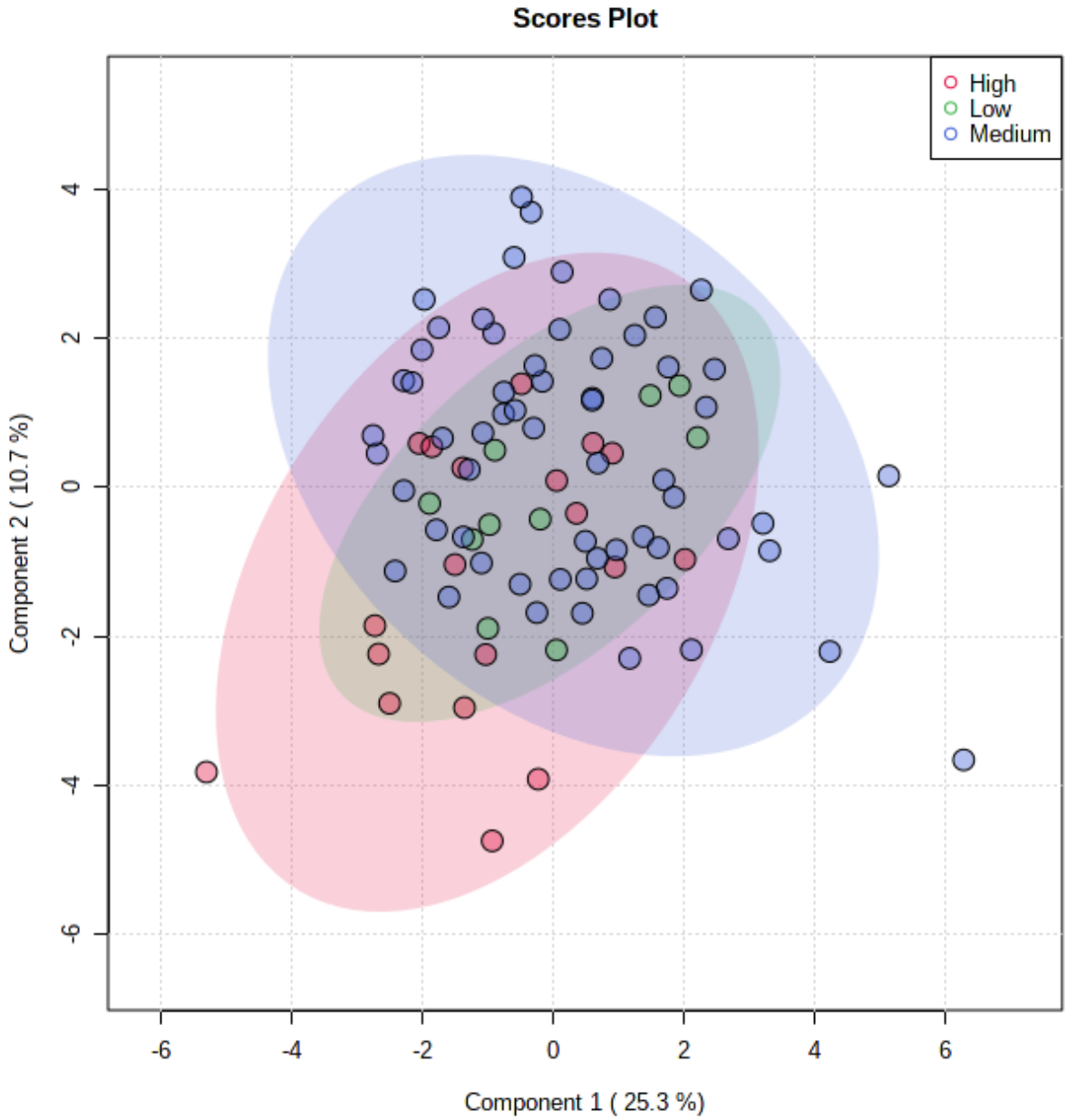

Figure 11. Significant features related to Average Daily Gain identified by PLS-DA: Scores plot for average daily gain between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is no significant separation between the groups.

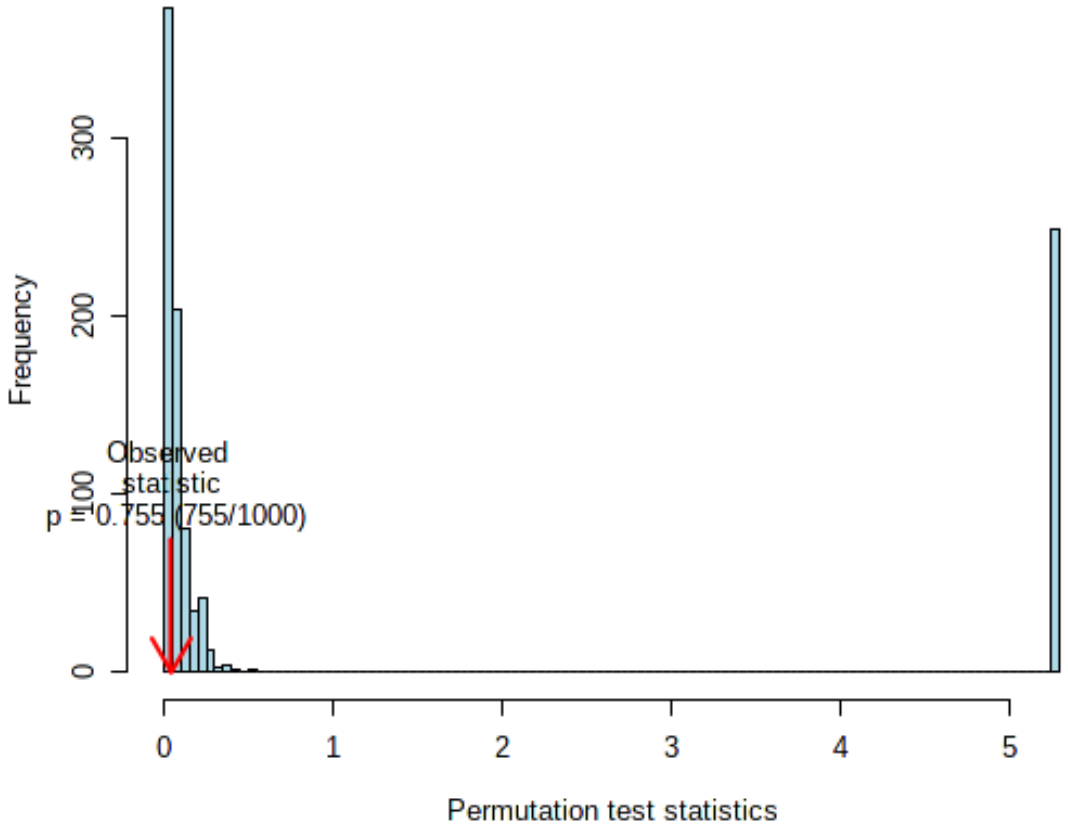

Figure 12. Significance related to Average Daily Gain identified by permutation test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is $P = 0.755$ (755/1000). With this *P*-value the model did not predict average daily gain classification. The reason for the spike on the right hand is this group is very uniform overall.

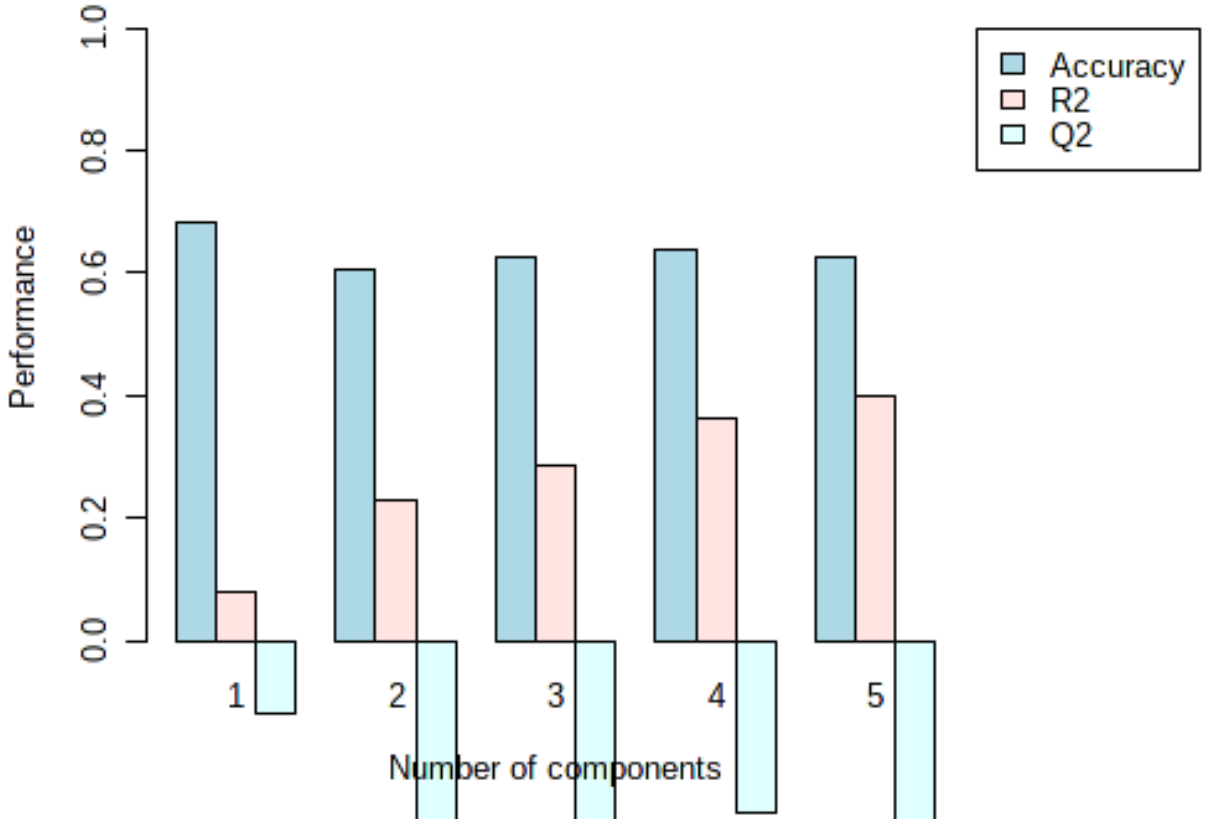

Figure 13. Significance related to Average Daily Gain identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when possible. The negative Q2 means there is not significance.

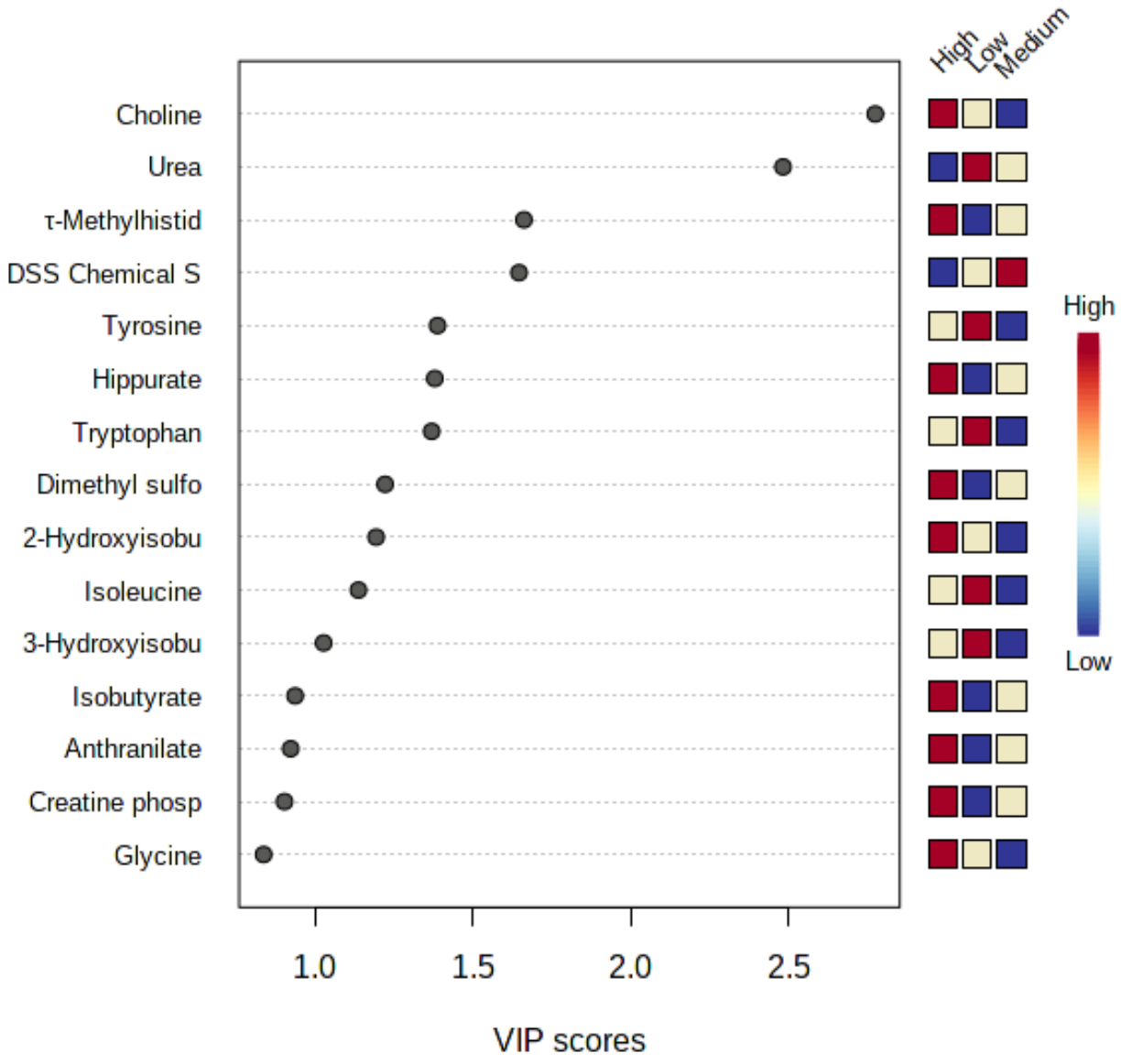

Figure 14. Significant metabolites related to Average Daily Gain identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each average daily gain class.

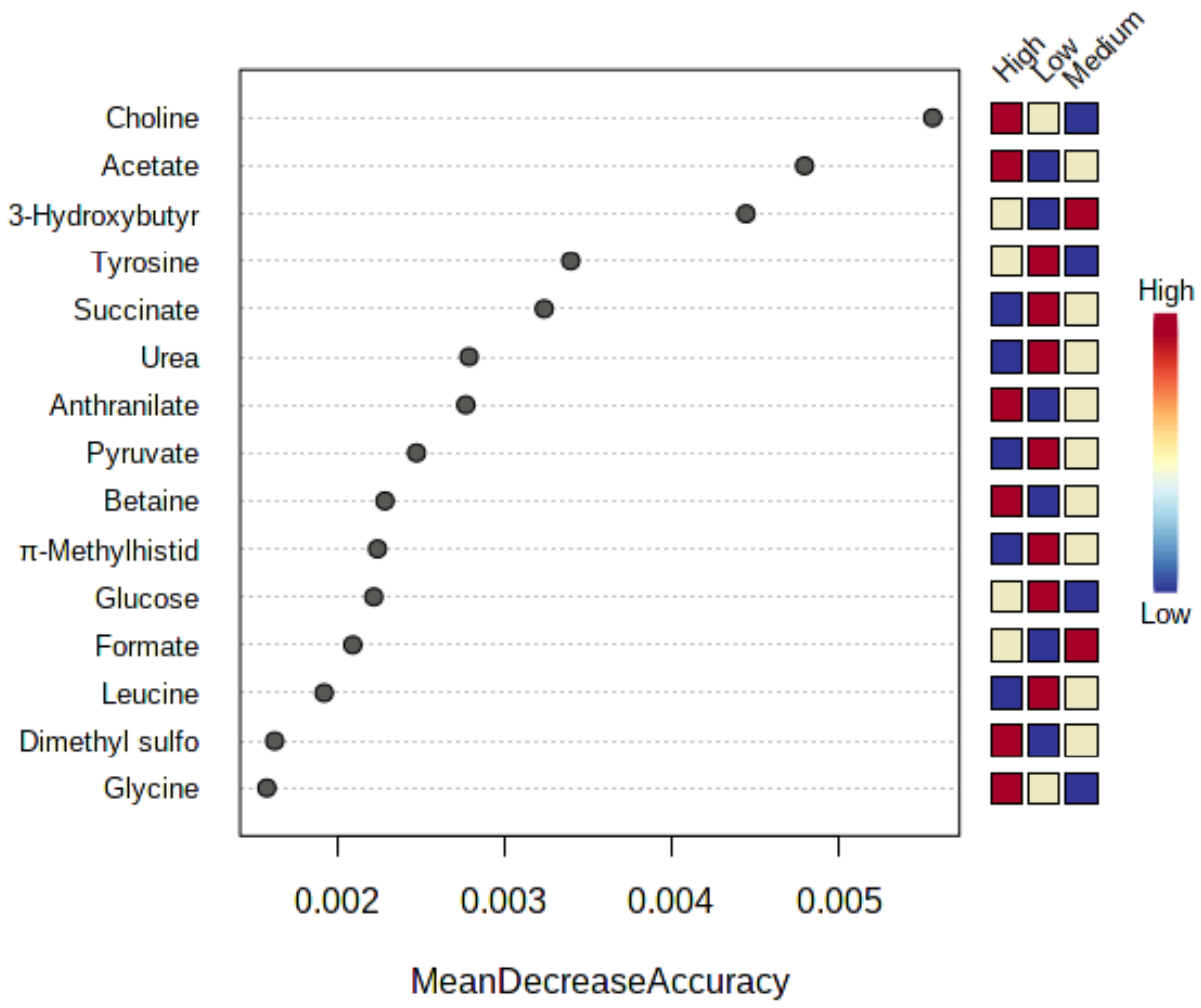

Figure 15. Significant Metabolites related to Average Daily Gain identified by Random Forest: Significant features identified by Random Forest. The features are ranked by the mean decreases in classification accuracy when they are permuted.

Dry Matter Intake NMR Data

Dry Matter Intake DMI (kg)

This is an estimate of the ration consumed by the animal prior to harvest. Data was normalized by sum using a row- wise procedure (Appendix Figure 16) and then log transformed. A one-way Analysis of Variance (ANOVA) was used to identify features that are different between DMI classes (Appendix Figure 17). PCA and PLSDA (Appendix Figure 18.19, respectively) showed little separation between DMI classification groups. The PLSDA validation with permutation (Appendix Figure 21) generated a p-value based on permutation of $P = 0.935$ (935/1000). The indicates that the model cannot predict dry matter intake class. Appendix Figure 20 shows the number of components that would allow the best classifications. It indicates that there is not viable classification possible for DMI. Appendix Figure 22 shows an ordered list of the most important features in discriminating between dry matter intake classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are acetate, urea, succinate, and isobutyrate. No patterns were evident in metabolite concentrations by DMI classification. Appendix Figure 23 shows a similar analysis using a Random Forest approach. The three most important features identified using this method were methanol, glycine, and 2-hydroxyisobutrate.

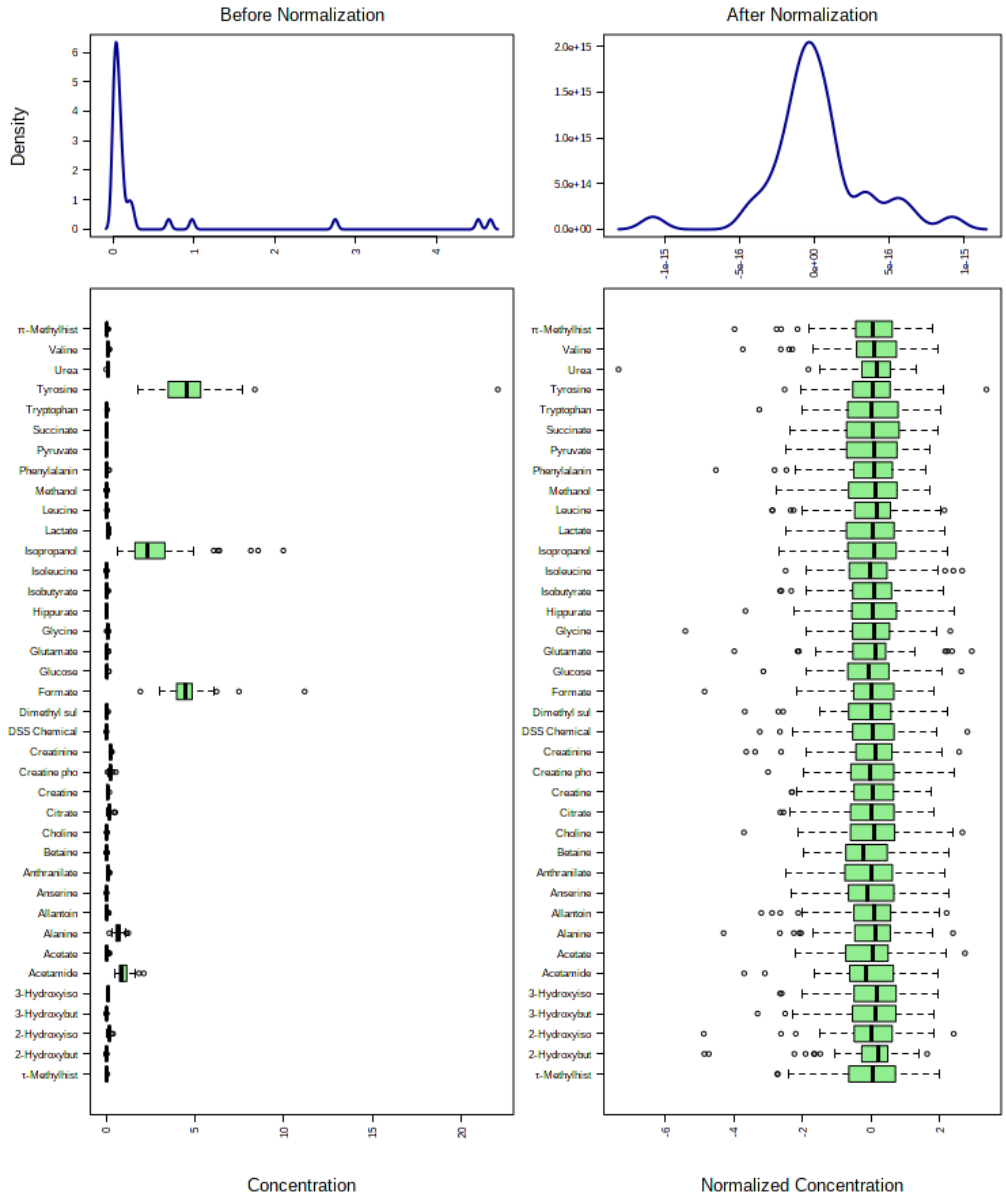

Figure 16. Dry Matter Intake data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

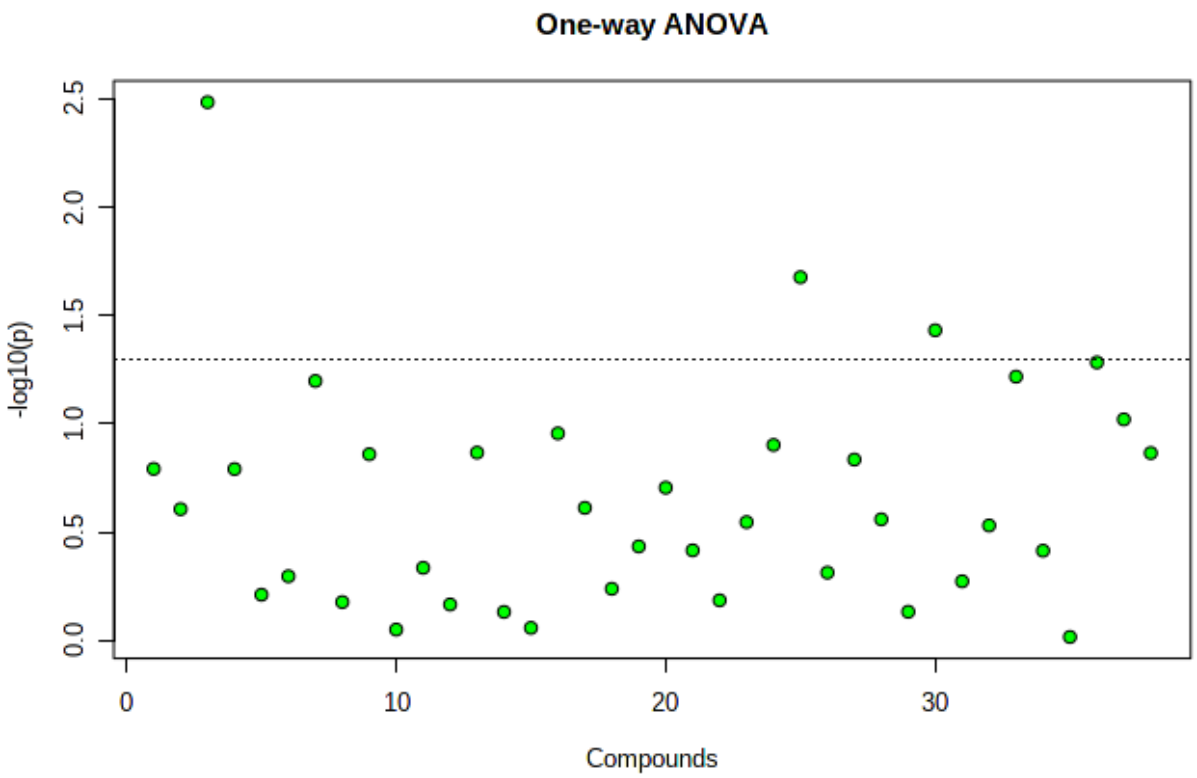

Figure 17. Significant features related to Dry Matter Intake identified by Analysis of Variance: Important metabolites identified by ANOVA for dry matter intake categories, *P*-value threshold of 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10 (*P*) which identifies level of significance of the. The reason for some metabolites shown as green even though they are at or above the level of significance is because of FDR adjustments.

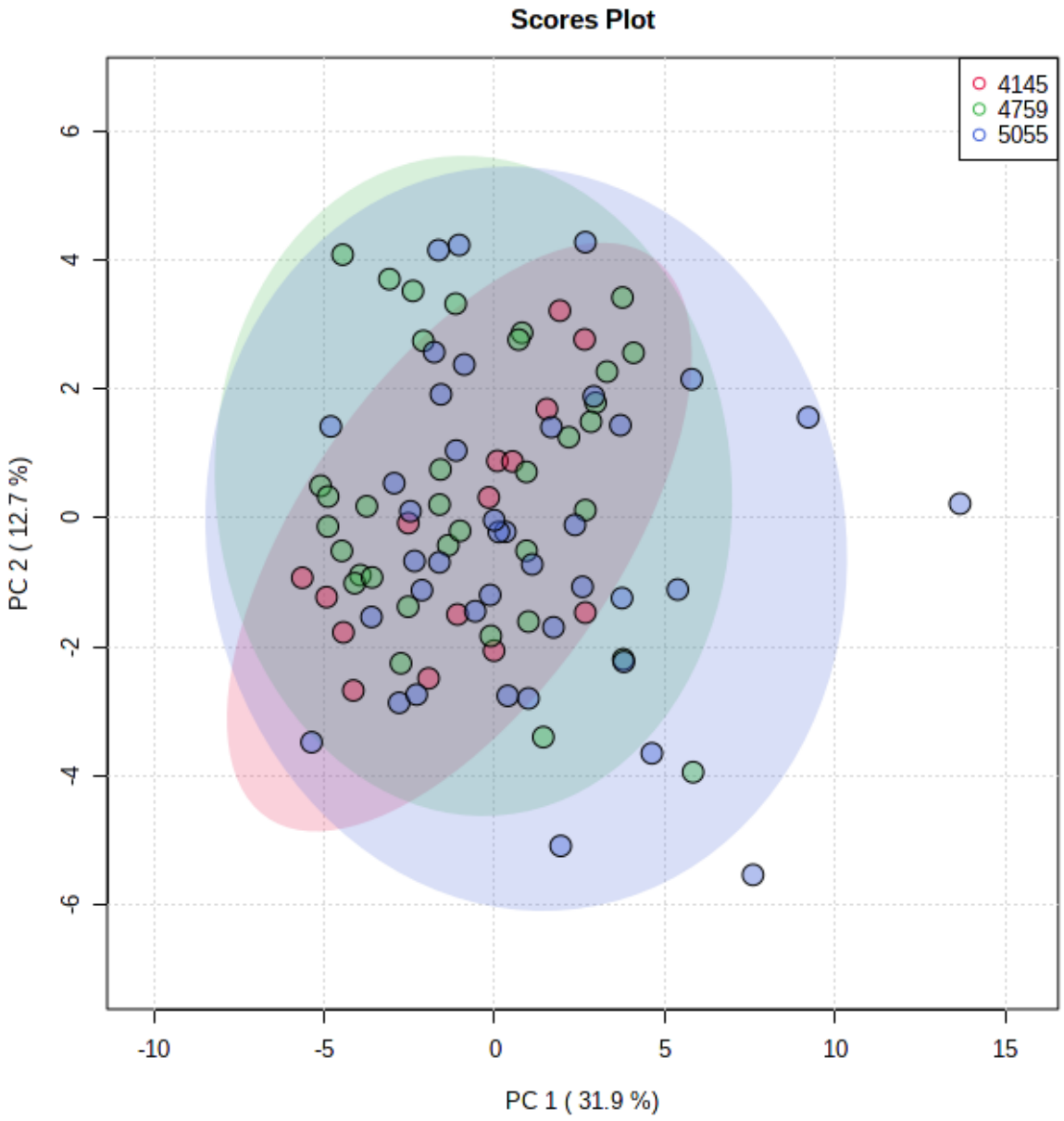

Figure 18. Significant features related to Dry Matter Intake identified by PCA: Scores plot between the selected principal components or group classifications for dry matter intake. The variation explained by the first and second principal components is in parentheses. There is no significant separation between the groups.

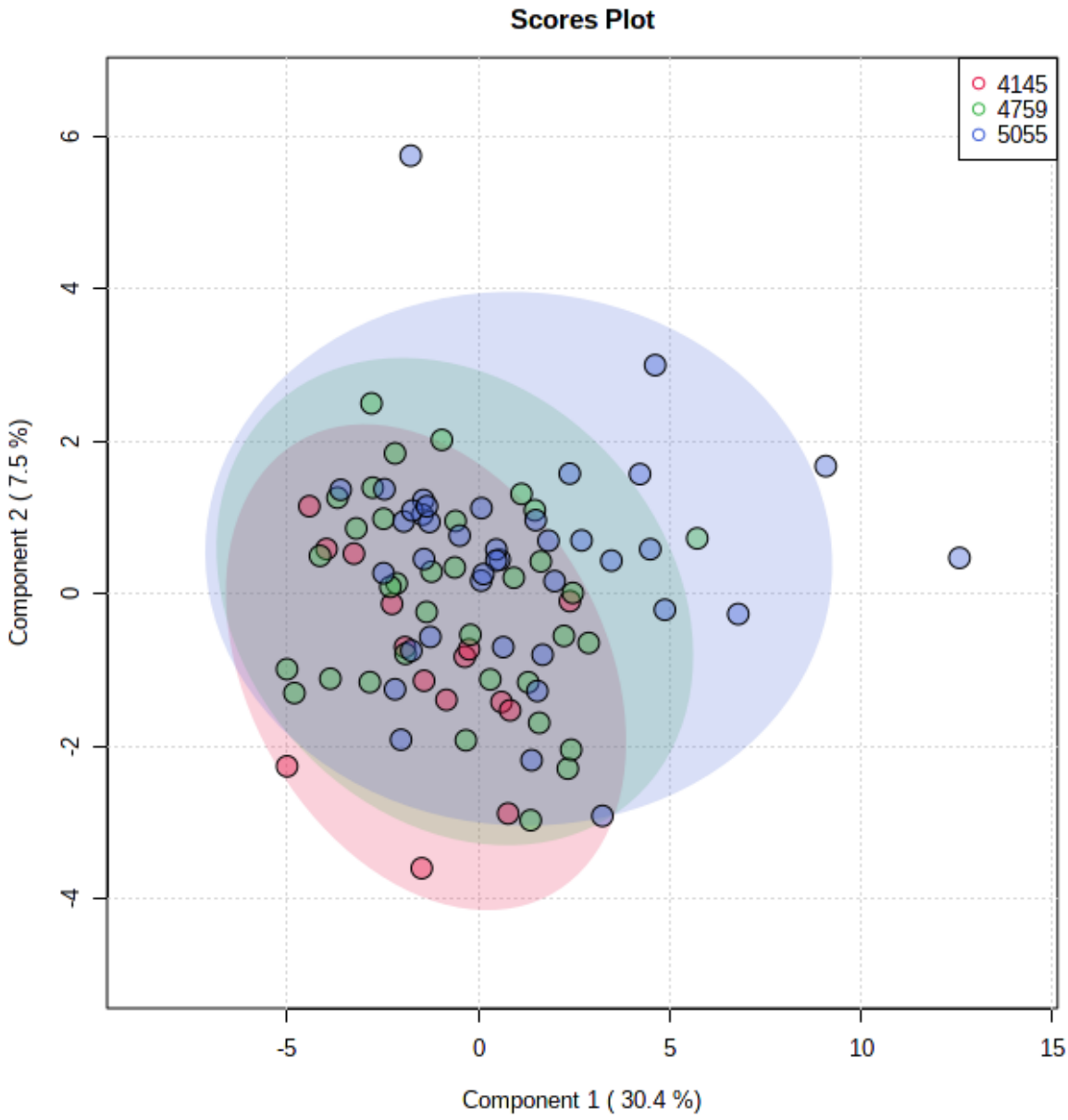

Figure 19. Significant features related to Dry Matter Intake identified by PLS-DA: Scores plot for dry matter intake between the selected principal components. The variance explained by the two principal components are shown in parentheses. There is no significant separation between the groups.

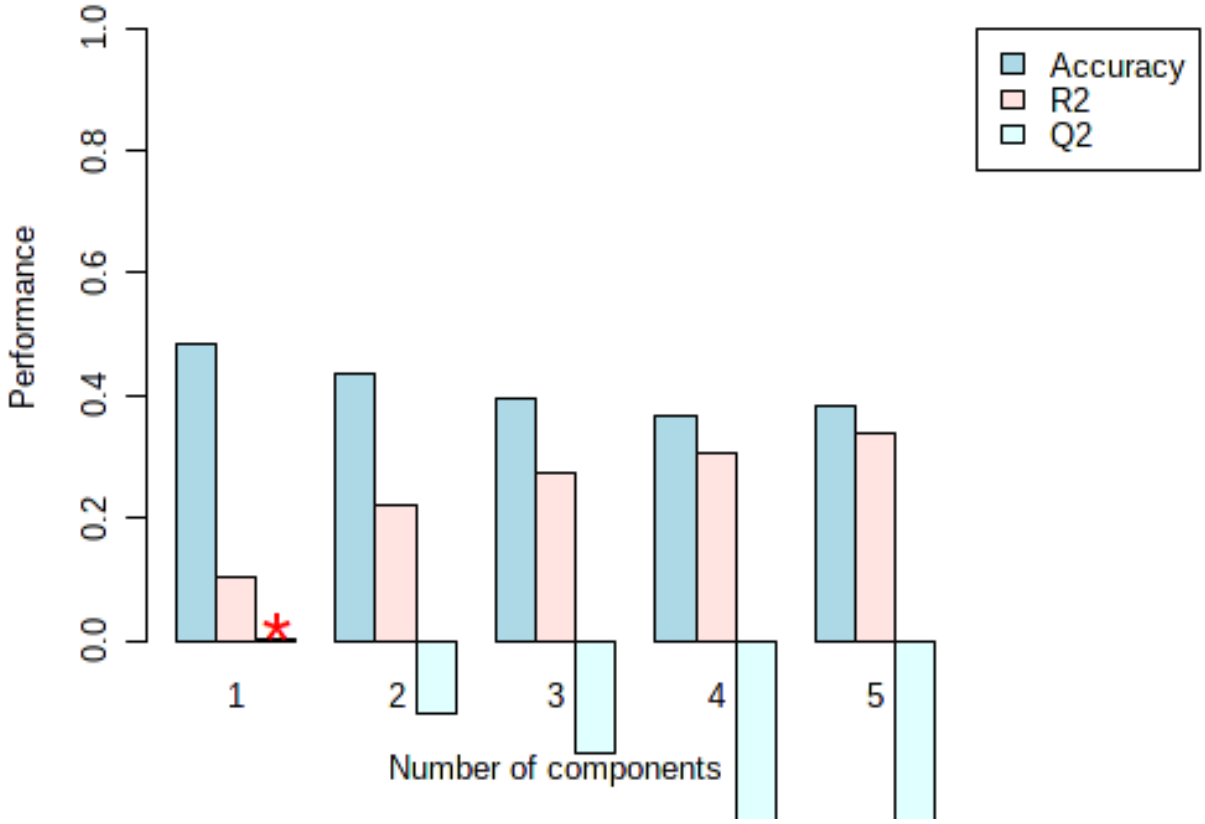

Figure 20. Significance related to Dry Matter Intake identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier. The negative Q2 shows a lack of significance.

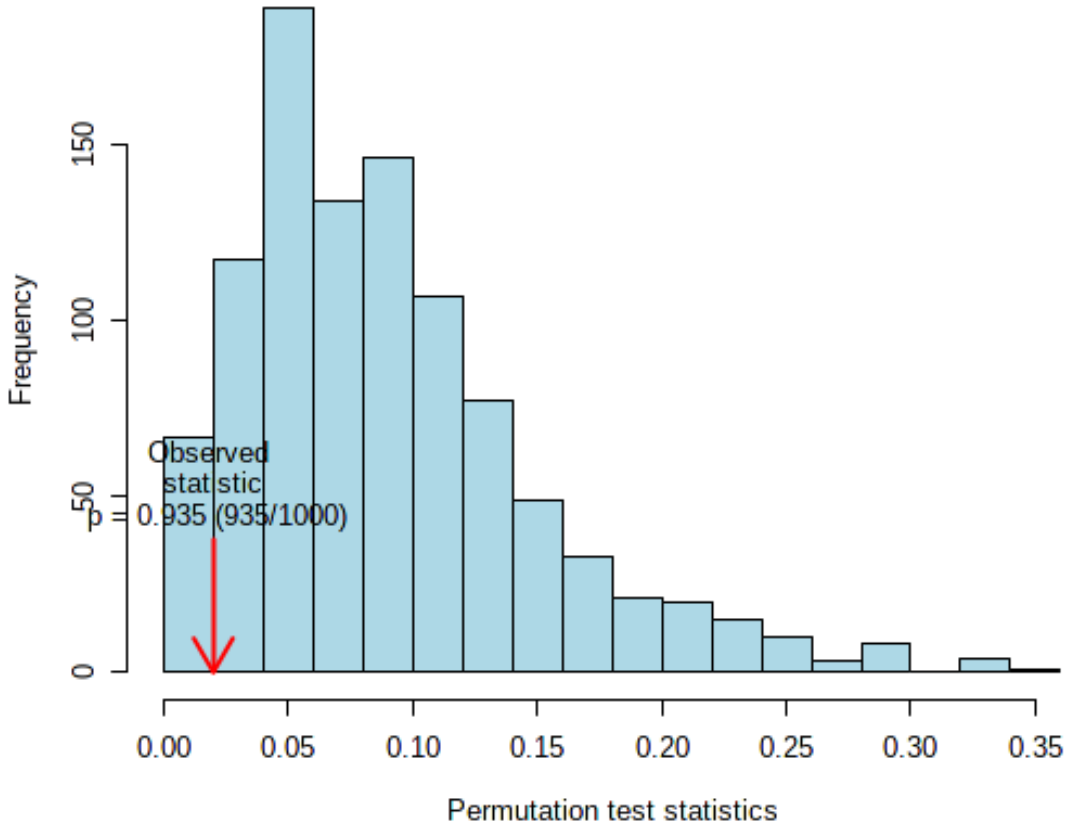

Figure 21. Significance related to Dry Matter Intake identified by Permutation Test: PLS-DA model validation by permutation test based on separation distance. The *P*-value based on permutation is *P* = 0.935 (935/1000). There is no significant difference.

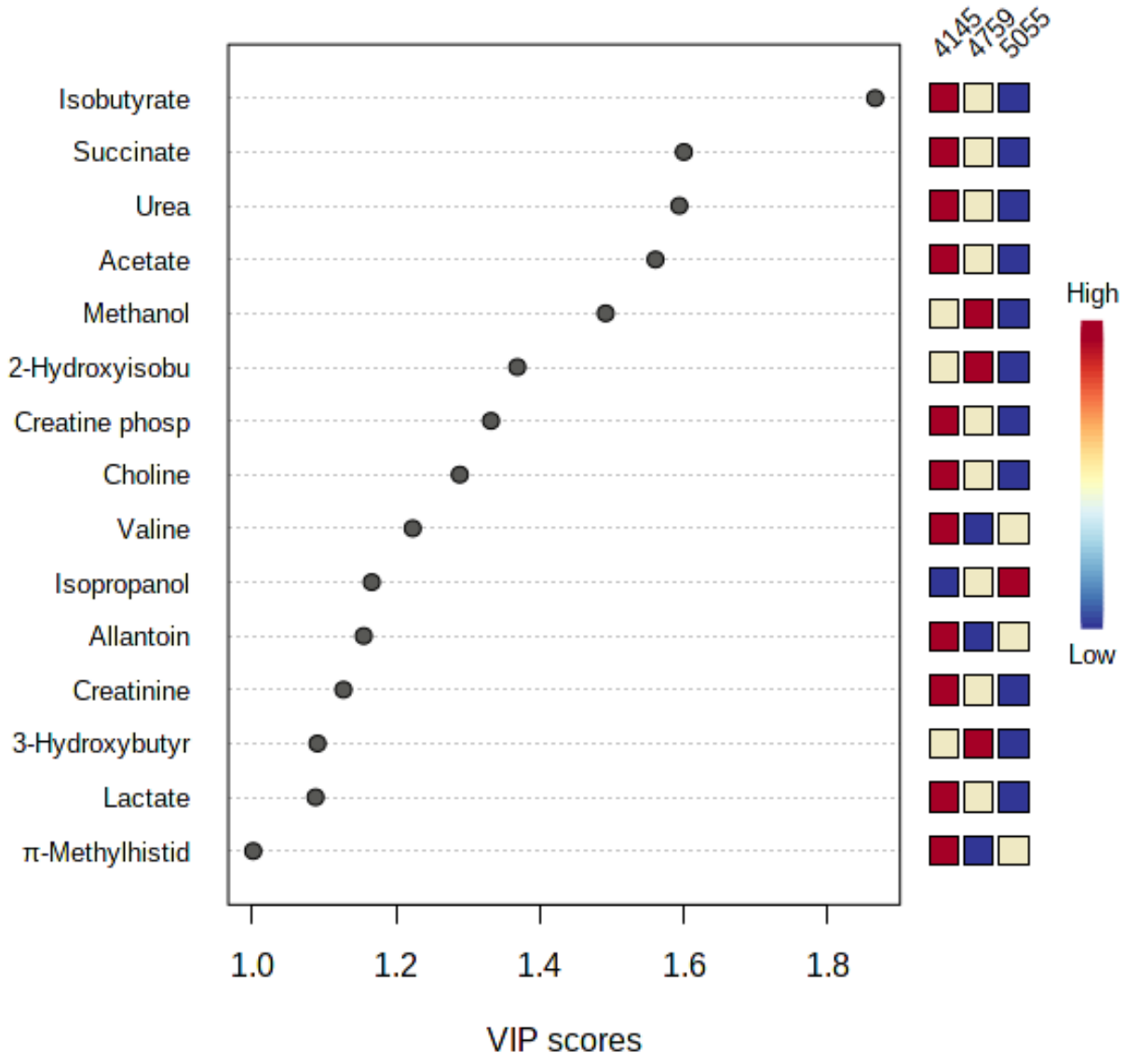

Figure 22. Significant Metabolites related to Dry Matter Intake identified by VIP Scores: Important metabolites identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each dry matter intake class.

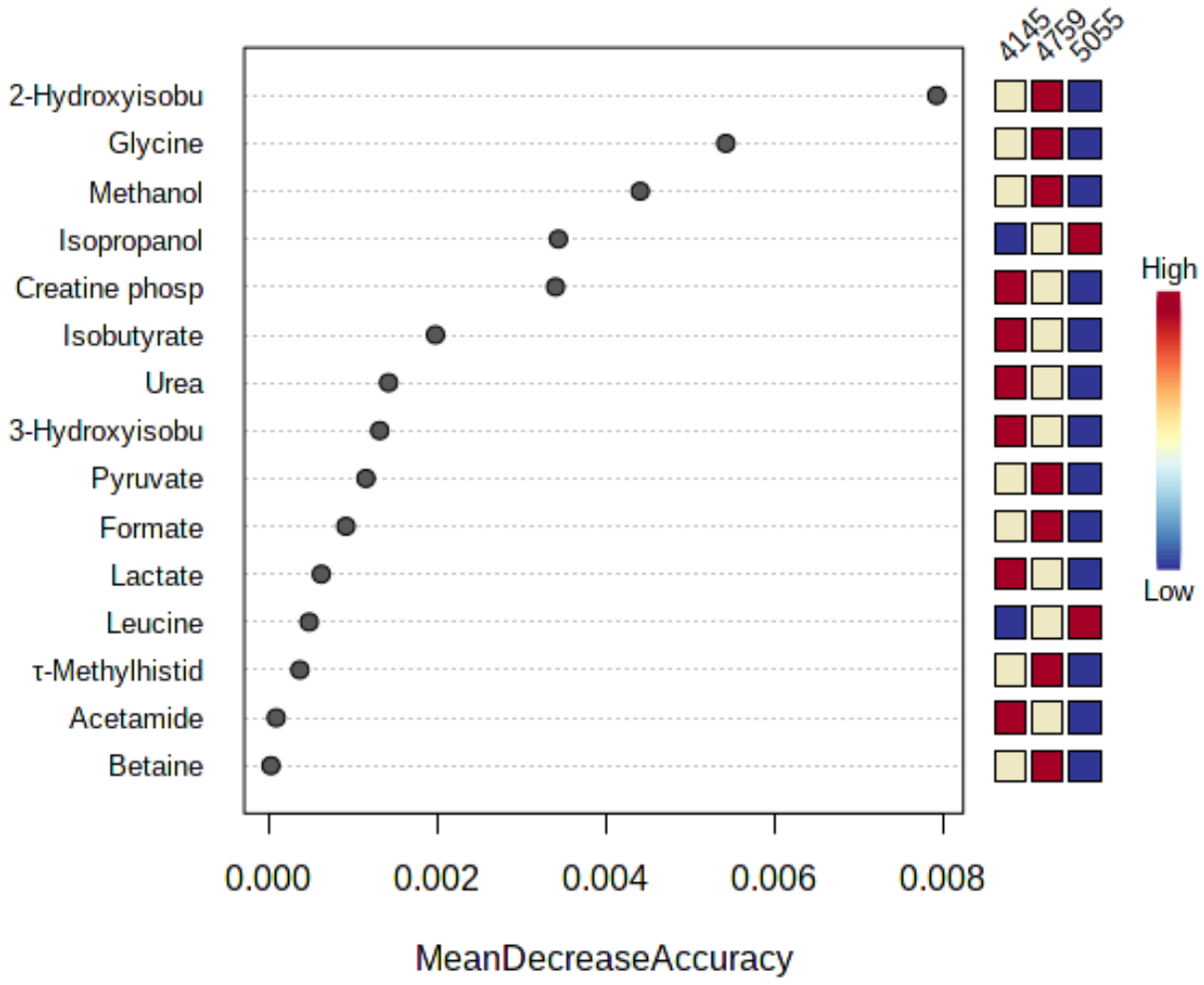

Figure 23. Significant Metabolites related to Dry Matter Intake identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decreased in classification accuracy when they are permuted.

Marbling Score NMR Data

Marbling Scores

Marbling score was a measure of the intermuscular fat in the ribeye measured between the 12th and 13th rib. The data normalization for marbling is shown in Appendix Figure 24. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was used to identify features that discriminate between marbling scores. Appendix Figure 25 shows the important features identified by ANOVA analysis. The post-hoc comparison column shows the comparisons between different levels that are significant given the *P*-value threshold. PCA and PLSDA shown in Appendix Figure 26 and Appendix Figure 27 respectively, show slight separation between marbling classification groups. The PLSDA validation with permutation shown in Appendix Figure 29 generated a p-value based on permutation of $P = 0.621$ (621/1000). This shows that there is no significant difference in marbling classes. Appendix Figure 28 looks at the number of components that would allow the best classifications. It indicates that there is viable classification based on metabolite features. Appendix Figure 30 shows an ordered list of the most important features in discriminating between marbling classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are 2-hydroxyisobutrate, betaine, π-methylhistidine, and valine. There were some patterns evident in metabolite concentrations. Appendix Figure 31 shows a similar analysis using a Random Forest approach. The single most important feature identified using this method was creatinine.

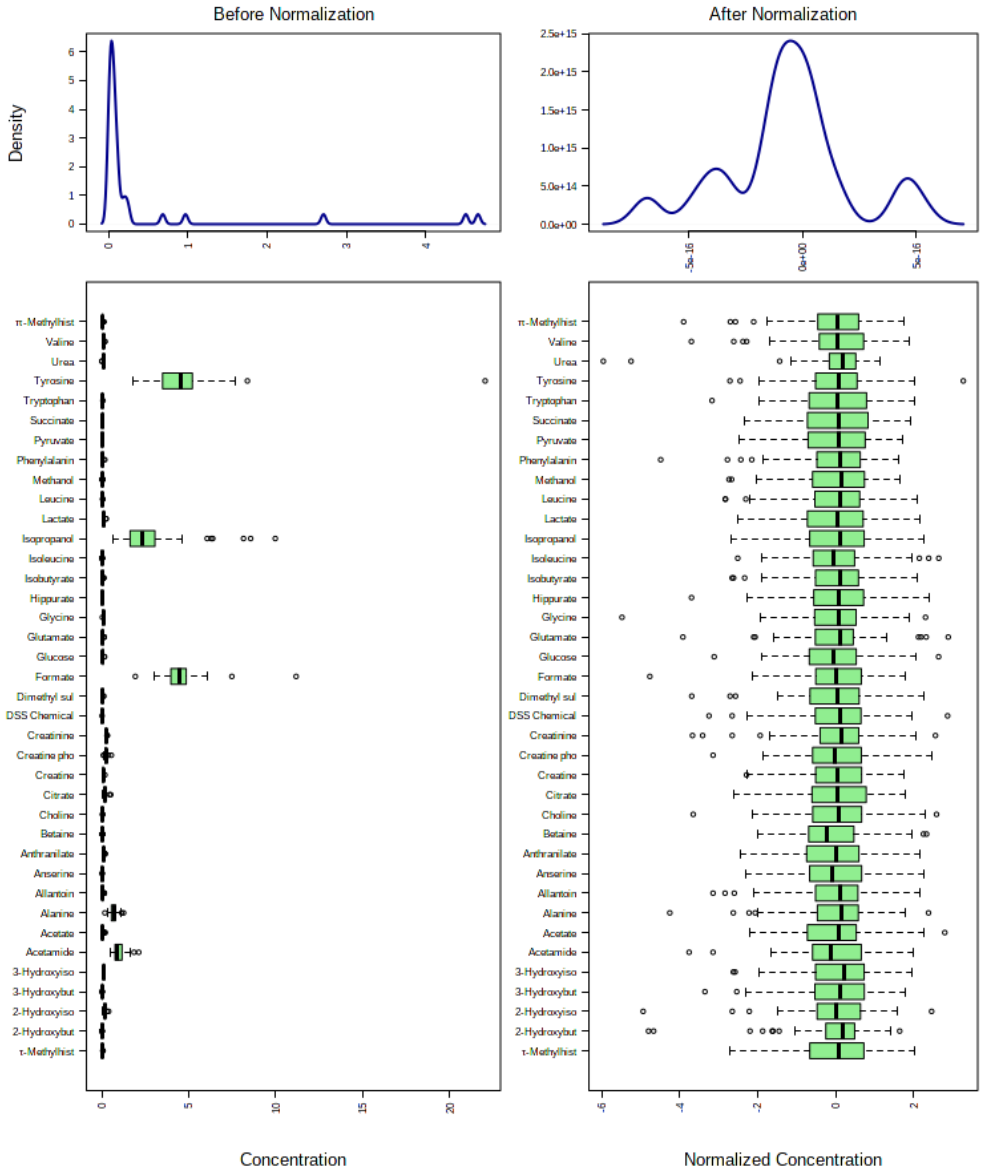

Figure 24. Marbling Score Data Before and After Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

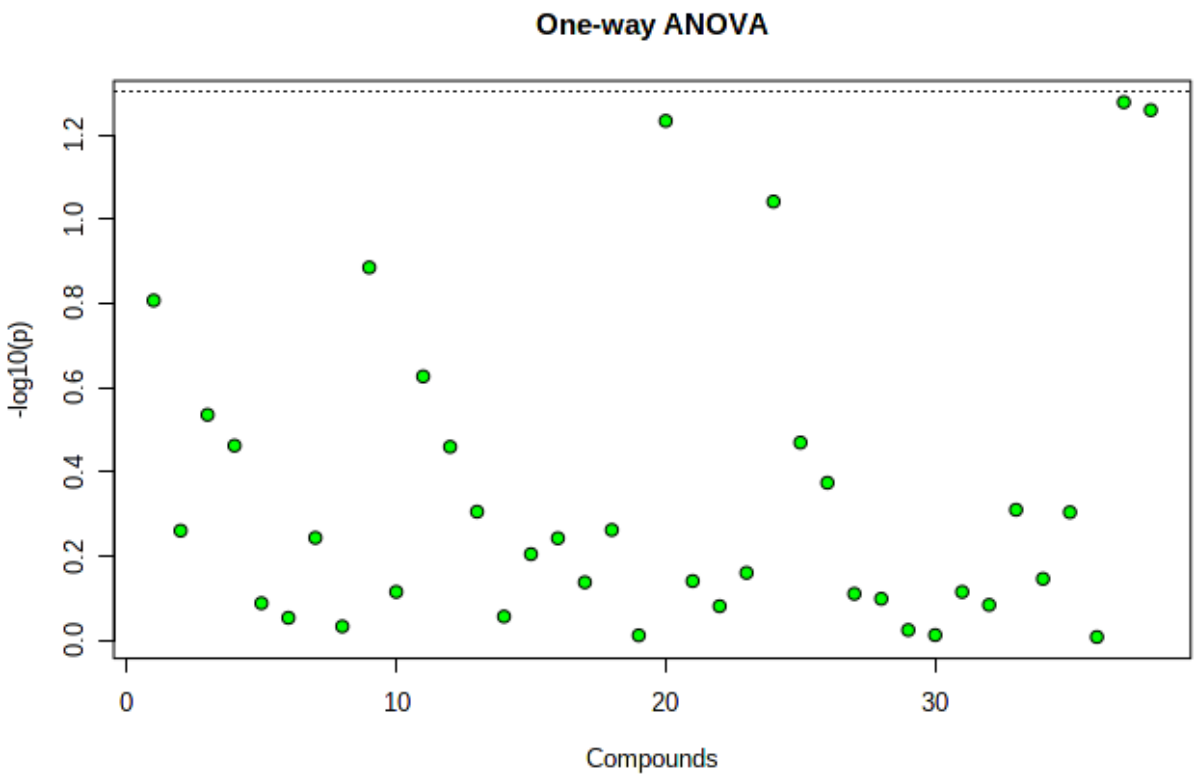

Figure 25. Significant features related to Marbling Score identified by Analysis of Variance: Important metabolites identified by ANOVA for marbling score categories, *P*-value threshold 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10(*P*) which identifies the level of significance for the metabolites. The reason for some metabolites shown as green even though they are at or above the level of significance is because were adjusted for FDR.

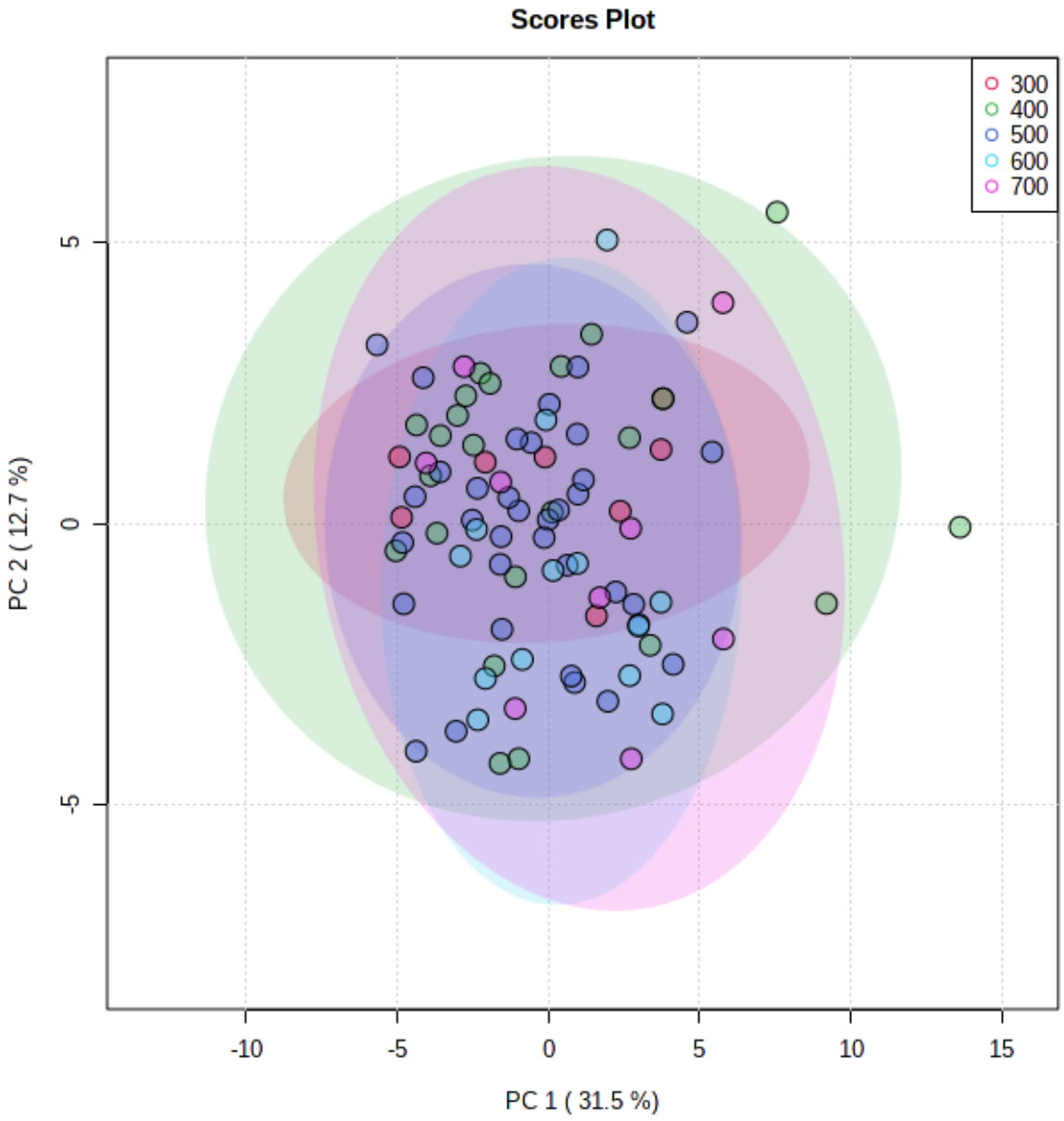

Figure 26. Significant features related to Marbling Score identified by PCA: Scores plot between the selected principal components or group classifications for marbling score. The variation explained by the first and second principal component is in parentheses.

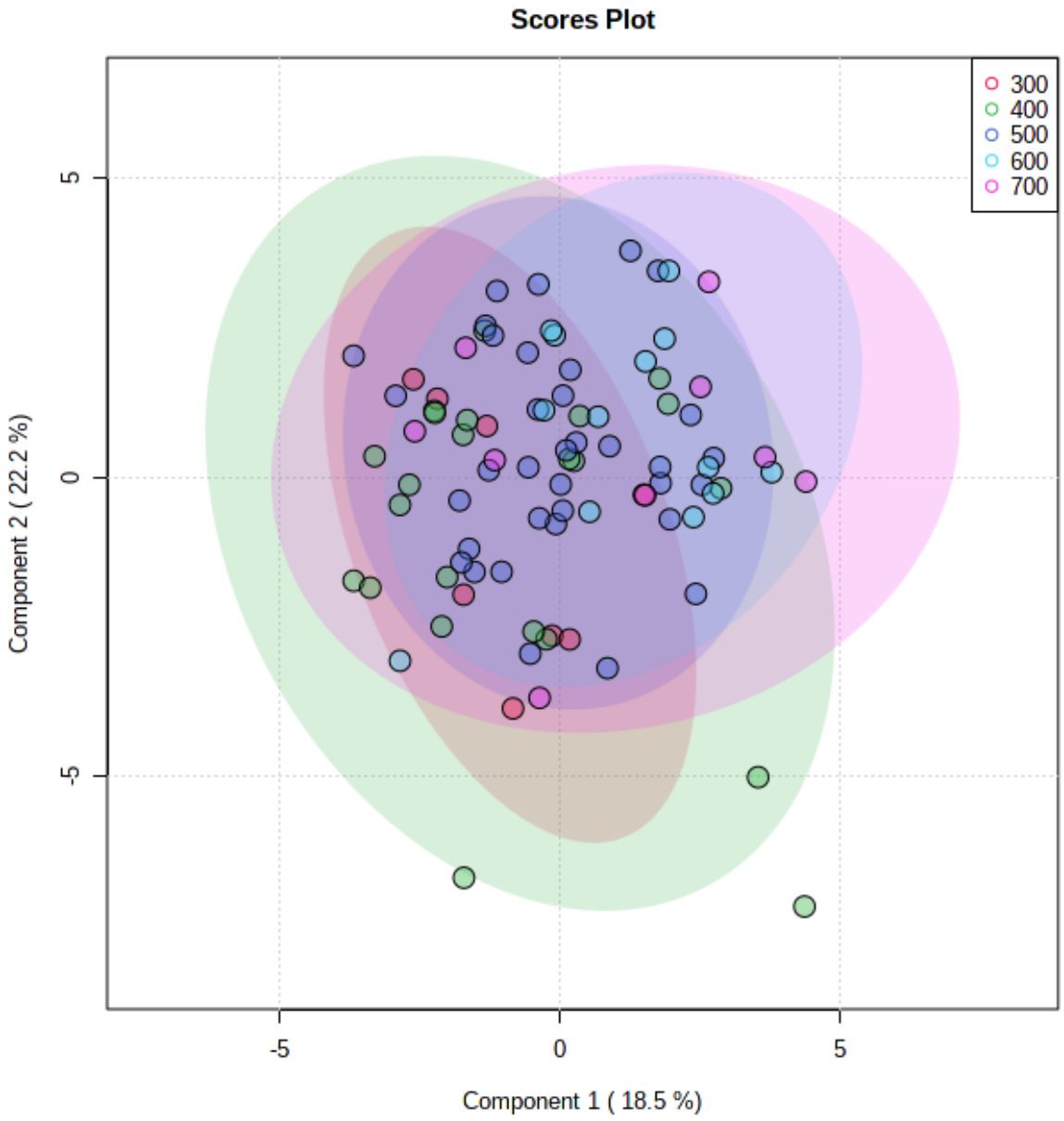

Figure 27. Significant features related to Marbling Score identified by PLS-DA: Scores plot for marbling scores between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is slight separation between the 5 groups of marbling scores.

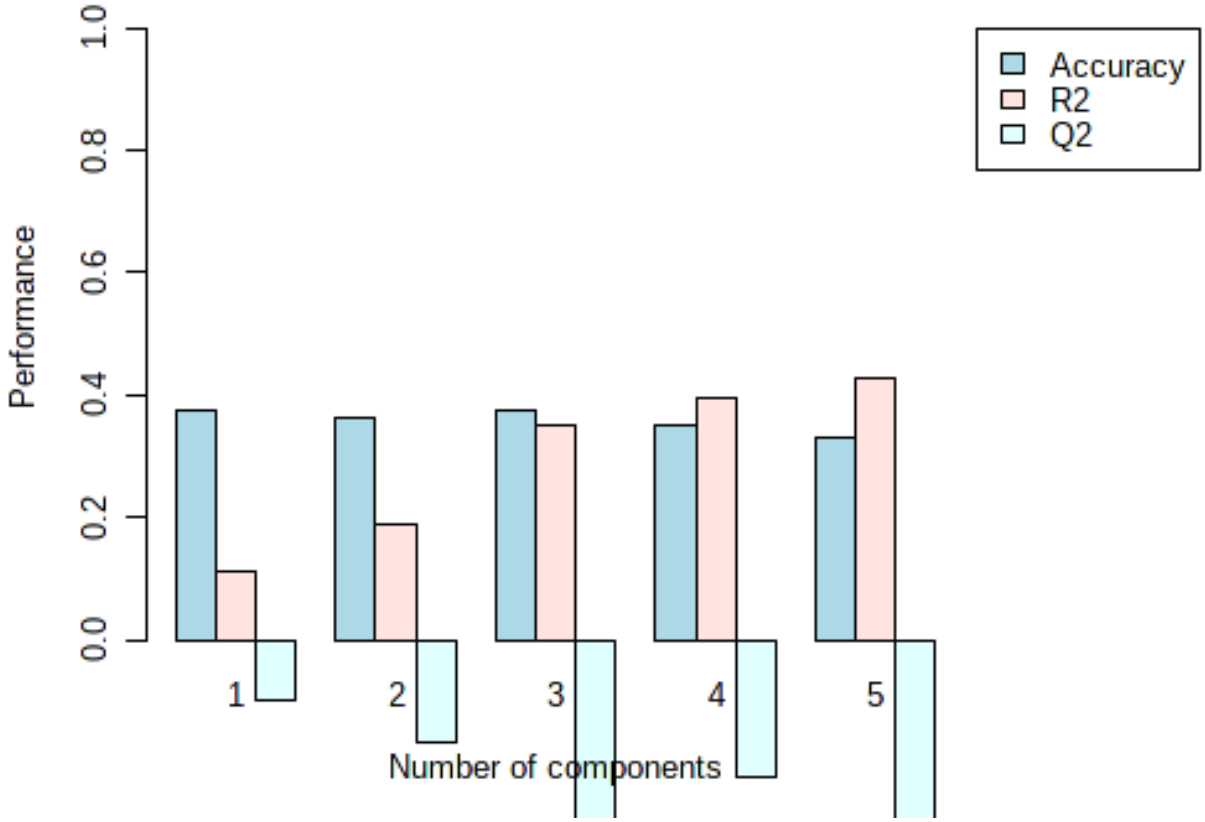

Figure 28. Significance related to Marbling Score identified by Components Test: PLS-DA classification using different numbers of components. The red star indicates the best classifier when present. The negative Q2 indicates that there is no significance between the classes.

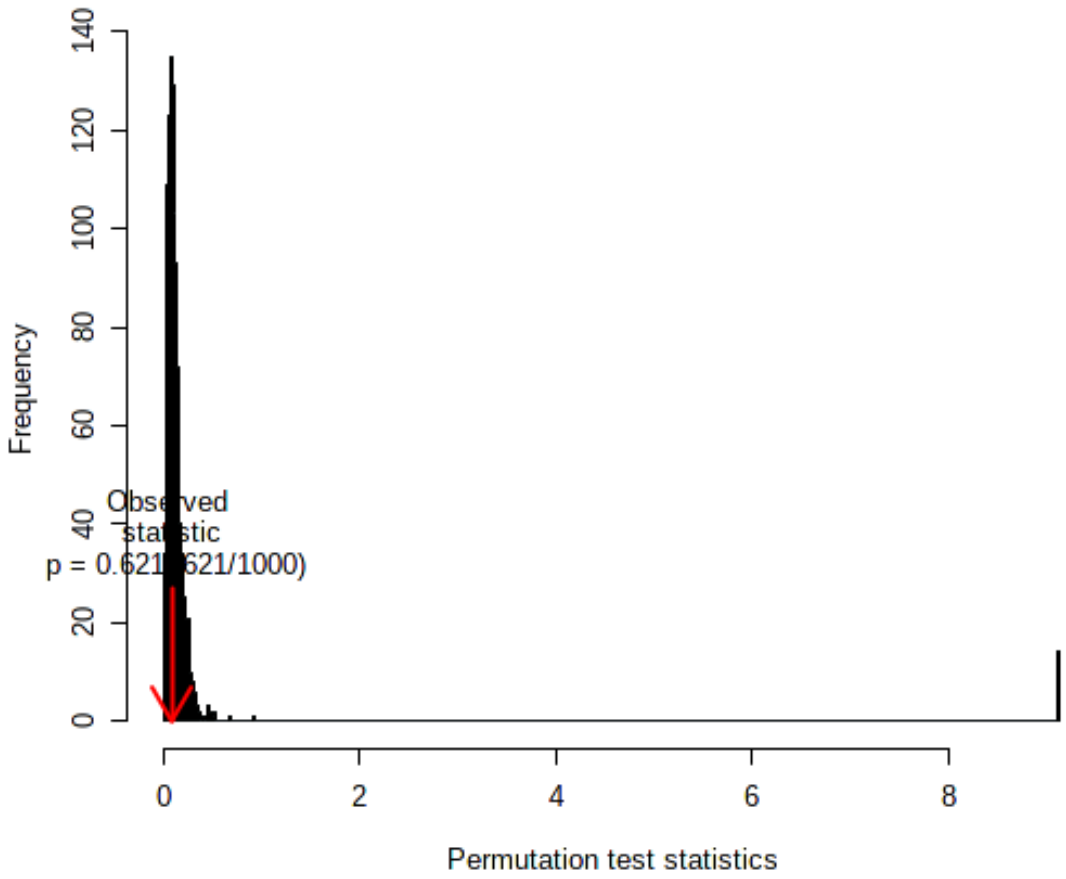

Figure 29. Significance related to Marbling Scores identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is *P* = 0.621 (621/1000). There is no significant evidence that there is a difference between the marbling classes.

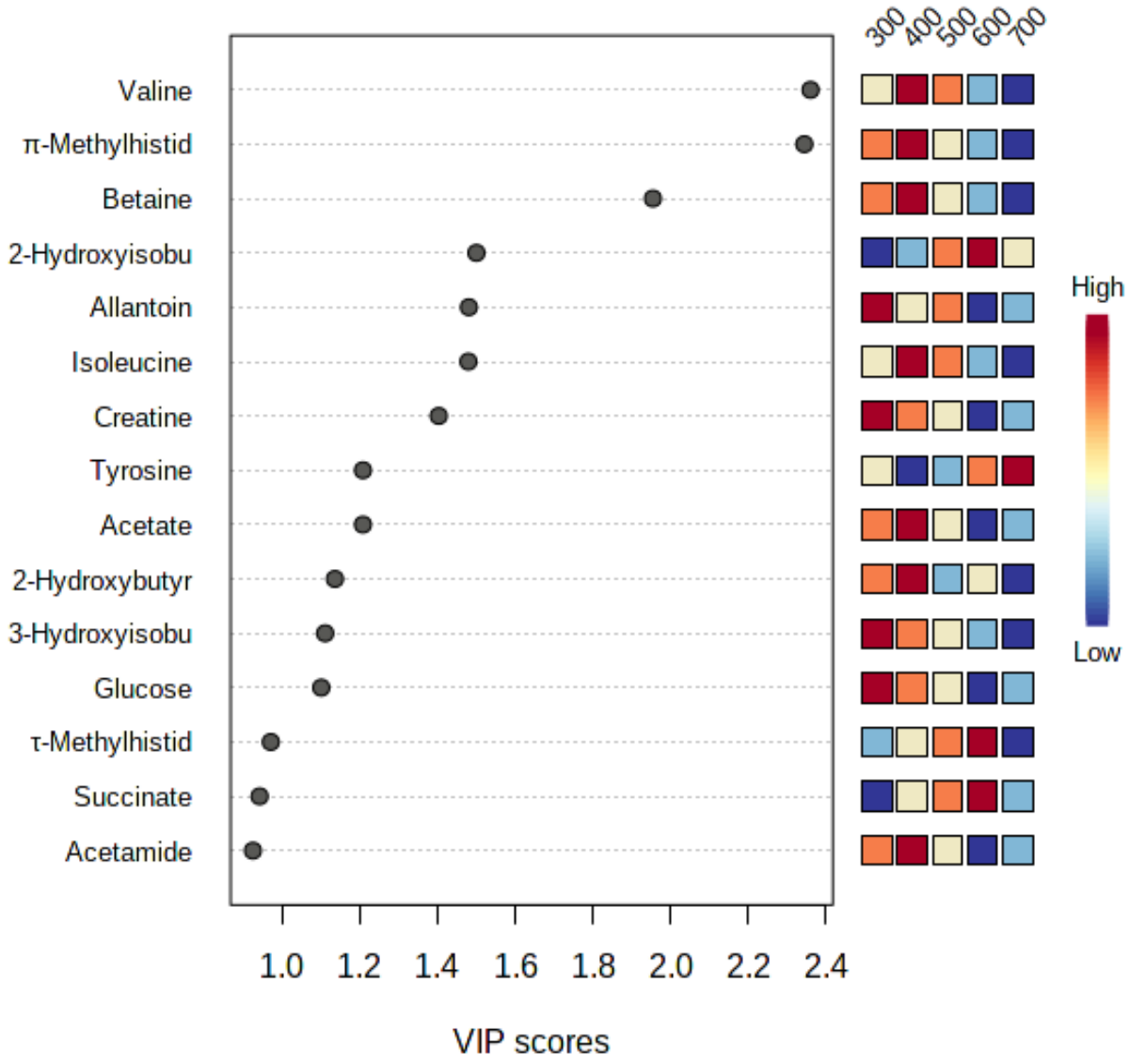

Figure 30. Significant Metabolites related to Marbling Score identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicated the relative concentrations of the corresponding metabolite in each marbling class.

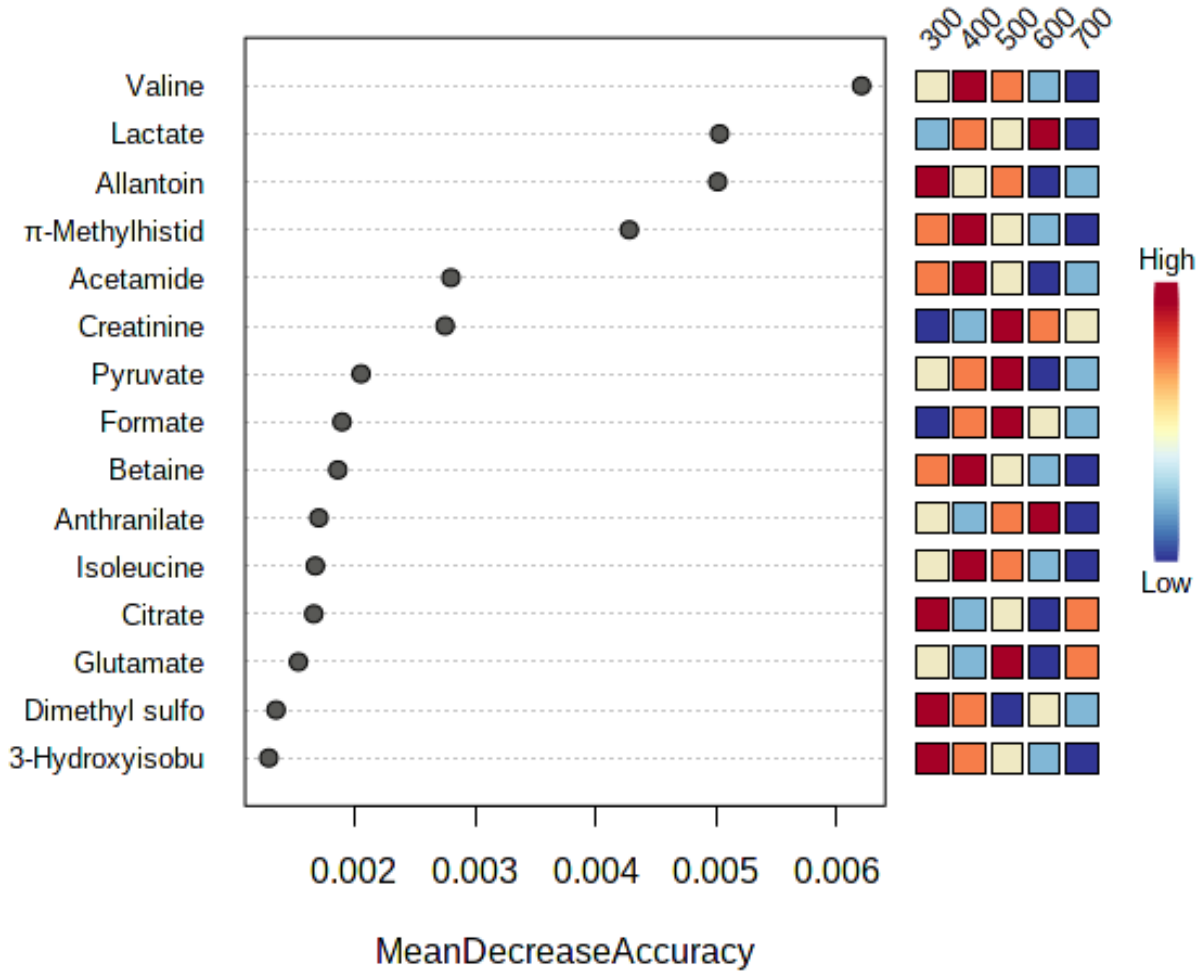

Figure 31. Significant Metabolites related to Marbling Score identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decreased in classification accuracy when they are permuted.

Quality Grade NMR Data

Quality Grade

Quality grade referred to USDA quality grade measured after harvest. This trait is scored based on physiological maturity and marbling. The data normalization for quality grade is shown in Appendix Figure 32. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was used to identify metabolite features that discriminate between quality grade classes. Appendix Figure 33 shows the features identified by ANOVA analysis, and no significant features were found. PCA and PLSDA shown in Appendix Figure 34 and Appendix Figure 35 respectively did not show separation between quality grade groups. The PLSDA validation with permutation shown in Appendix Figure 37 generated a *P*-value based on permutation of $P = 0.177$ (177/1000). This demonstrates that the model cannot differentiate based on quality grade class. Appendix Figure 36 looks at the number of components that would allow the best classifications. It indicates that there is no viable classification based on metabolite features possible for quality grade. Appendix Figure 38 shows an ordered list of the most important features in discriminating between back fat classifications from the PLSDA analysis. Feature with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are creatine, isopropanol, isobutyrate, succinate, and formate. No patterns were evident in the concentrations of metabolites by quality grade classification. Appendix Figure 39 shows a similar analysis using a Random Forest approach. The two most important features identified using this method were valine and glycine.

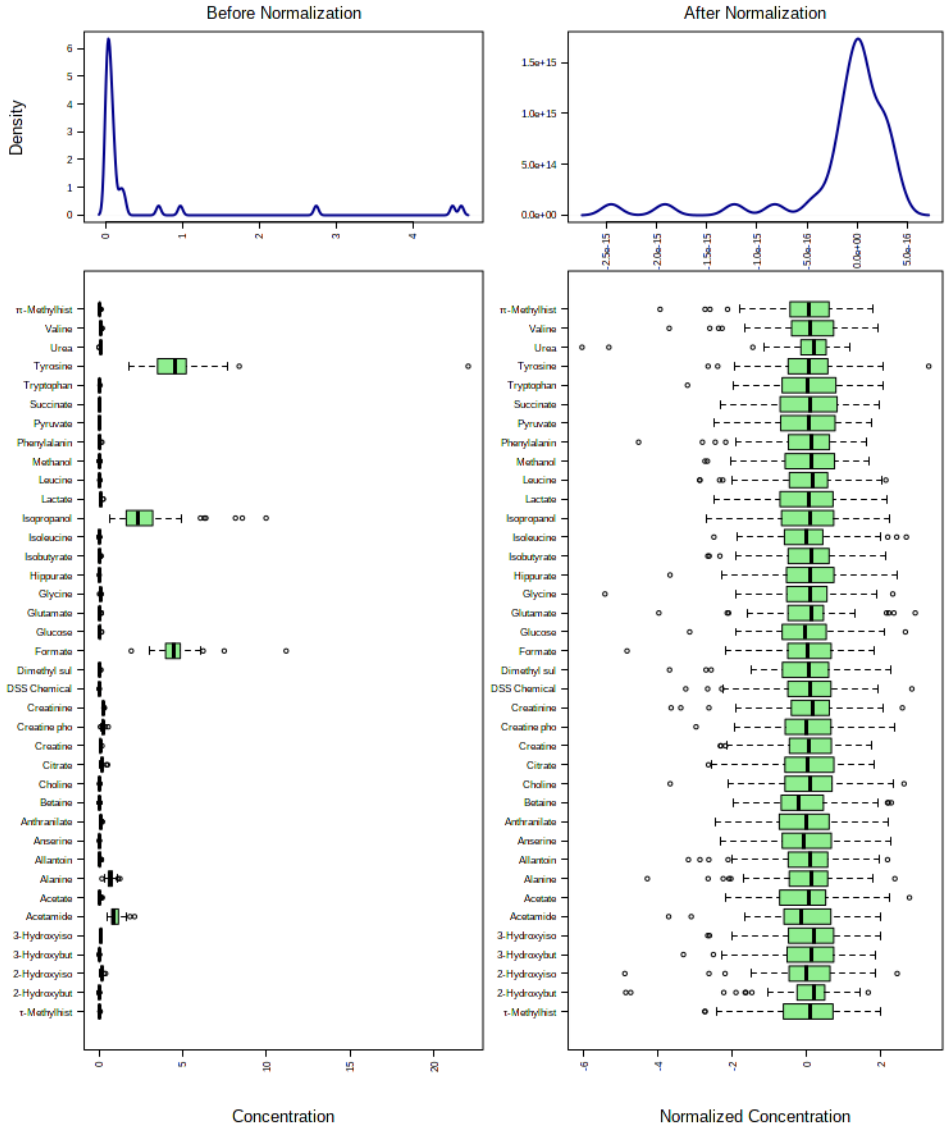

Figure 32. Quality Grade Data Before and After Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

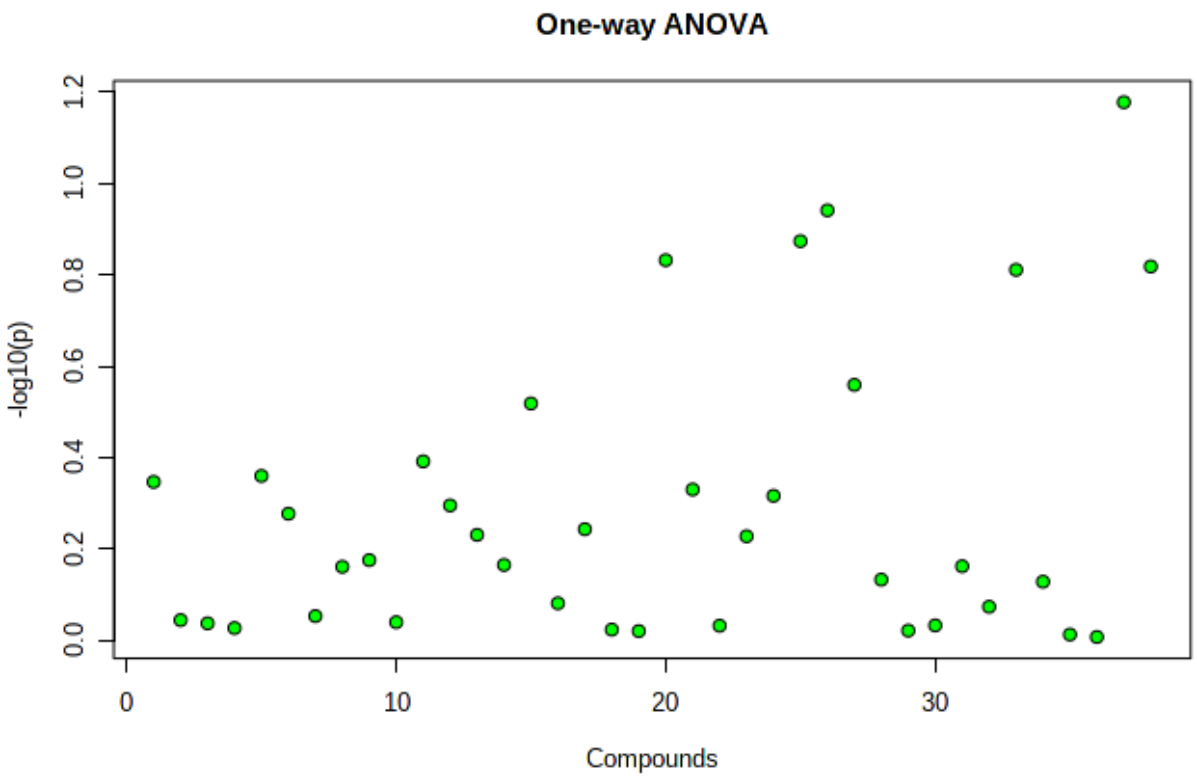

Figure 33. Significant features related to Quality Grade identified by Analysis of Variance: Important metabolites identified by ANOVA for Quality Grade categories, *P*-value threshold 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10 (*P*) which shows the significance of the metabolite for discriminating between quality grade classes. The reason for some metabolites shown as green even though they are at or above the level of significance due to FDR adjustment.

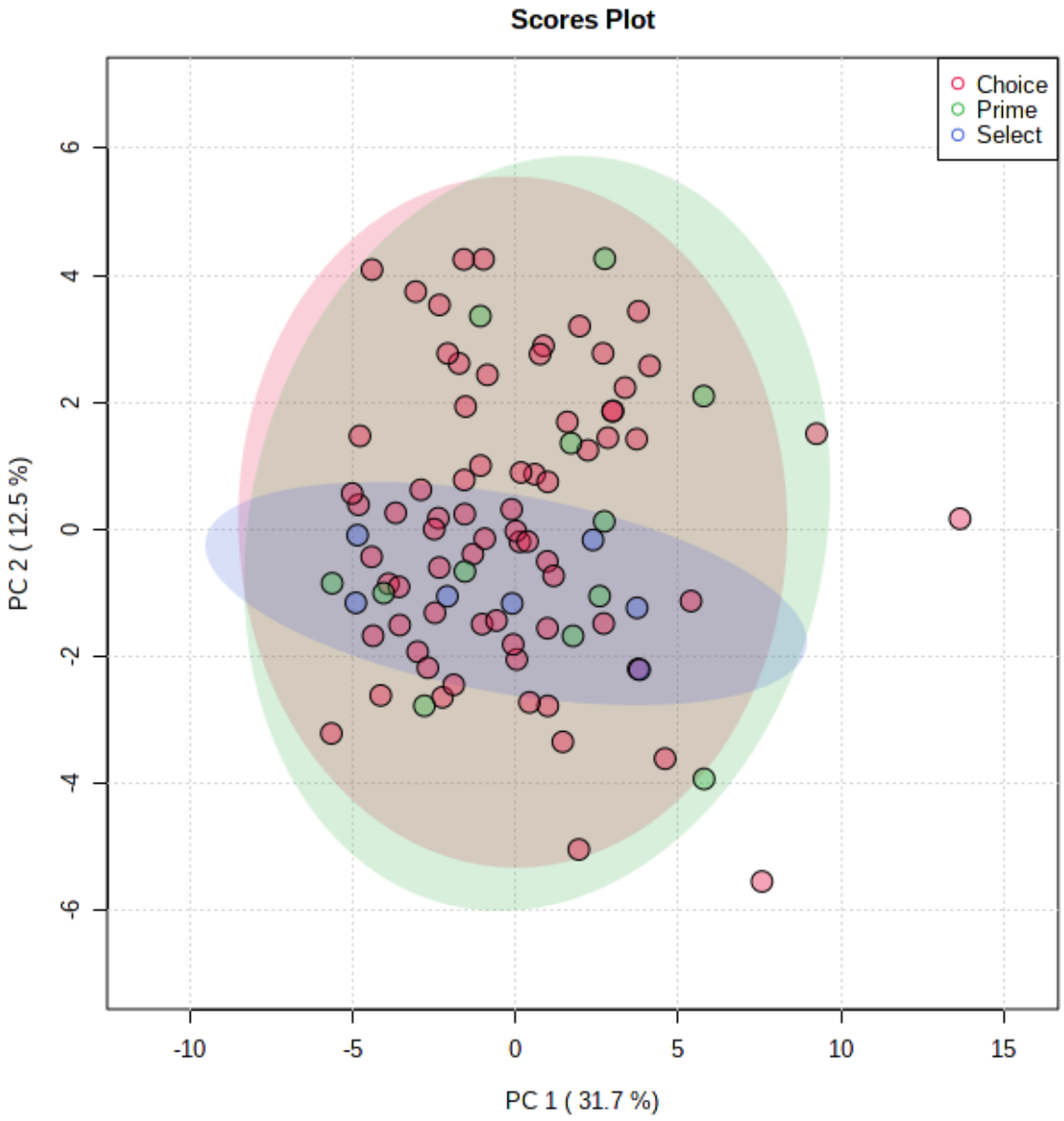

Figure 34. Significant features related to Quality Grade identified by PCA: Scores plot between the selected principal components or group classifications for quality grade. The variation explained by the first and second principal component is in parentheses.

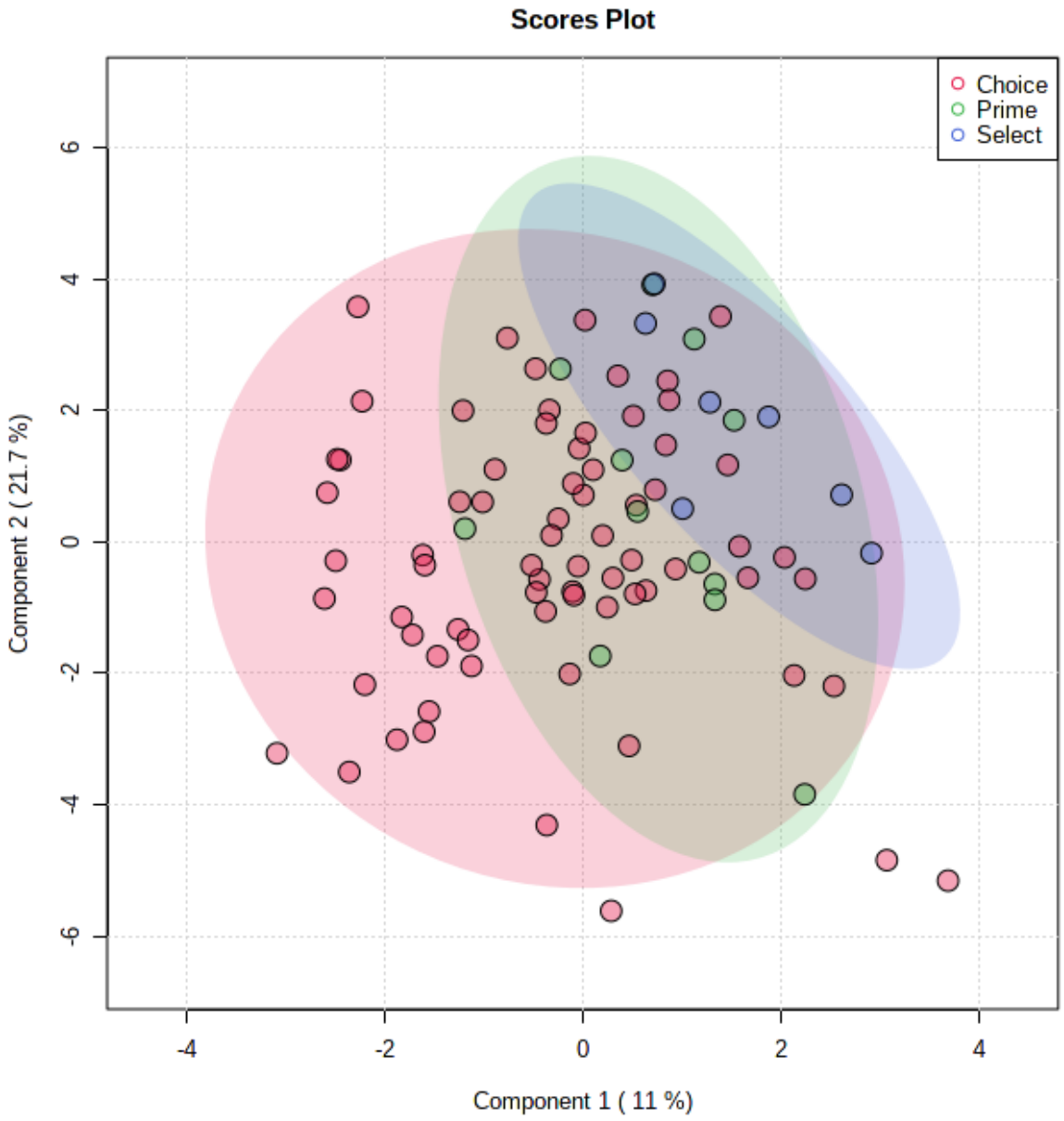

Figure 35. Significant features related to Quality Grade identified by PLS-DA: Scores plot for quality grade between selected principal components. The variance explained by the two principal components are shown in parentheses. In this image you can see some separation between classes of quality grade.

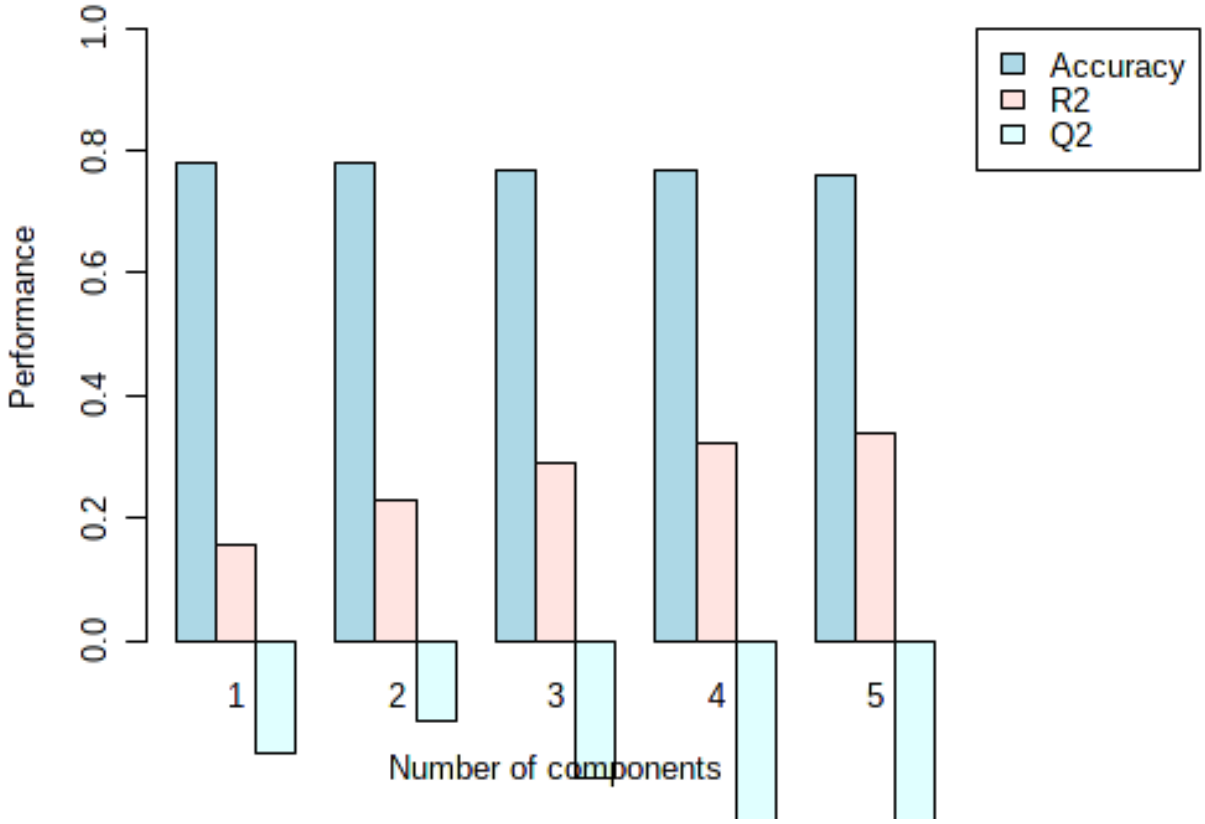

Figure 36. Significance related to Quality Grade identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present. The negative Q2 shows a lack of significance.

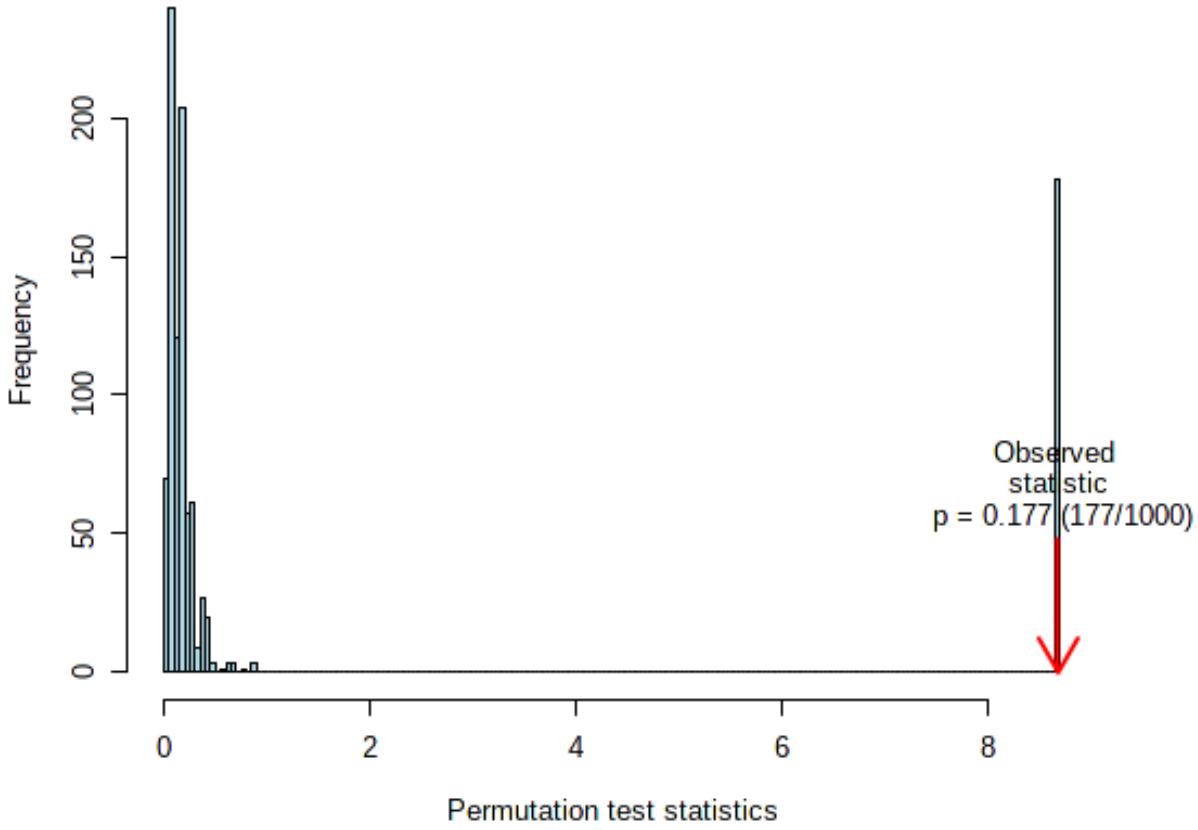

Figure 37. Significance related to Quality Grade identified by Permutation Test: PLS-DA model validation by permutation test based on separation distance. The *P*-value based on permutation is $P = 0.177$ (177/1000). There is no significant difference between quality grade classes in this analysis. The reason for the spike on the right hand is that this group is very uniform overall.

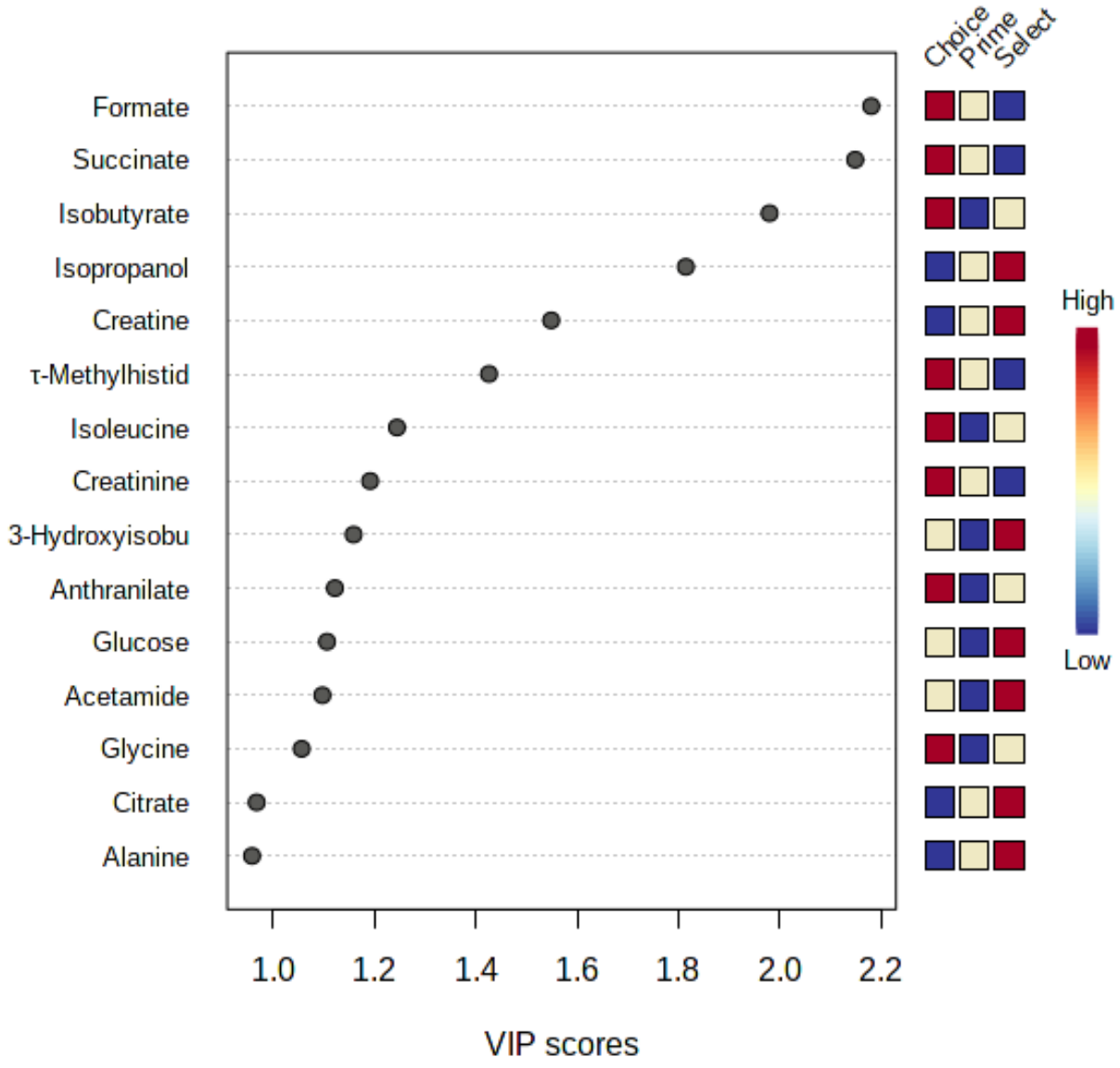

Figure 38. Significant Metabolites related to Quality Grade identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each quality grade class.

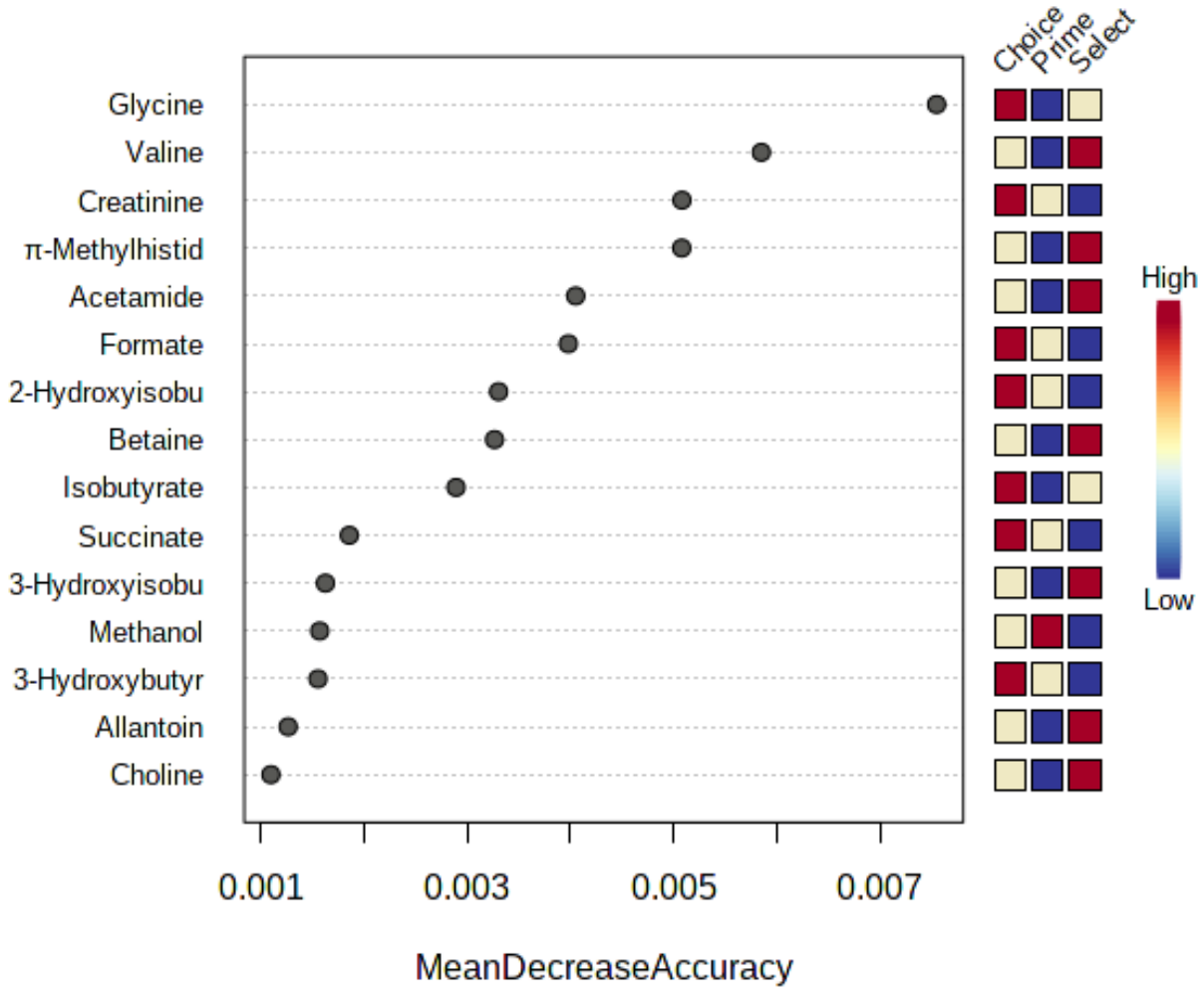

Figure 39. Significant Metabolites related to Quality Grade identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decreased in classification accuracy when they are permuted.

Residual Feed Intake NMR Data

Residual Feed Intake (RFI)

Another trait analyzed was residual feed intake (RFI). This a measure of feed efficiency and is calculated based on expected intake for the rate of growth and the actual individual intake of the animals as measured in GrowSafe feedbunks. The data normalization for RFI is shown in Appendix Figure 40. Data was normalized by sum using a row-wise procedure and then log transformed. PCA and PLSDA shown in Appendix Figure 41 and Appendix Figure 42 respectively did not show separation between RFI classification groups. The PLSDA validation with permutation shown in Appendix Figure 44 generated a *P*-value based on permutation $P = 0.997$ (997/1000). This shows that the model cannot predict RFI classification. Appendix Figure 43 looks at the number of components that would allow the best classifications. It indicates that there is no viable classification based on metabolite features possible for RFI. Appendix Figure 45 shows an ordered list of the most important features in discriminating between RFI classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are isobutyrate, urea, alanine, and glutamate. All metabolite features with the expectation of Alanine were highest in the high RFI class (low feed efficiency). Appendix Figure 46 shows a similar analysis using a Random Forest approach. The single most important feature identified using this method was glutamate.

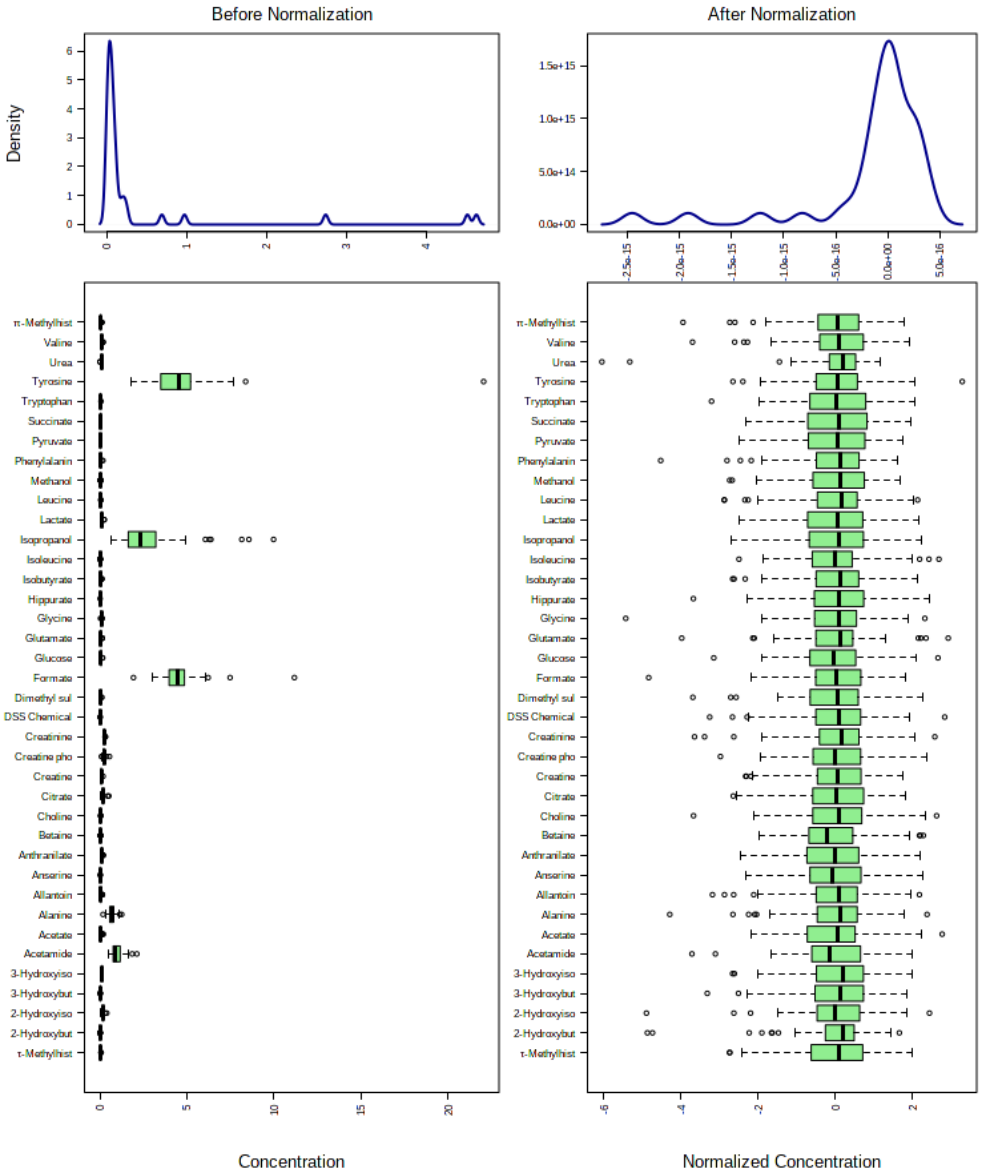

Figure 40. Residual Feed Intake (RFI) Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

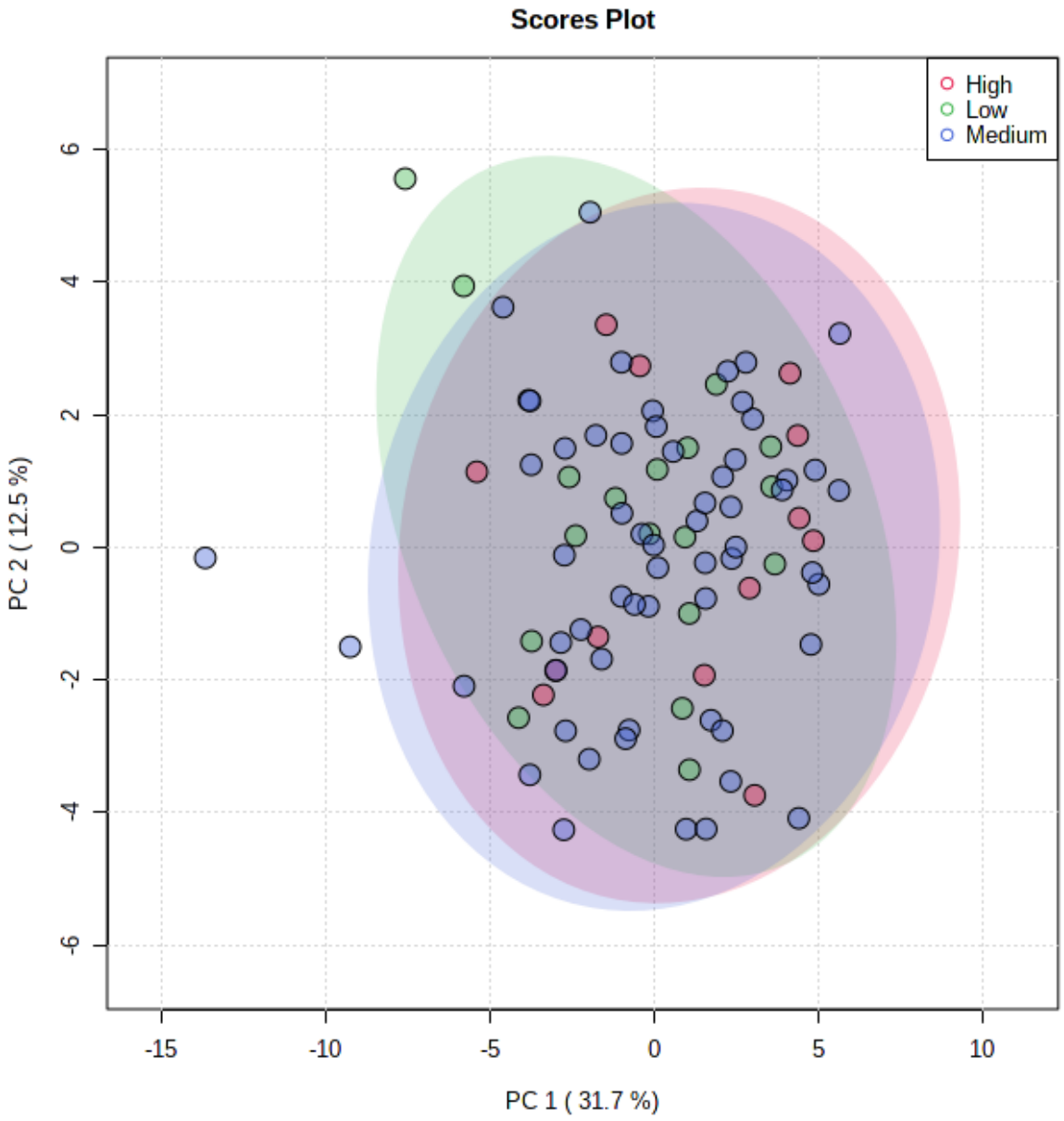

Figure 41. Significant features related to RFI identified by PCA: Scores plot between the selected principal components or group classifications for RFI. The variation explained by the first and second principal component is in parentheses.

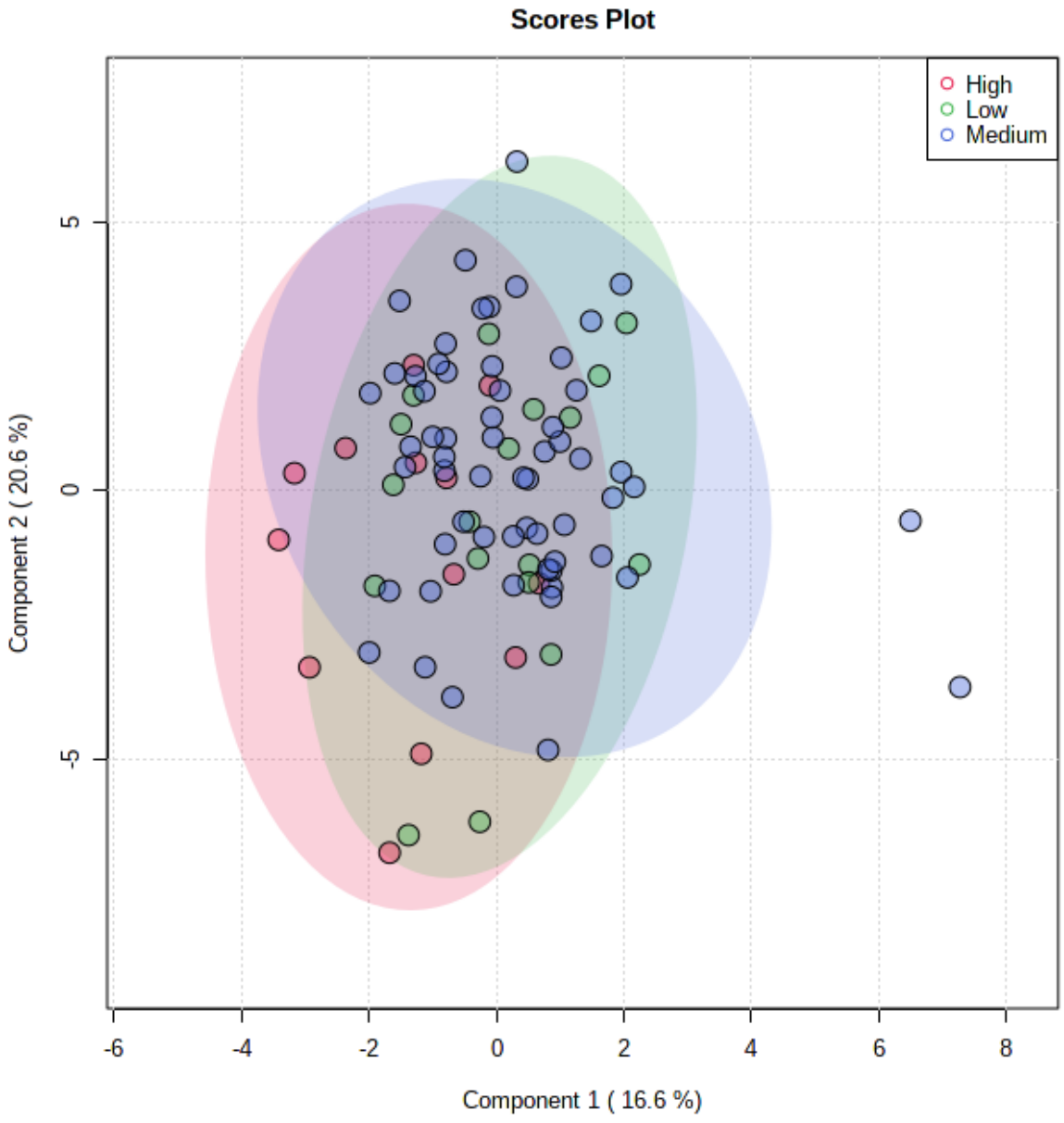

Figure 42. Significant features related to RFI identified by PLS-DA: Scores plot for RFI between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image you can see slight separation between the RFI groups.

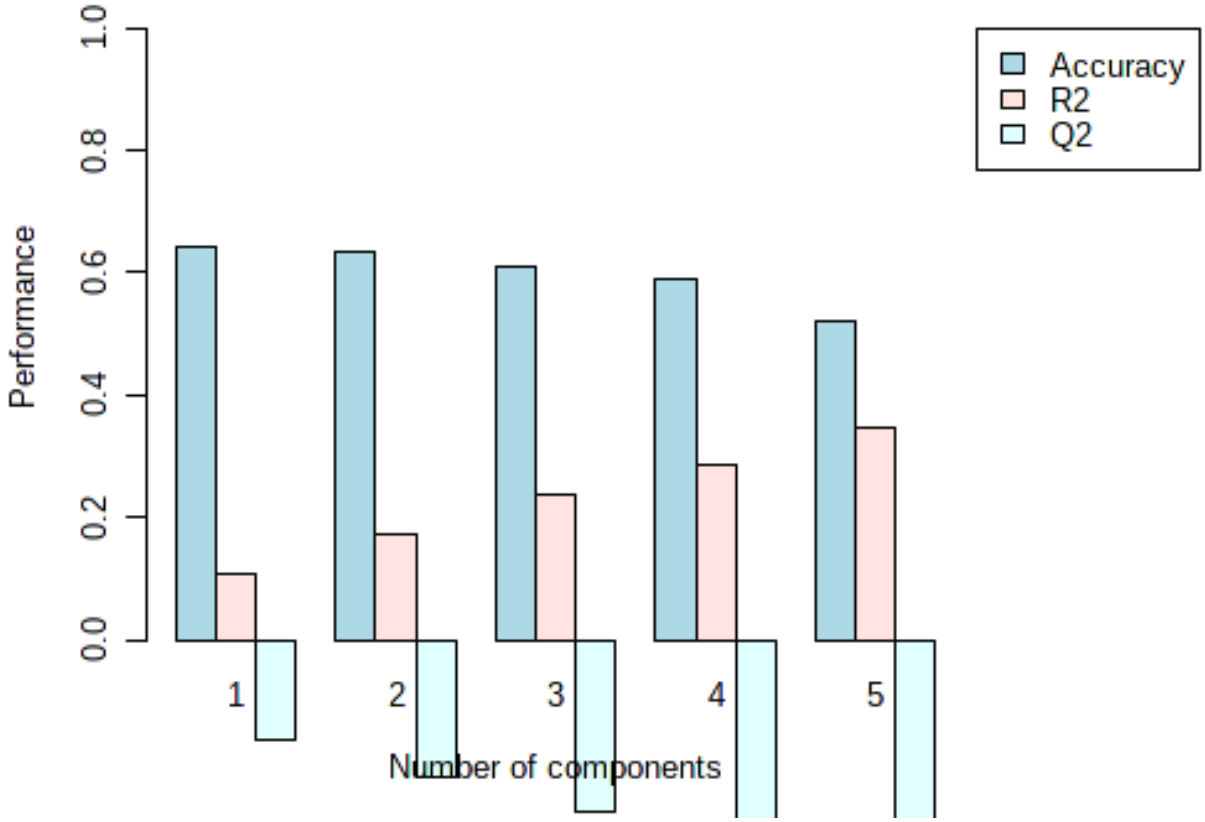

Figure 43. Significance related to RFI identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present, the negative Q2 indicates a lack of significance.

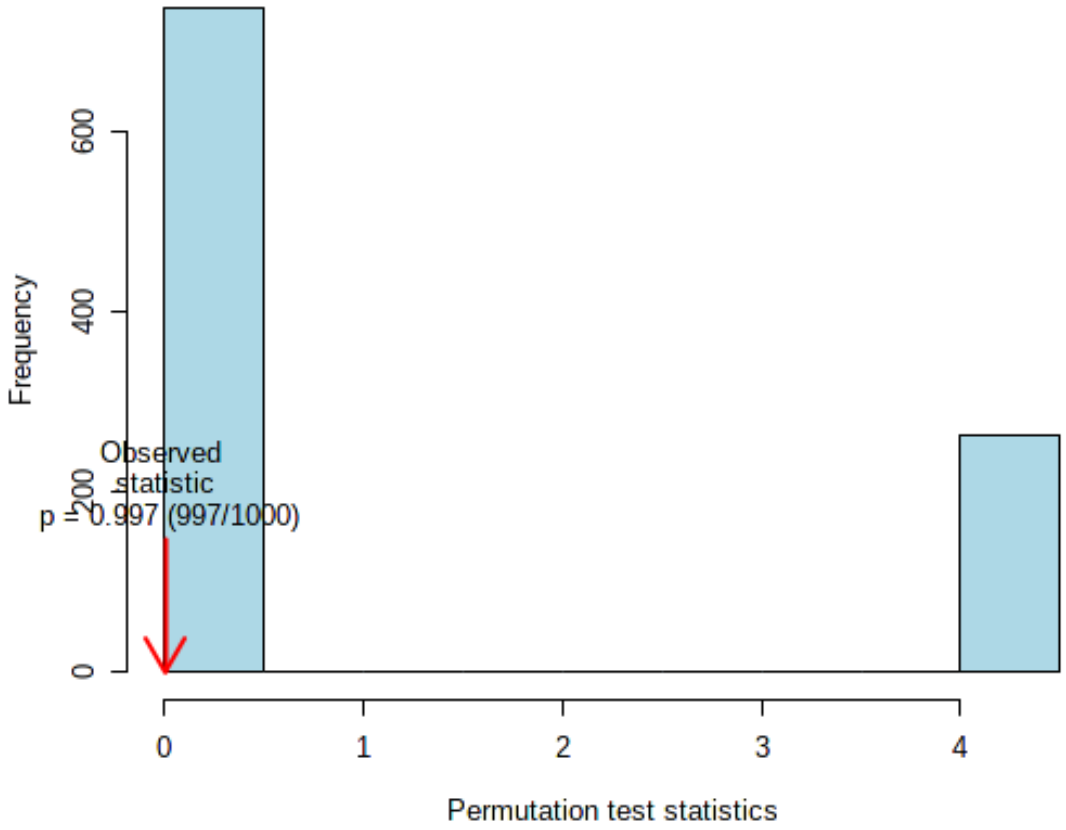

Figure 44. Significance related to RFI identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P* value based on permutation is *P* = 0.997 (997/1000). The *P*-value shows that there no difference between RFI classes in this analysis.

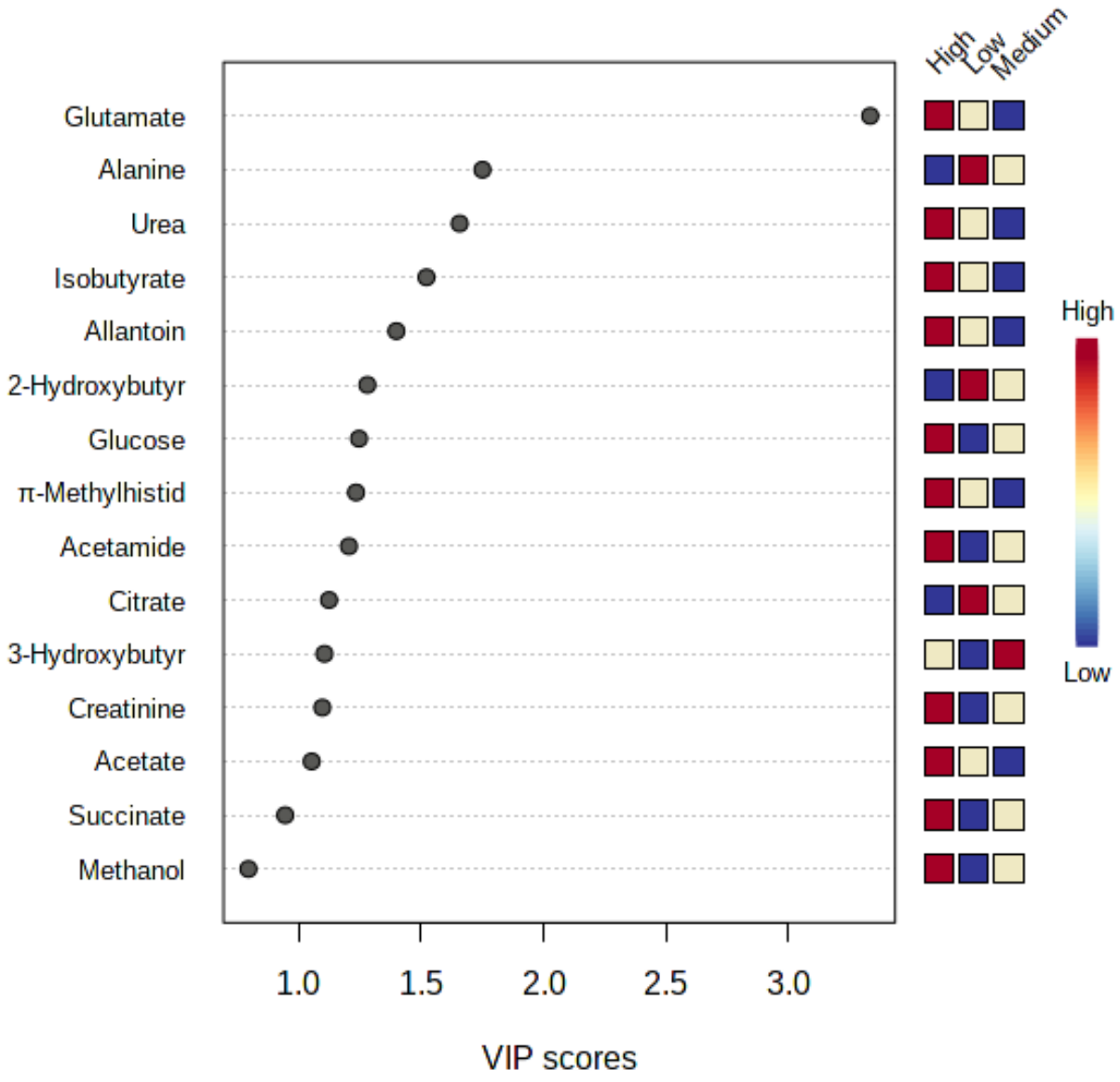

Figure 45. Significant Metabolites related to RFI identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each RFI class.

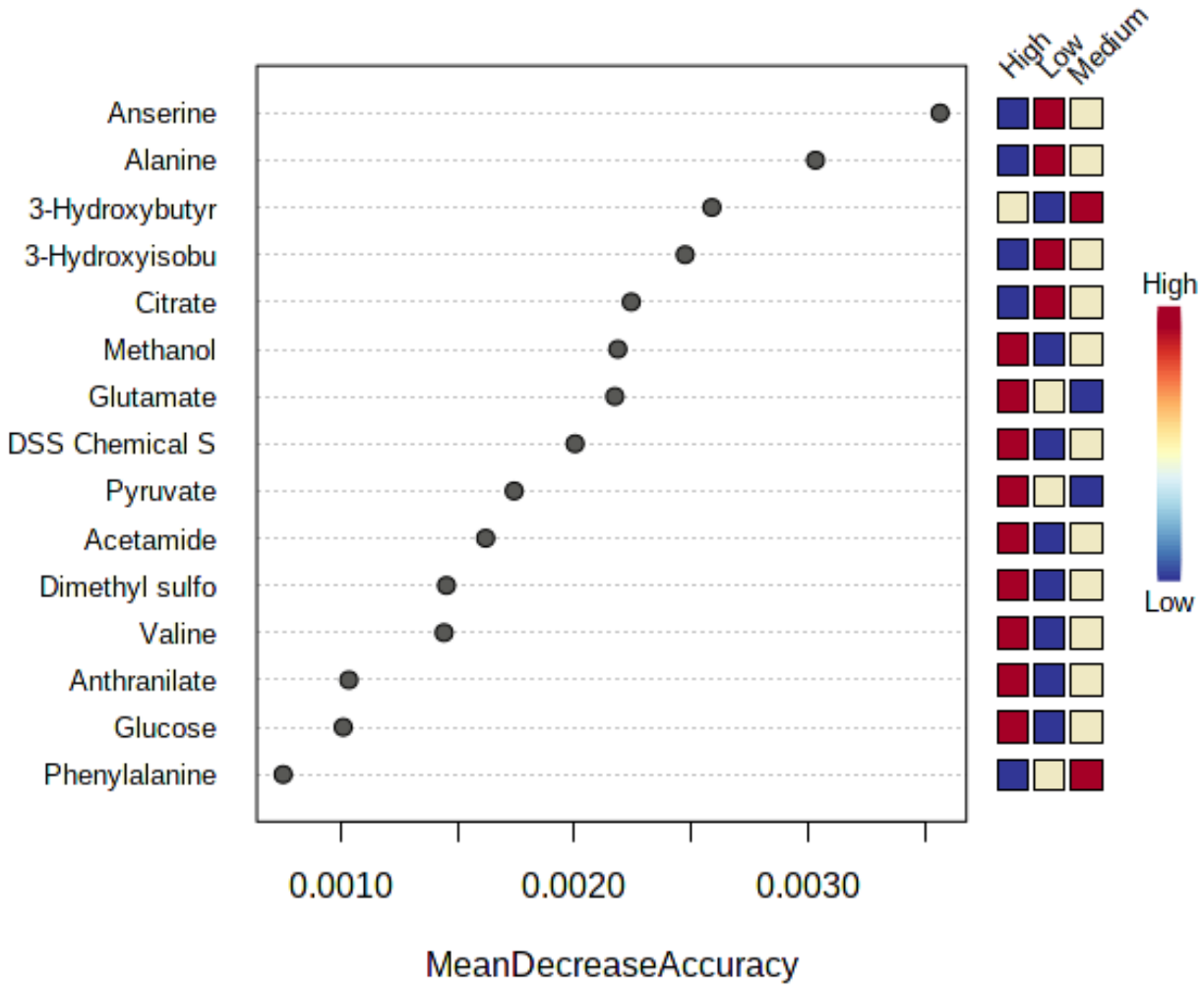

Figure 46. Significant metabolites related to RFI identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Yield Grade NMR Data

Yield Grade

Yield grade referred to USDA carcass yield grade measured after harvest. The data normalization for yield grade is shown in Appendix Figure 47. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was used to identify features that discriminate between yield grade classes. Appendix Figure 48 shows the important features identified by ANOVA analysis. There were no significant features identified for yield grade class. PCA and PLSDA shown in Appendix Figure 49 and Appendix Figure 50 respectively showed little to no separation between yield grade classification groups. The PLSDA validation with permutation shown in Appendix Figure 52 generated a *P*-value based on permutation of $P = 0.439$ (439/1000). This shows that the model cannot differentiate yield grade classes. Appendix Figure 51 looks at the number of components that would allow the best classifications. It indicates that there is no viable classification based on metabolite features possible for yield grade. Appendix Figure 53 shows an ordered list of the most important features in discriminating between yield grade classifications from the PLSDA analysis. Features with a VIP score above 1.2 are considered important in this analysis. The features above this threshold are betaine, dimethyl sulfoxide, hippurate, 2-hydroxybutyrate, valine, urea, tryptophan, succinate, allantoin, creatinine, methanol, and phenylalanine. No patterns were evident in concentrations of metabolites by yield grade classification. Appendix Figure 54 shows a similar analysis using a random forest approach. The two most important features identified using this method were phenylalanine and valine.

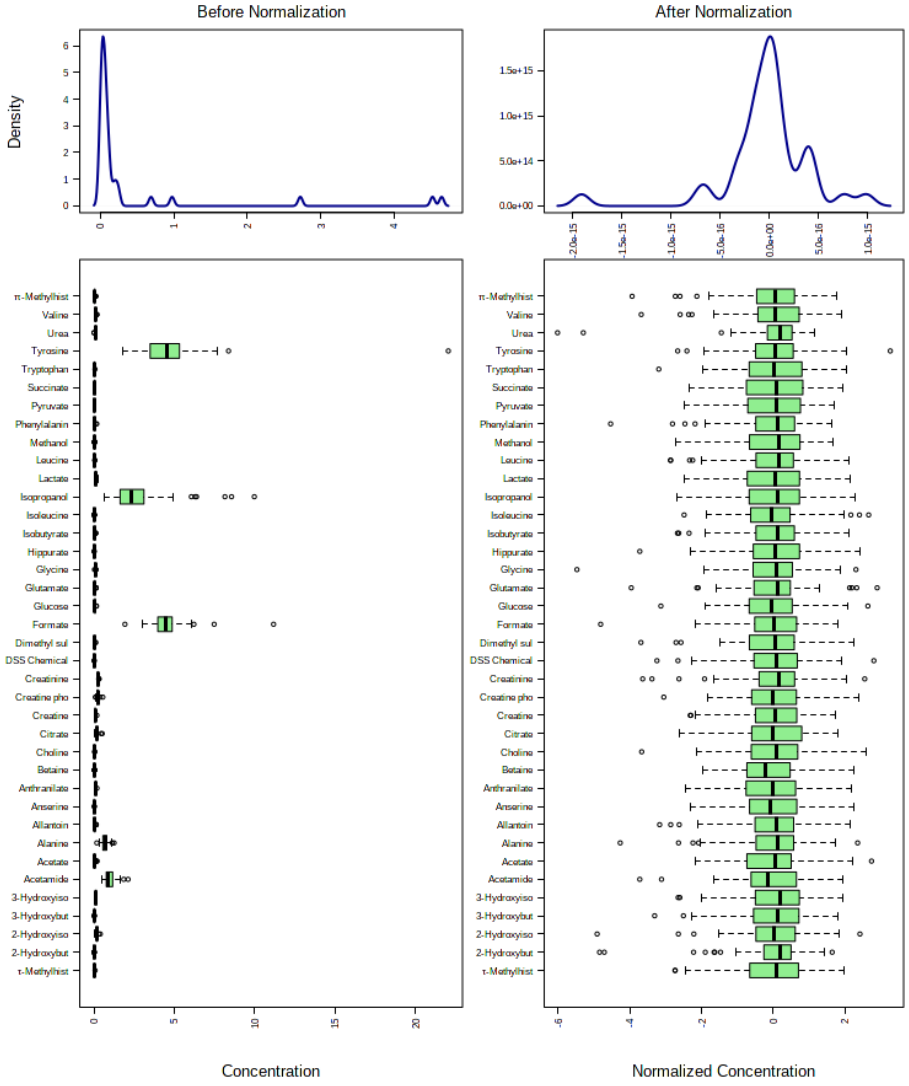

Figure 47. Yield Grade Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

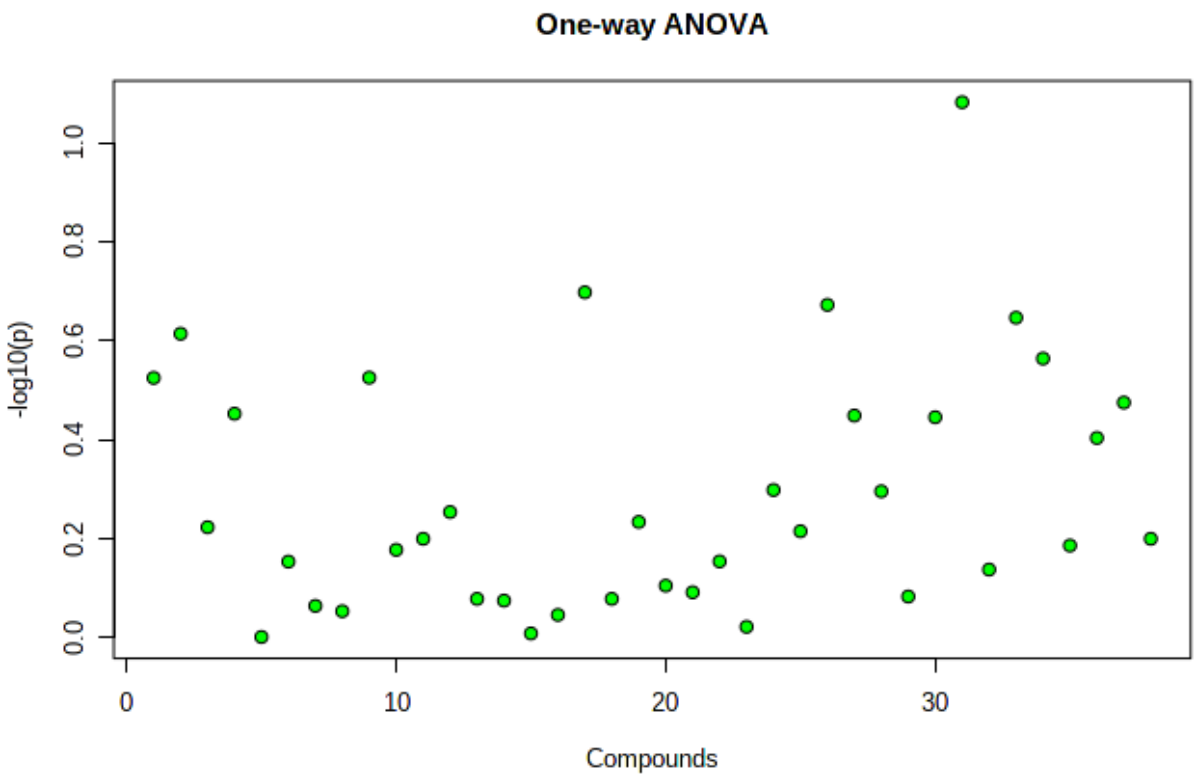

Figure 48. Significant features related to Yield Grade identified by Analysis of Variance: Important metabolites identified by ANOVA for yield grade categories, *P*-value threshold 0.05. . On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10 (*P*) which shows significance of the metabolites for predicting yield grade class. The reason for some metabolites shown as green even though they are at or above the level of significance because they were adjusted for FDR.

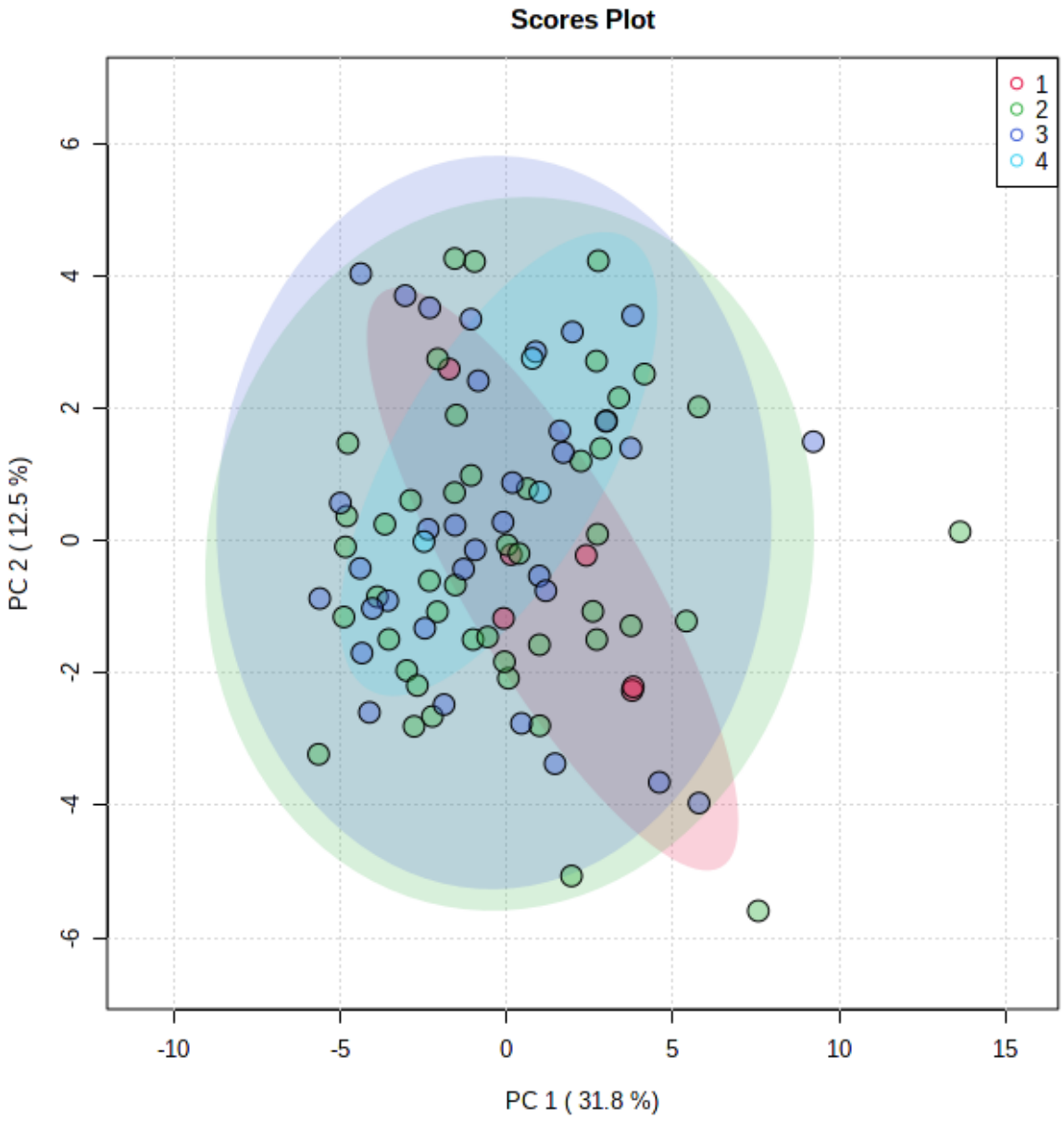

Figure 49. Significant features related to Yield Grade identified by PCA: Scores plot between the selected principal components or group classifications for yield grade. The variation explained by the first and second principal component is in parentheses.

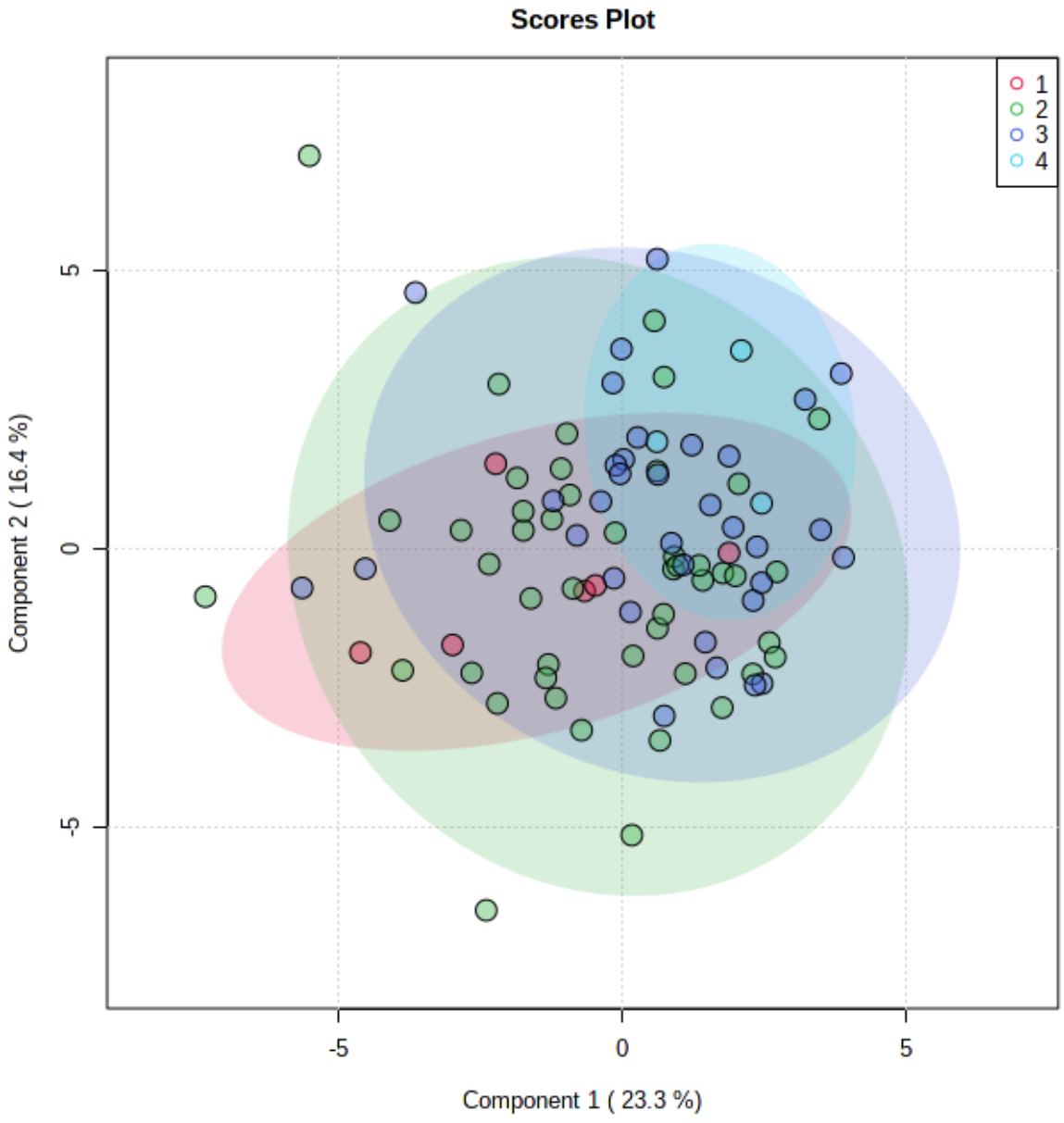

Figure 50. Significant features related to Yield Grade identified by PLS-DA: Scores plot for yield grade between the selected principal components. The variance explained by the two principal components are shown in parentheses. In the image there is no significant separation between the yield grade groups.

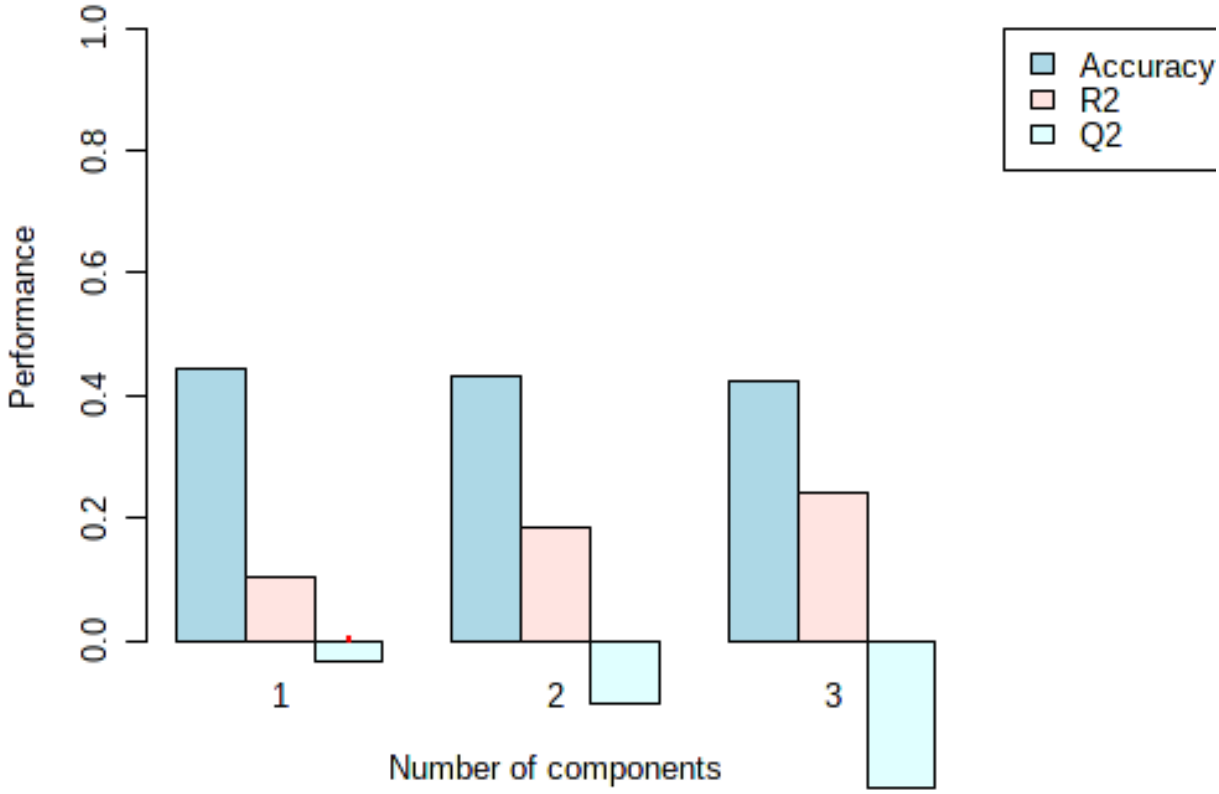

Figure 51. Significance related to Yield Grade related to Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present, the negative Q2 shows a lack of significance.

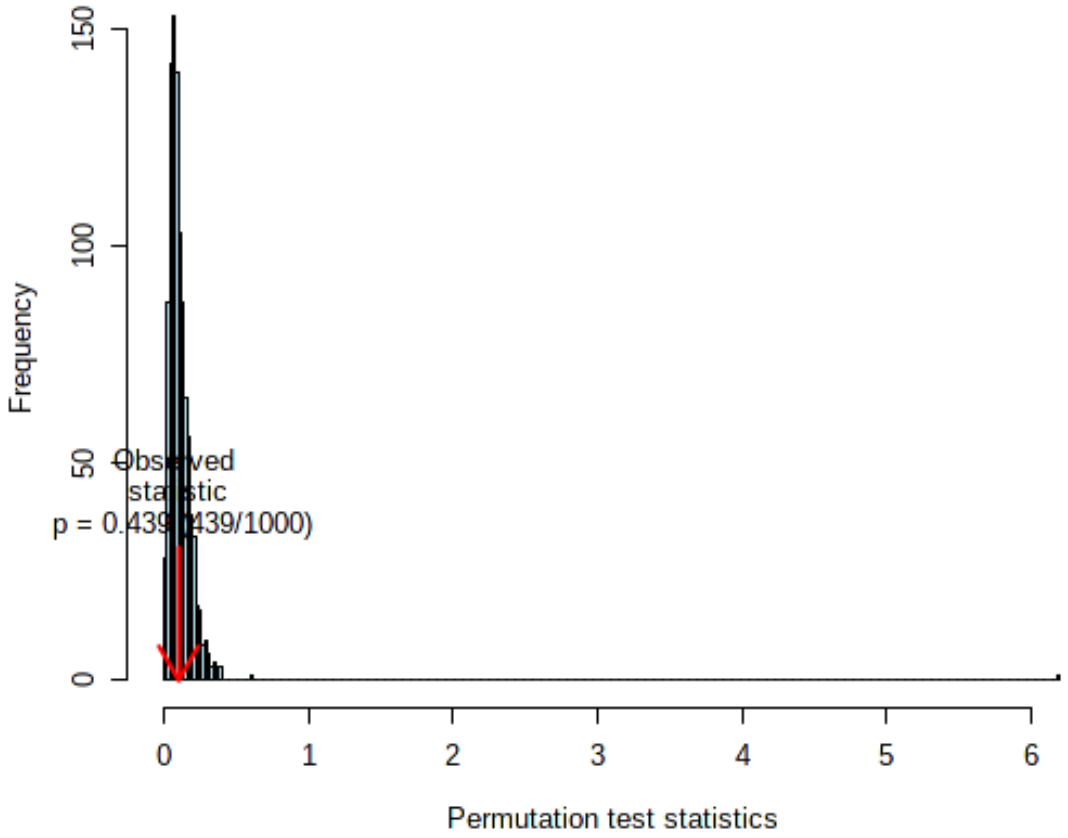

Figure 52. Significance related to Yield Grade identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is $P = 0.439$ (439/1000). The *P*-value shows that there is no difference between yield grade classes.

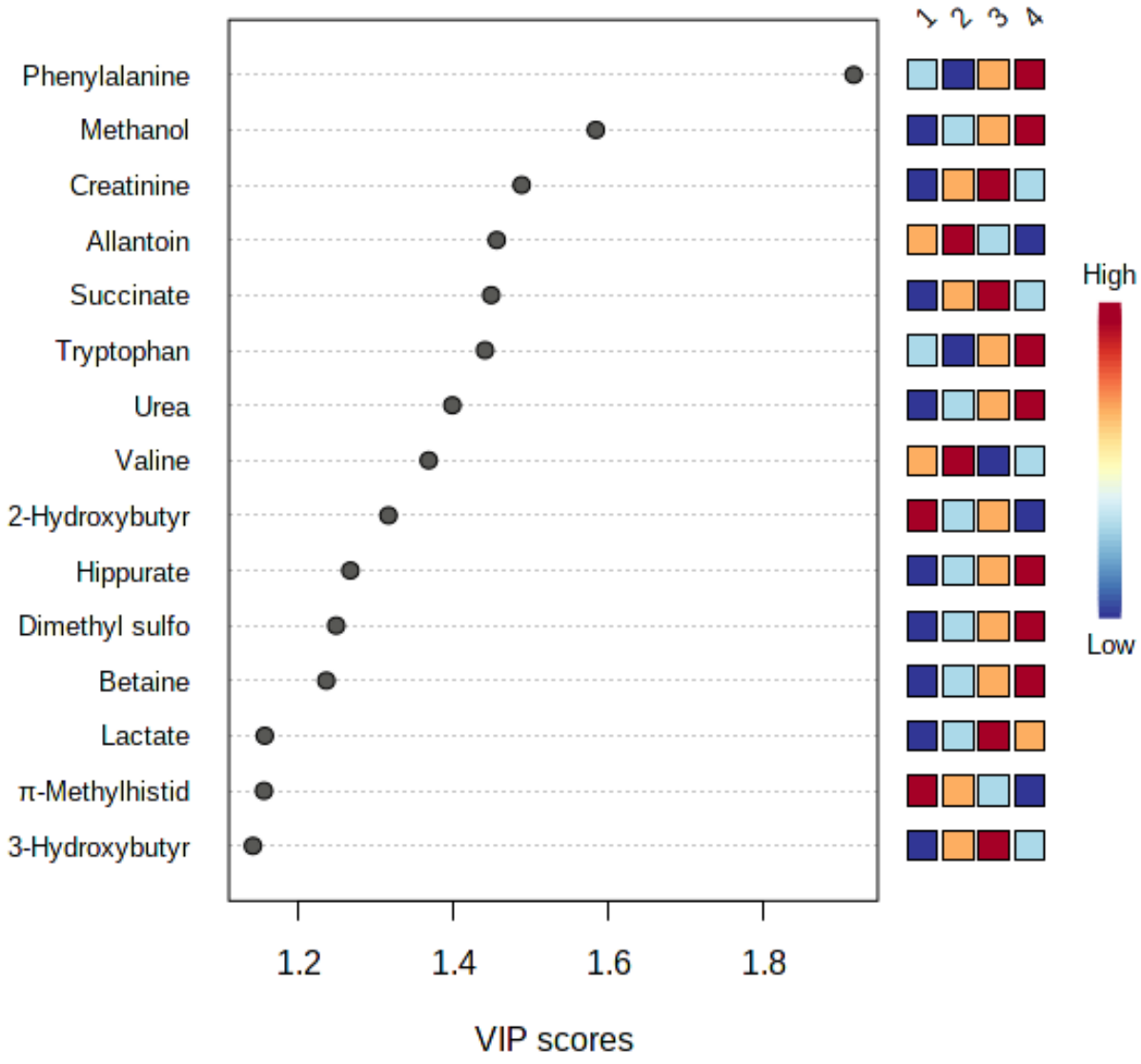

Figure 53. Significant Metabolites related to Yield Grade identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each yield grade class.

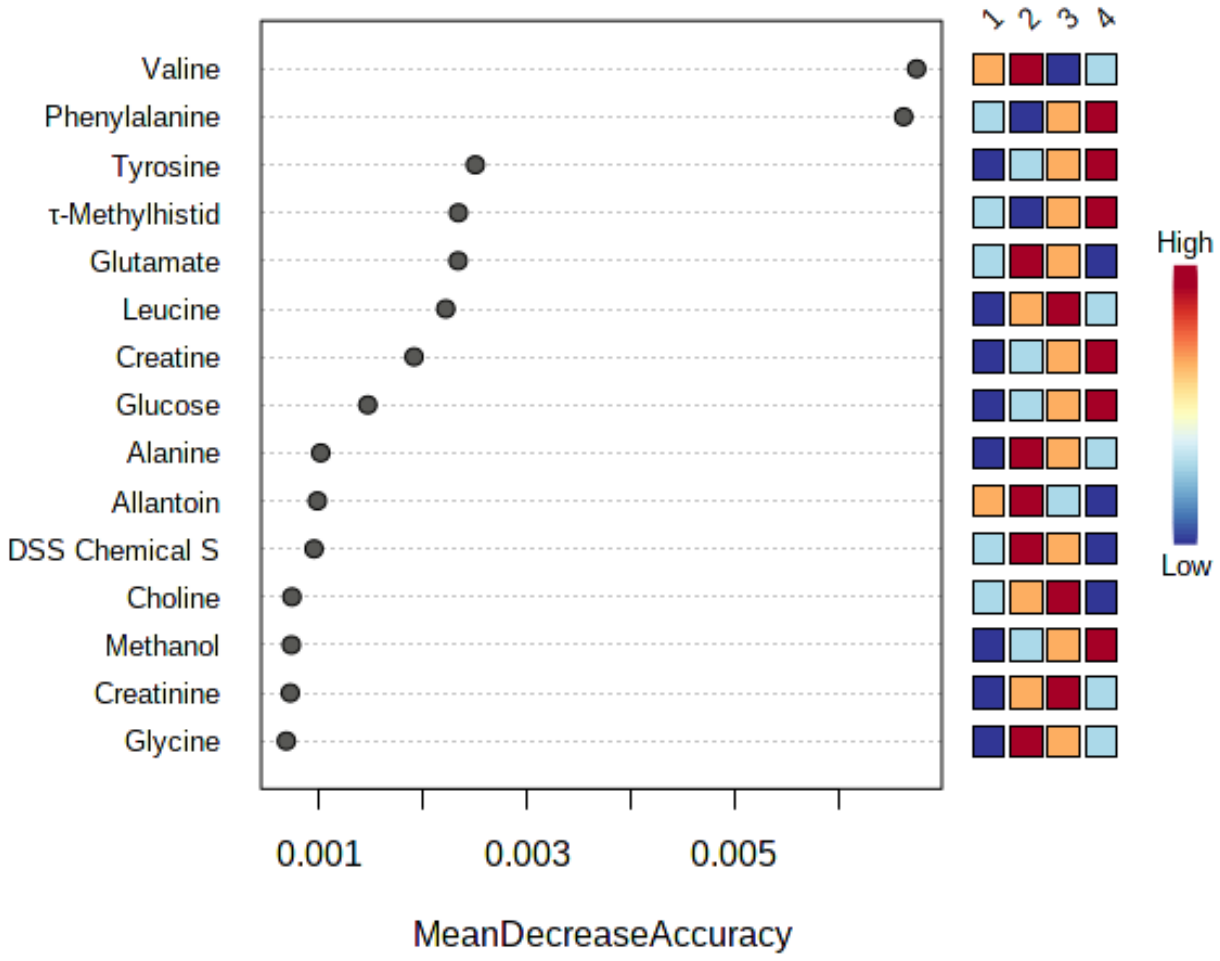

Figure 54. Significant Metabolites related to Yield Grade identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Treatment Costs NMR Data

Treatment Costs of BRD

Treatment costs was one way animal health was assessed in this dataset. The cost of treatment over the time in the feedlot was reported. No reported treatment costs represented a health animal, low treatment costs represented a single treatment and higher treatment costs represented animals that required multiple treatments. The data normalization for treatment costs is shown in Appendix Figure 55. Data was normalized by sum using a row-wise procedure and then log transformed. A one-way Analysis of Variance (ANOVA) was first used to identify features that discriminated between treatment cost classes. Appendix Figure 56 shows the important features identified by ANOVA analysis. PCA and PLSDA shown in Appendix Figure 57 and Appendix Figure 58 respectively and showed little to no separation between treatment cost classification groups. The PLSDA validation with permutation shown in Appendix Figure 60 generated a *P*-value based on permutation of $P = 0.69$ (690/1000). This showed that we were unable to differentiate treatment cost classifications. Appendix Figure 59 looks at the number of components that would allow the best classifications. It indicates that there is no viable classification based on metabolites for treatment costs. Appendix Figure 61 shows an ordered list of the most important features in discriminating between treatment costs classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are choline, anserine, citrate, alanine, and phenylalanine. A pattern was seen for concentrations for phenylalanine, alanine, citrate, and anserine with the lowest concentrations in the high treatment cost group. Appendix Figure 62 shows a similar analysis using a random forest approach. The five most important features identified using this method were pyruvate, π-methylhistidine, isobutyrate, creatinine, and lactate.

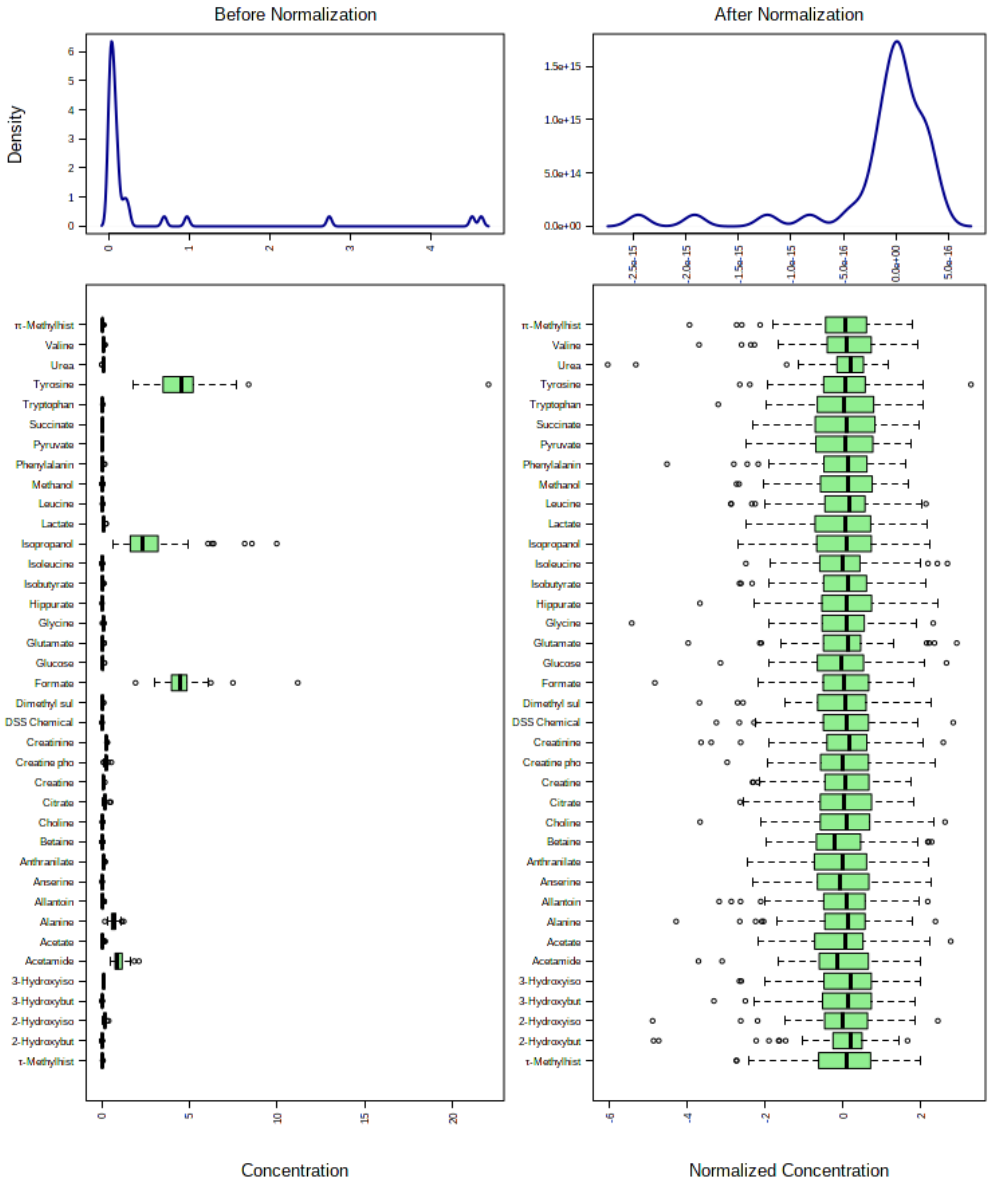

Figure 55. Treatment Costs Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

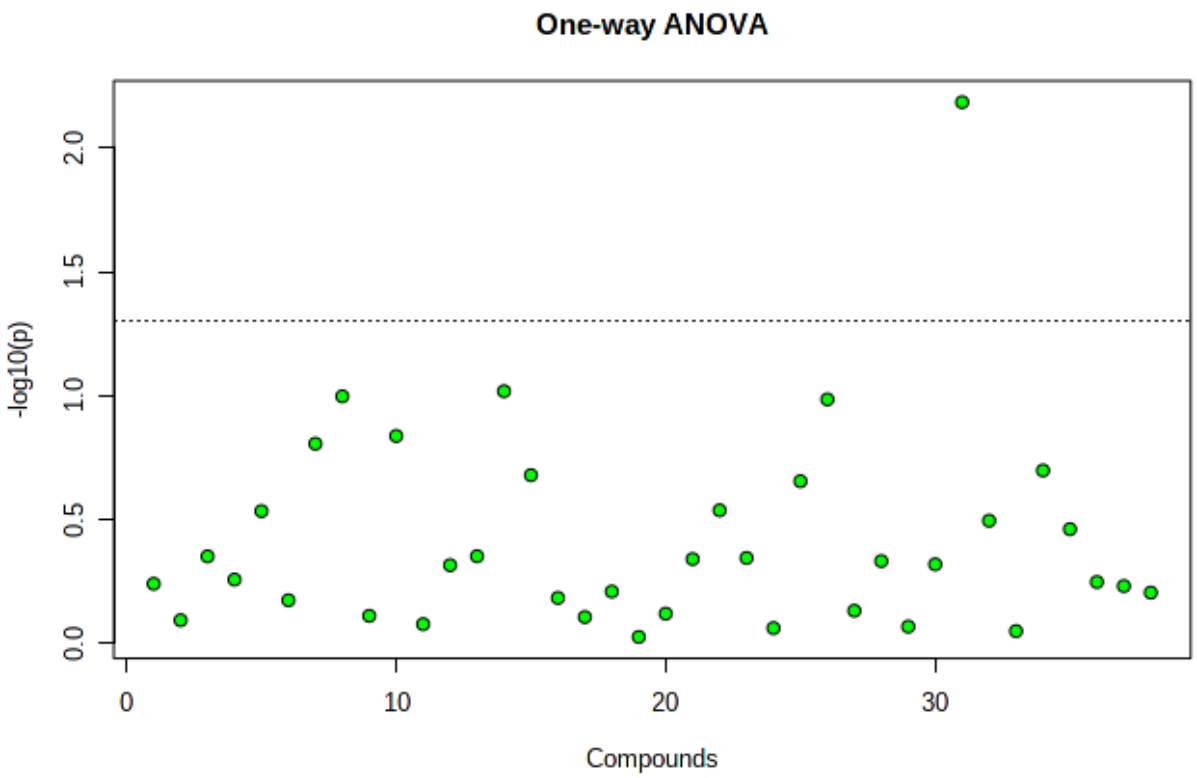

Figure 56. Significant features related to Treatment Costs identified by Analysis of Variance: Important metabolites identified by ANOVA for treatment costs categories, *P*-value threshold 0.05. On the x-axis are the number of metabolites being looked at in this ANOVA, the y-axis is the –log10(*P*) which is the adjustment made to find the level of significance of the metabolites. The reason for some metabolites shown as green even though they are at or above the level of significance is because they were adjusted for FDR.

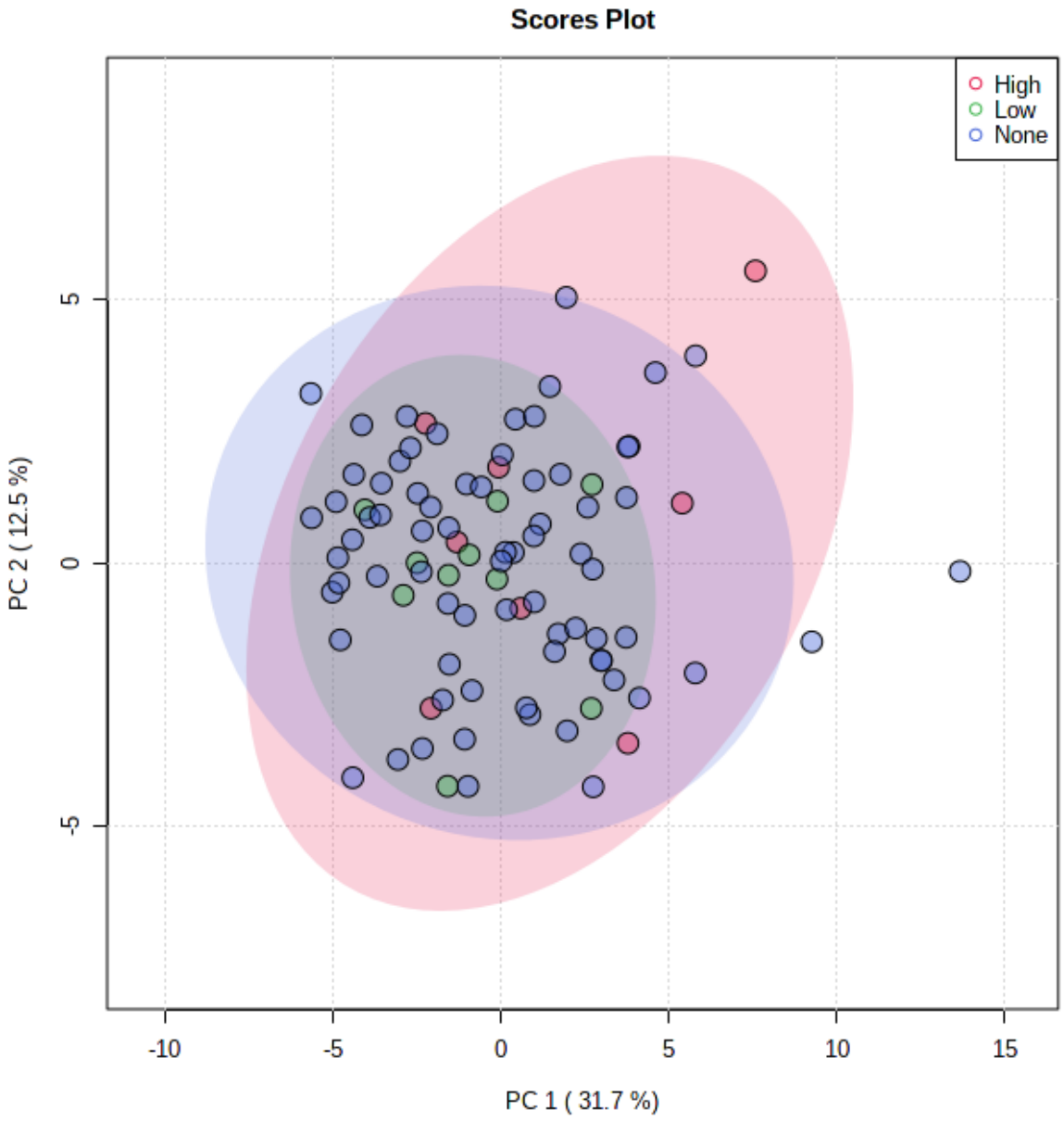

Figure 57. Significant features related to Treatment Costs identified by PCA: Scores plot between the selected principal components or group classifications for treatment costs. The variation explained by the first and second principal component is in parentheses. In the image above you see there is no significant separation between the groups of high, low, and none.

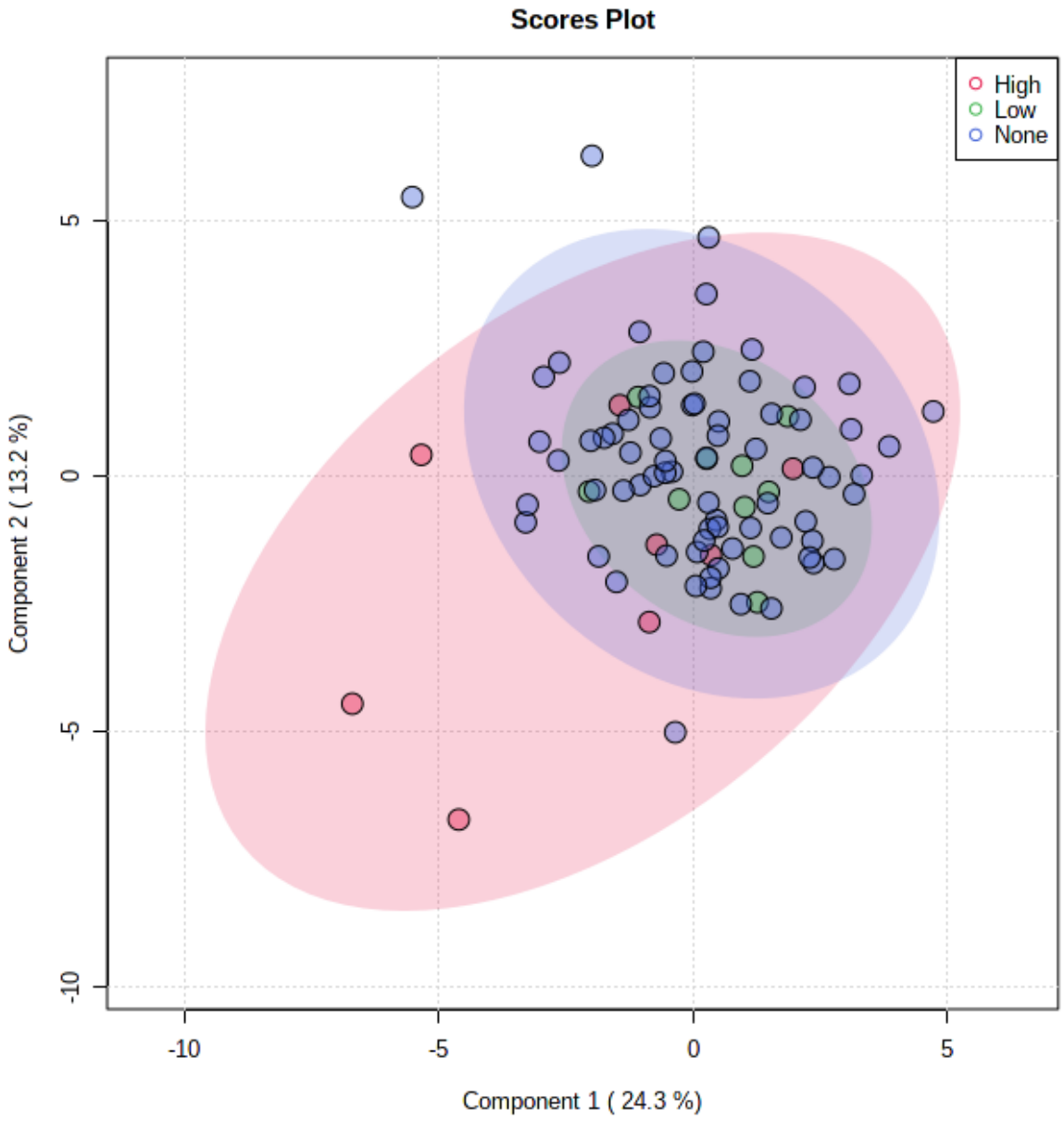

Figure 58. Significant features related to Treatment Costs identified by PLS-DA: Scores plot between for treatment costs between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is no significant separation between the three groups high, low, and none.

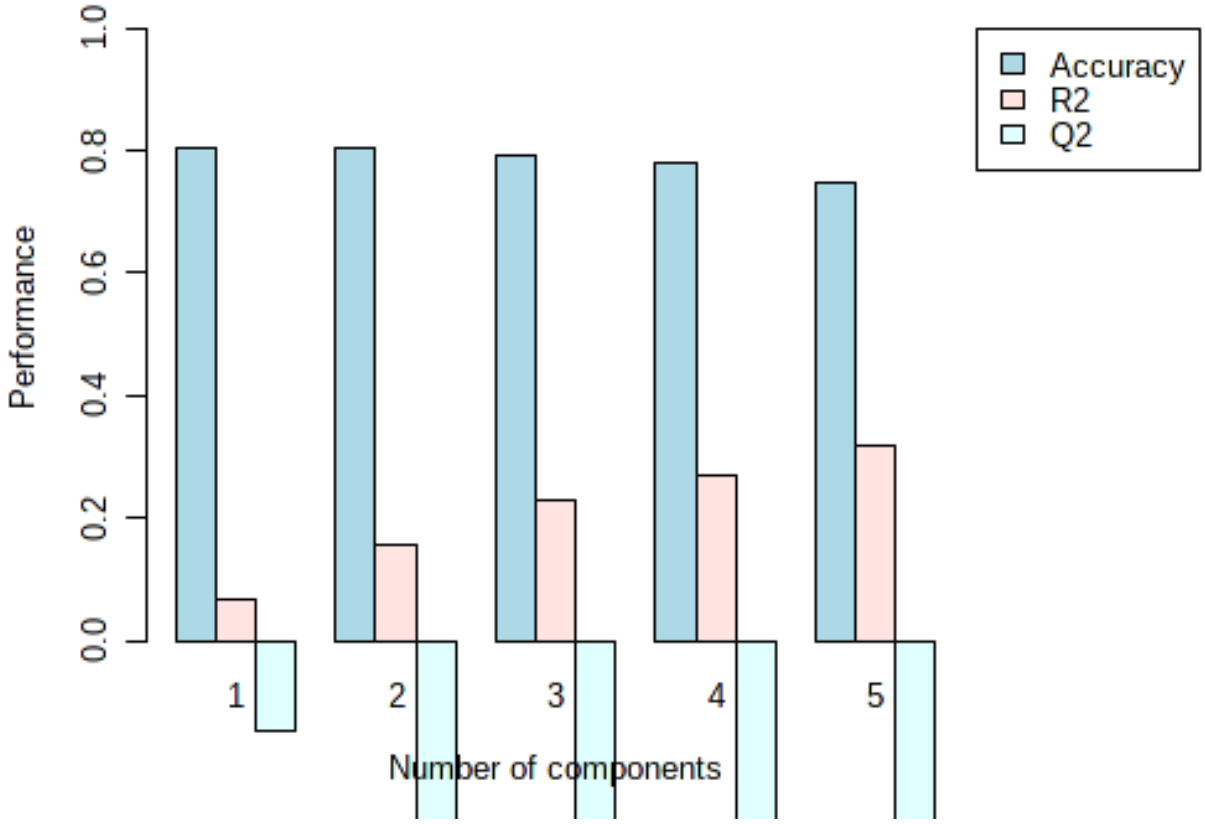

Figure 59. Significance related to Treatment Cost identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present, the negative Q2 shows a lack of significance.

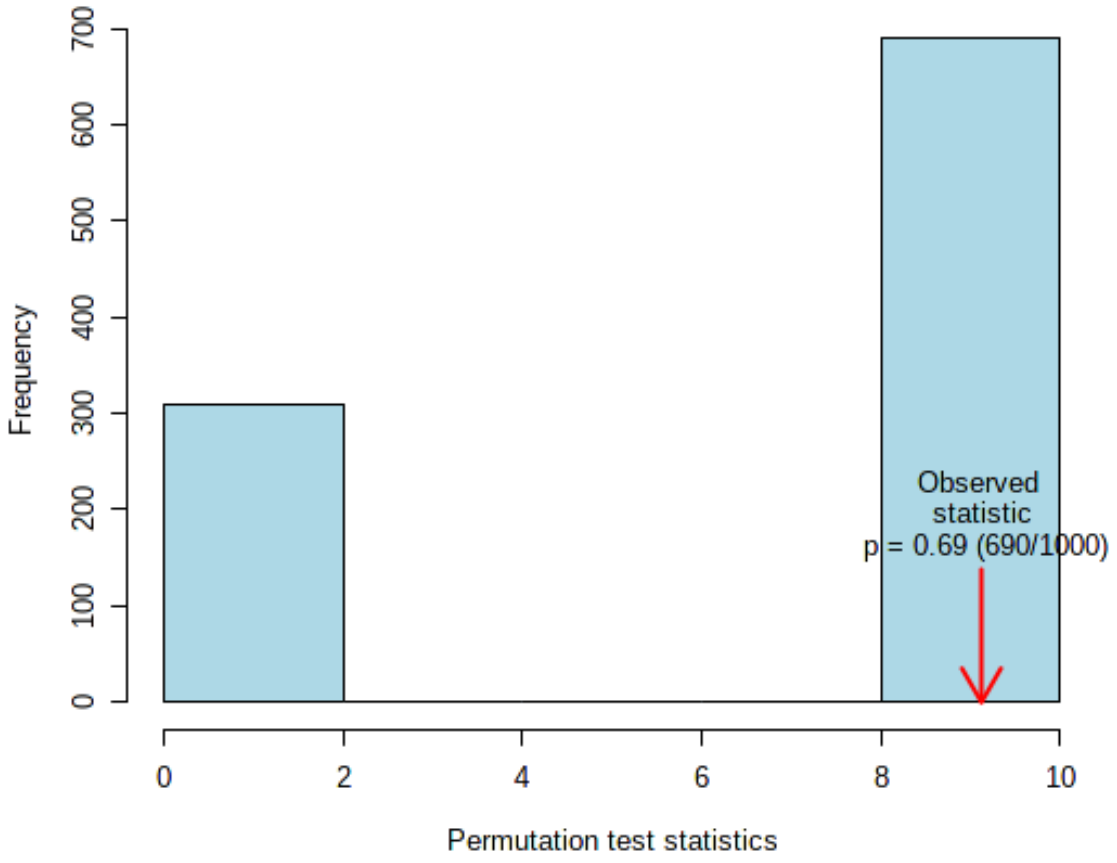

Figure 60. Significance related to Treatment Cost identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is $P = 0.69$ (690/1000). The *P*-value shows that there is no significant difference between the treatment cost classes.

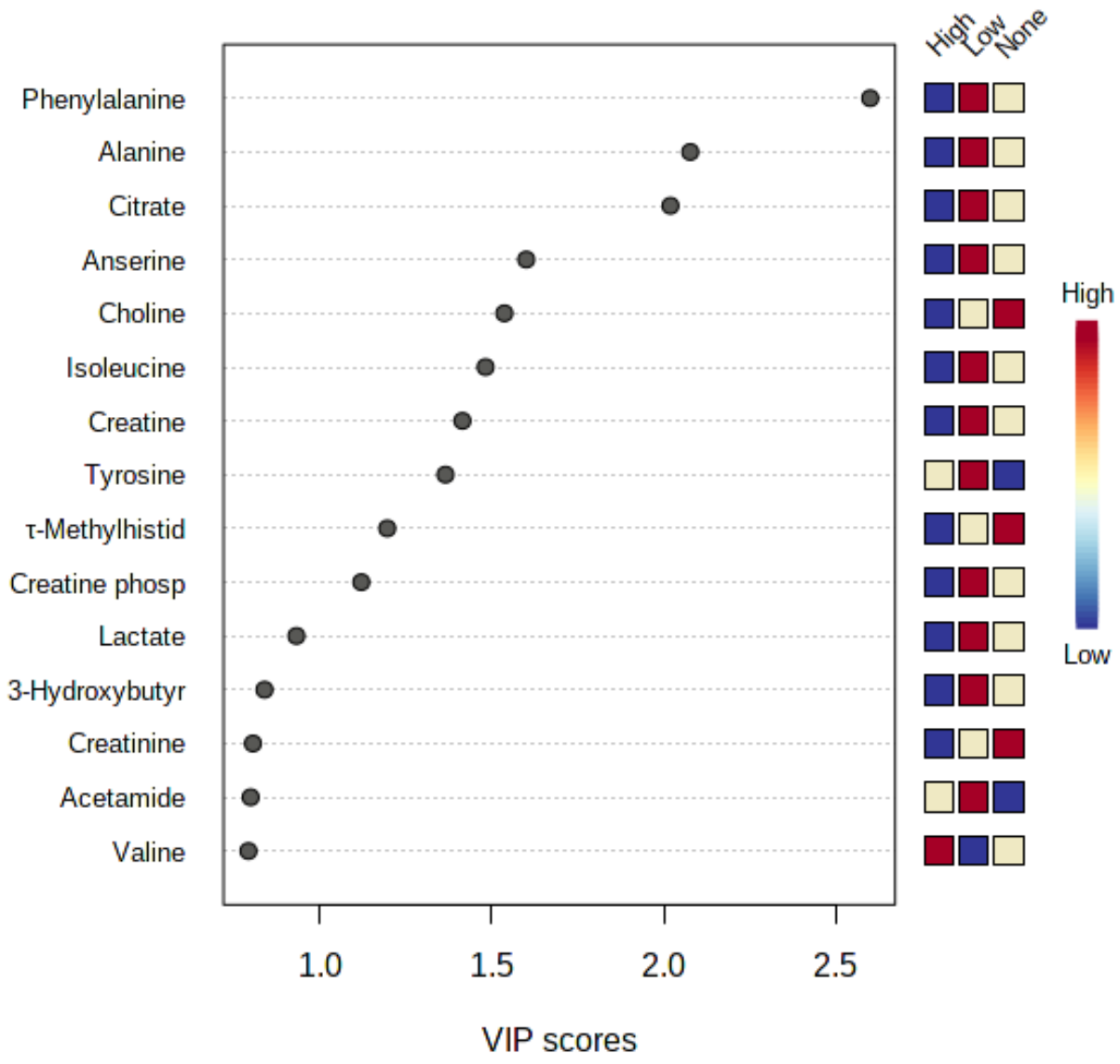

Figure 61. Significant Metabolites related to Treatment Cost identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each treatment cost class.

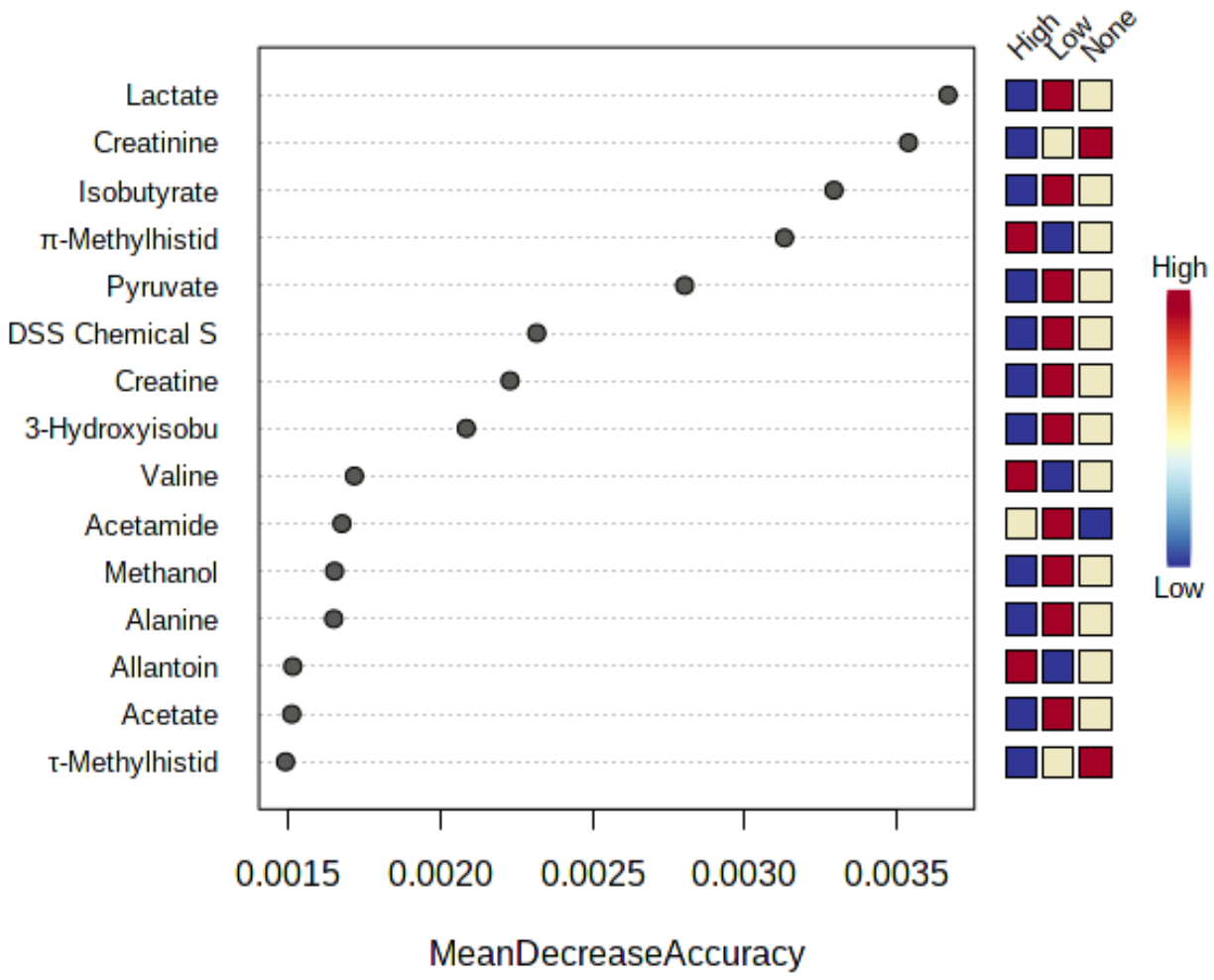

Figure 62. Significant Metabolites related to Treatment Cost identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Treatment Costs High vs. Low NMR Data

Treatment Cost High vs. Low

In addition to the treatment costs analysis above a comparison was run of high vs. low treatment costs in only treated animals. The data normalization for treatment costs is shown in Appendix Figure 63. Data was normalized by sum using a row-wise procedure and then log transformed. PCA and PLSDA analyses are shown in Appendix Figure 64 and Appendix Figure 65 respectively, and they showed slight separation between treatment costs of high vs low classification groups. The PLSDA validation with permutation shown in Appendix Figure 67 generated a *P*-value based on permutation of $P = 0.9$ (900/1000). This shows that the model cannot differentiate between high and low treatment costs groups. Appendix Figure 66 looks at the number of components that would allow the best classifications. It indicates that there is no viable classification based on metabolite features for treatment costs of high vs. low. Appendix Figure 68 shows an ordered list of the most important features in discriminating between treatment costs high vs. low classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are citrate, isoleucine, phenylalanine, and glutamate. A pattern was seen in the concentration of glutamate with a higher concentration in the high treatment cost class. Appendix Figure 69 shows a similar analysis using a random forest approach. The two most important features identified using this method were citrate and π-methylhistidine.

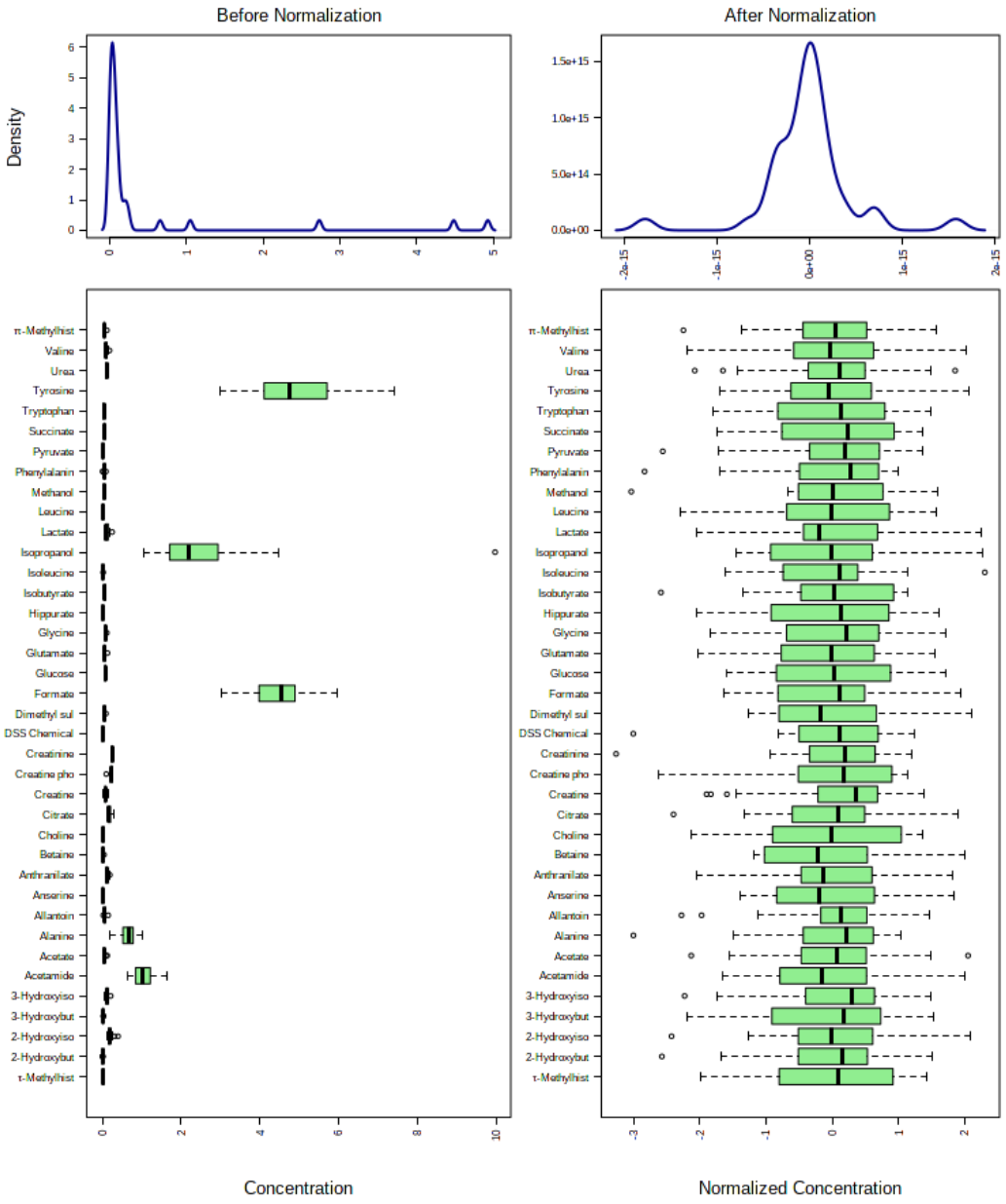

Figure 63. Cost High vs Low Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

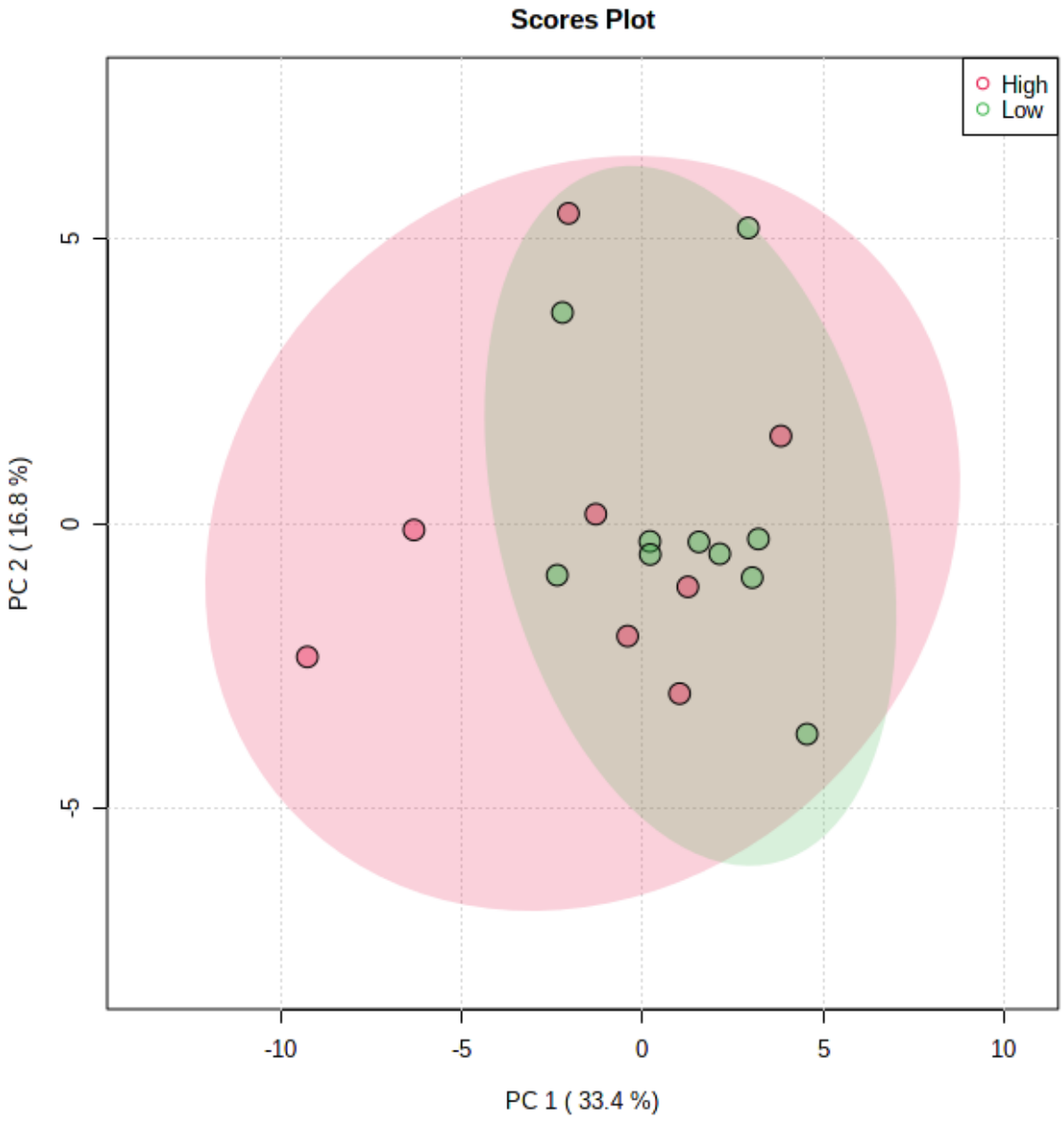

Figure 64. Significant features related to Cost High vs Low identified by PCA: Scores plot between the selected principal components or group classifications. The variation explained by the first and second principal component is in parentheses. In this image there is no significant separation between the groups.

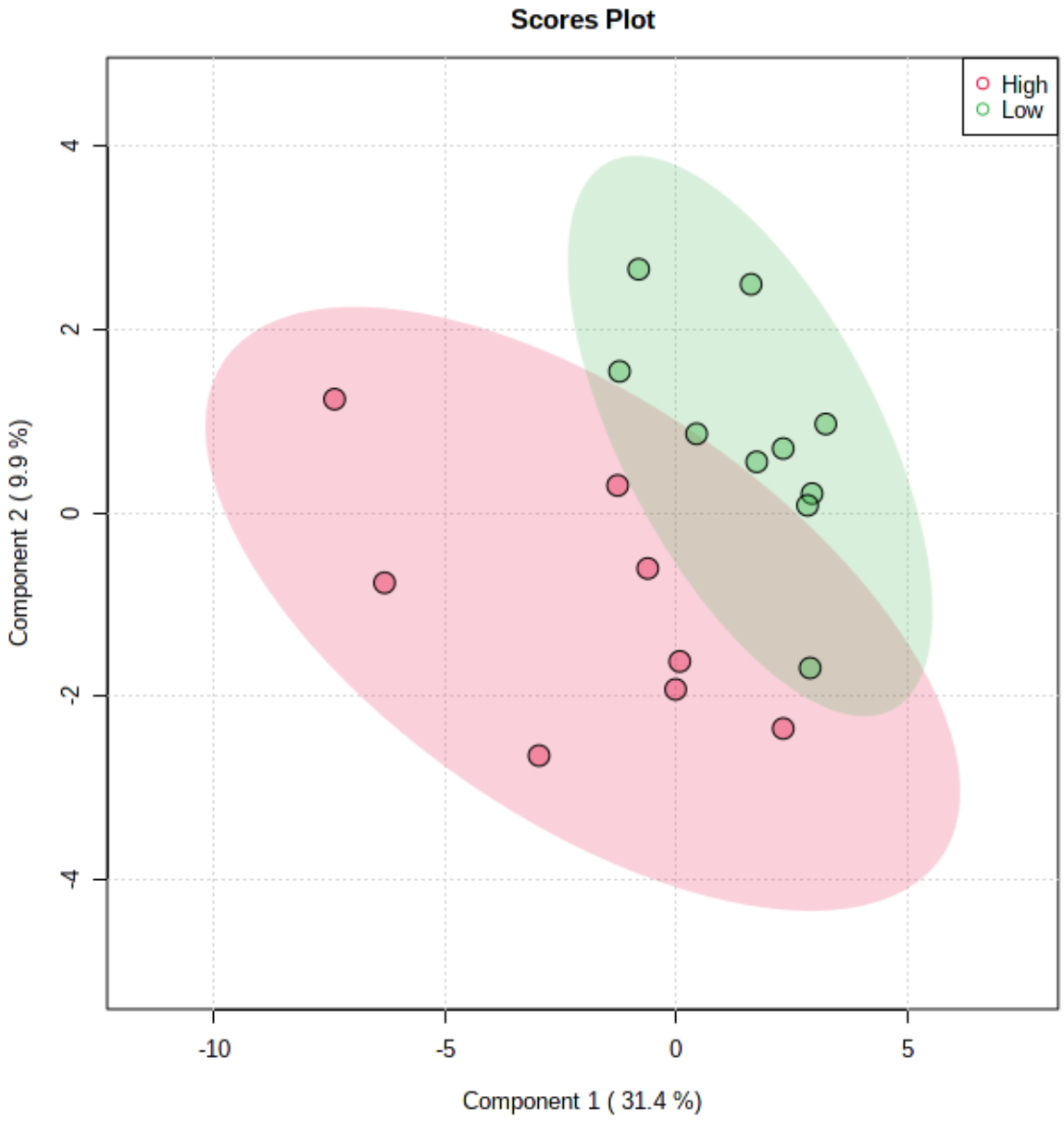

Figure 65. Significant features related to Cost High vs Low identified by PLS-DA: Scores plot for cost high vs. low between the selected principal components. The variance explained by the two principal components are shown in parentheses. There is some separation seen between the groups.

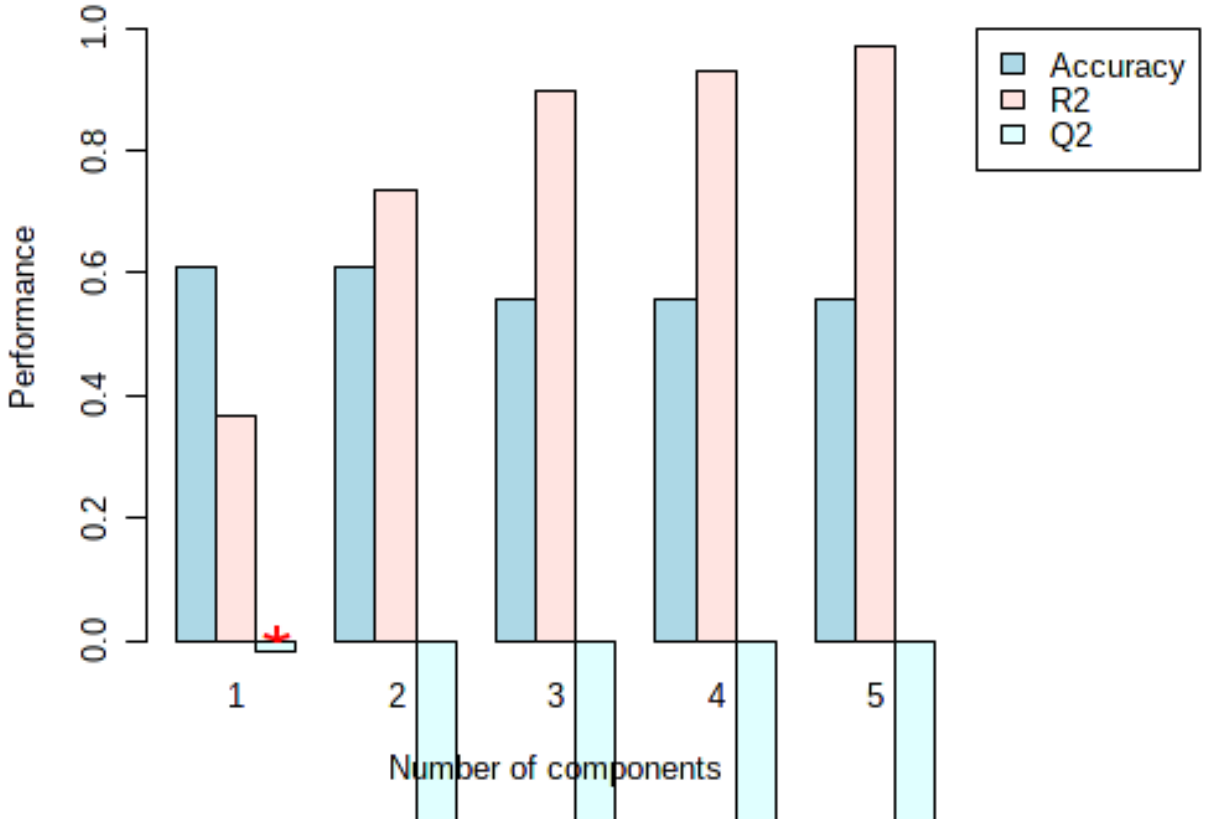

Figure 66. Significance related to Cost High vs Low identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present, the negative Q2 indicates a lack of significance.

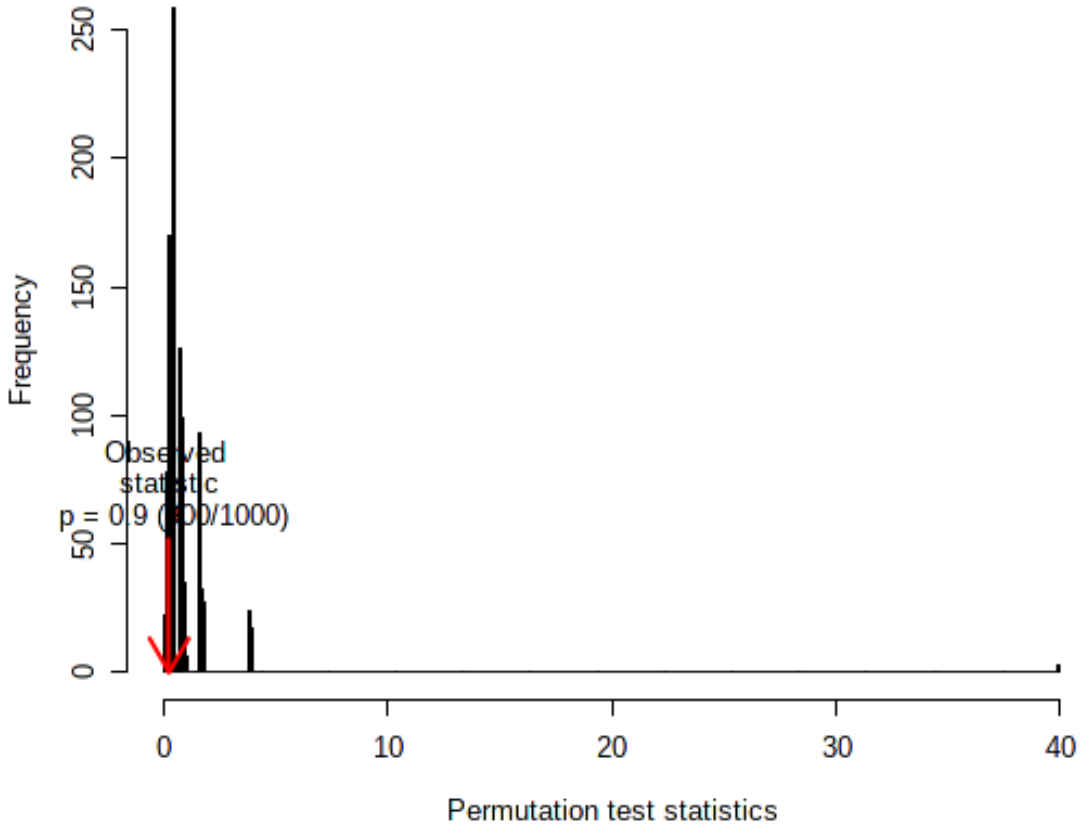

Figure 67. Significance related to Cost High vs Low identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is $P = 0.9$ (900/1000). The *P*-value shows that there is no significant difference between treatment costs groups.

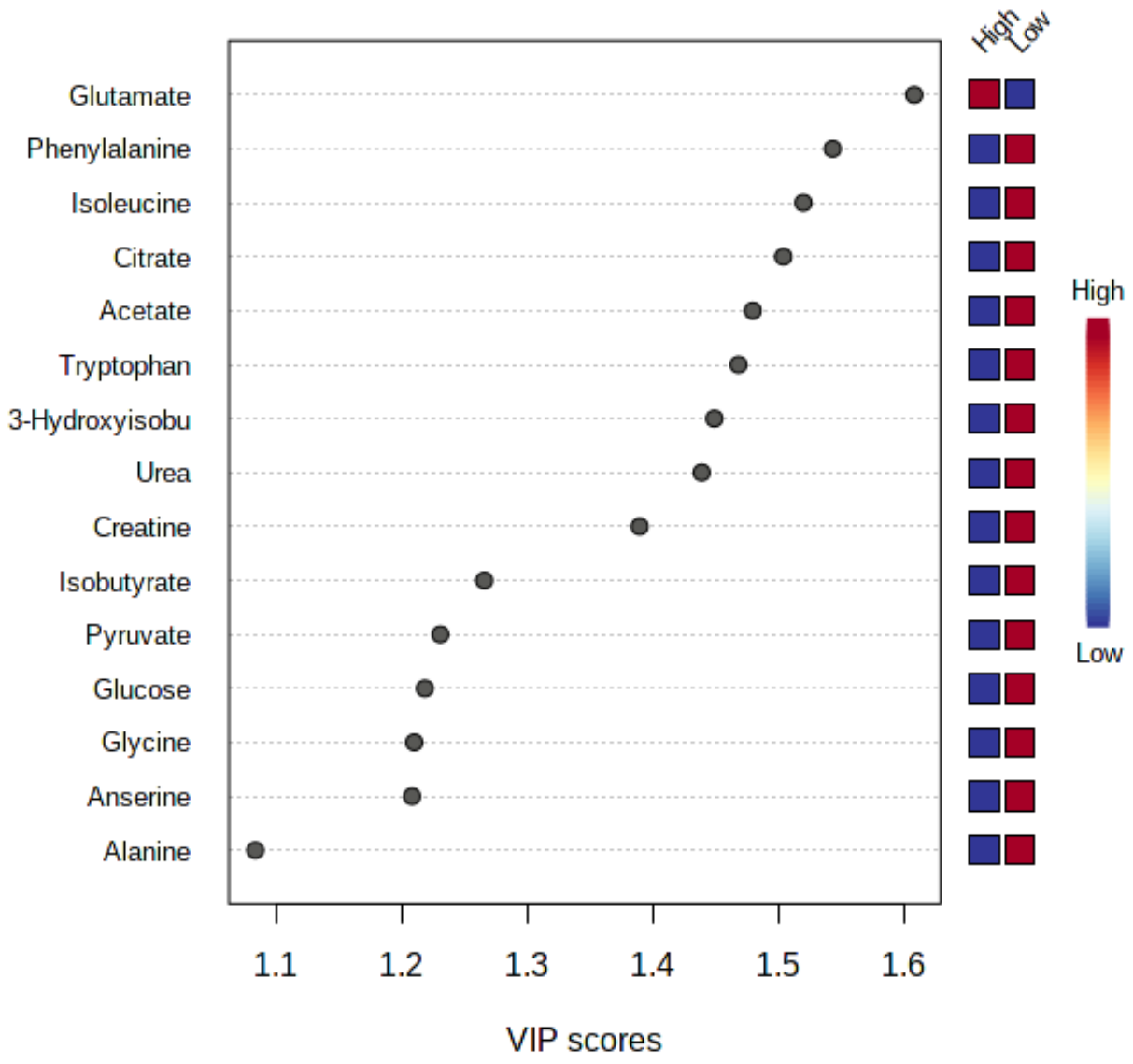

Figure 68. Significant Metabolites related to Cost High vs Low identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each treatment cost class.

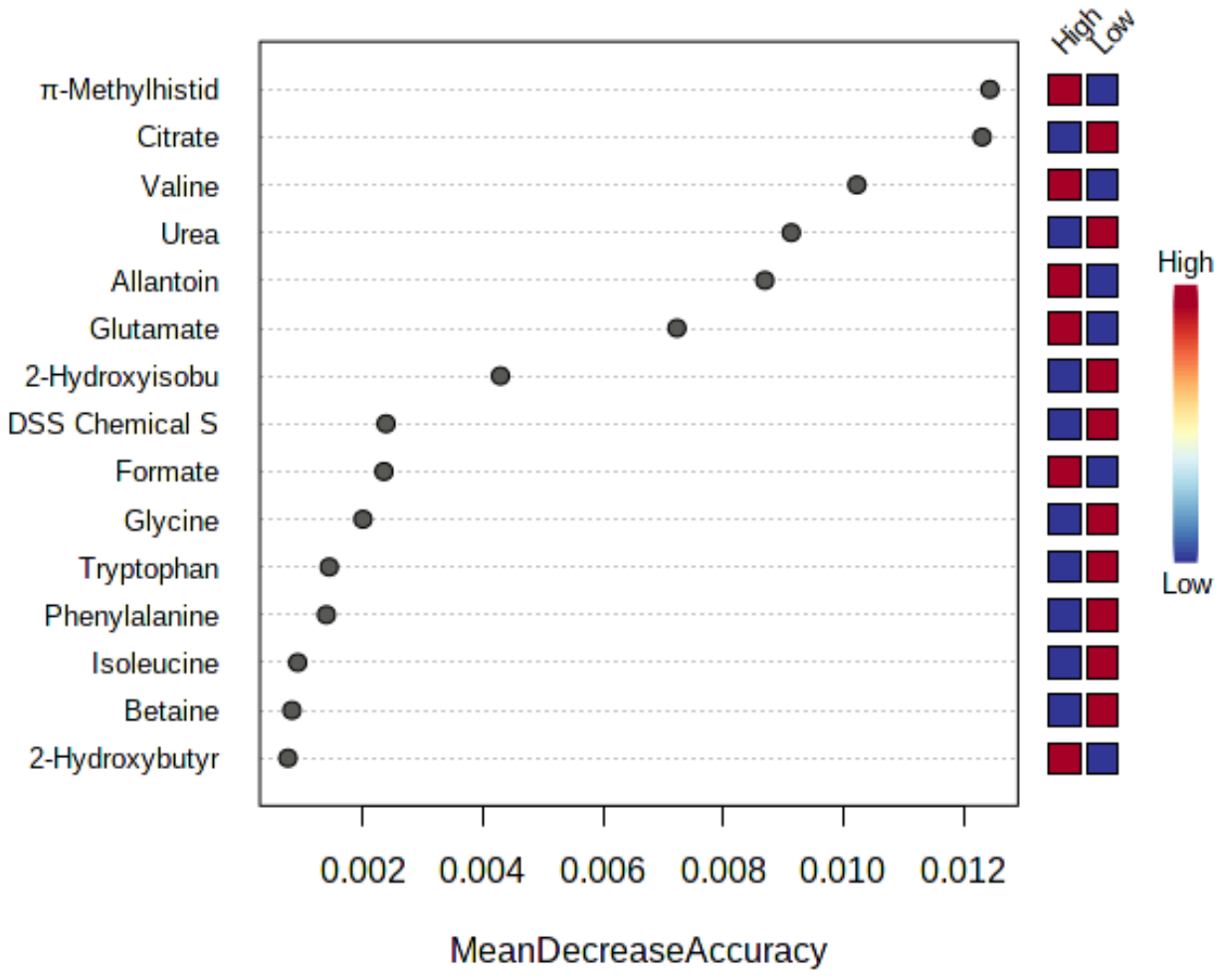

Figure 69. Significant metabolites related to Cost High vs Low identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Treatment Cost High vs no treatment NMR Data

Treatment Costs High vs. None

This analysis looked at differences between animals that had high reported treatment costs and those that had no reported treatment cost. The data normalization for treatment costs is shown in Appendix Figure 70. Data was normalized by sum using a row-wise procedure and then log transformed. PCA and PLSDA shown in Appendix Figure 71 and 72 respectively, and showed little to no separation between the treatment cost classes of high vs. none. The PLSDA validation with permutation shown in Appendix Figure 74 generated a *P*-value based on permutation of $P = 0.839$ (839/1000). This shows that the model differentiate between treatment cost classifications. Appendix Figure 73 looks at the number of components that would allow the best classifications. It indicates that there are no viable classifications based on metabolite features possible for this comparison of treatment costs. Appendix Figure 75 shows an ordered list of the most important features in discriminating between treatment costs classifications from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are anserine, isoleucine, citrate, alanine, and phenylalanine. All these features expressed a similar pattern for concentration with lower concentrations in the high treatment cost class. Appendix Figure 76 shows a similar analysis using a random forest approach. The three most important features identified using this method were lactate, alanine, and isoleucine.

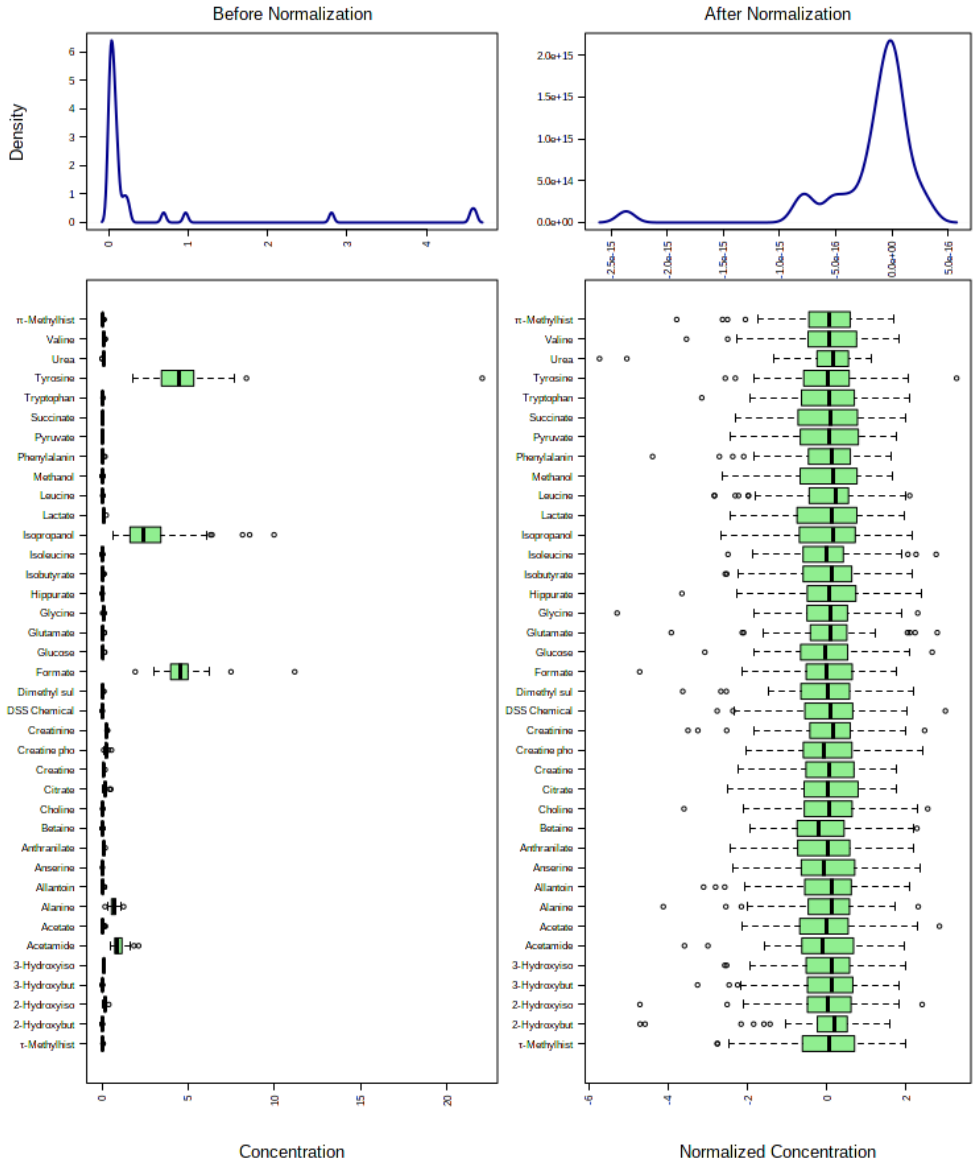

Figure 70. Cost High vs None Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

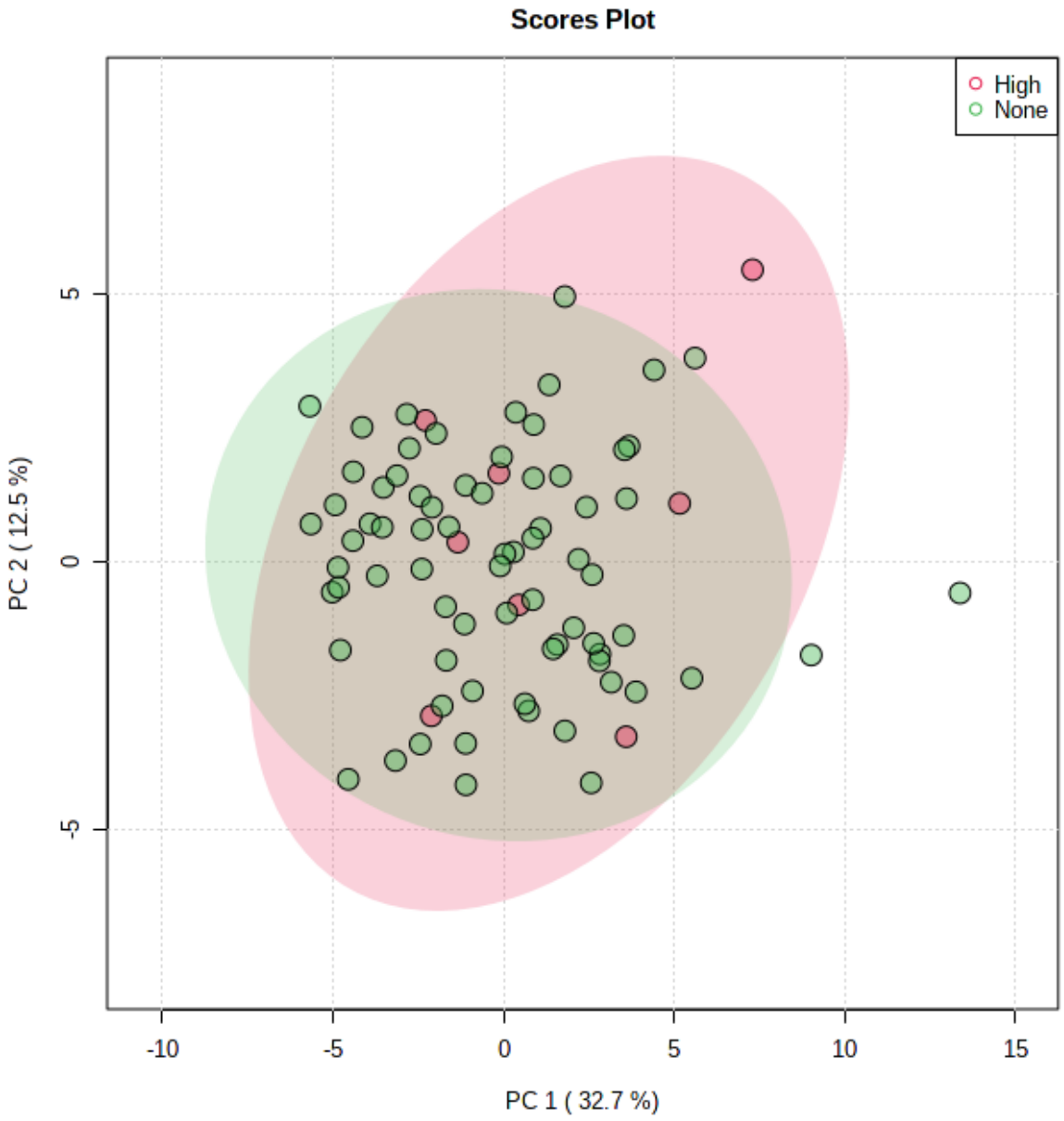

Figure 71. Significant features related to Cost High vs None identified by PCA: Scores plot between the selected principal components or group classifications for cost high vs. none. The variation explained by the first and second component is in parentheses.

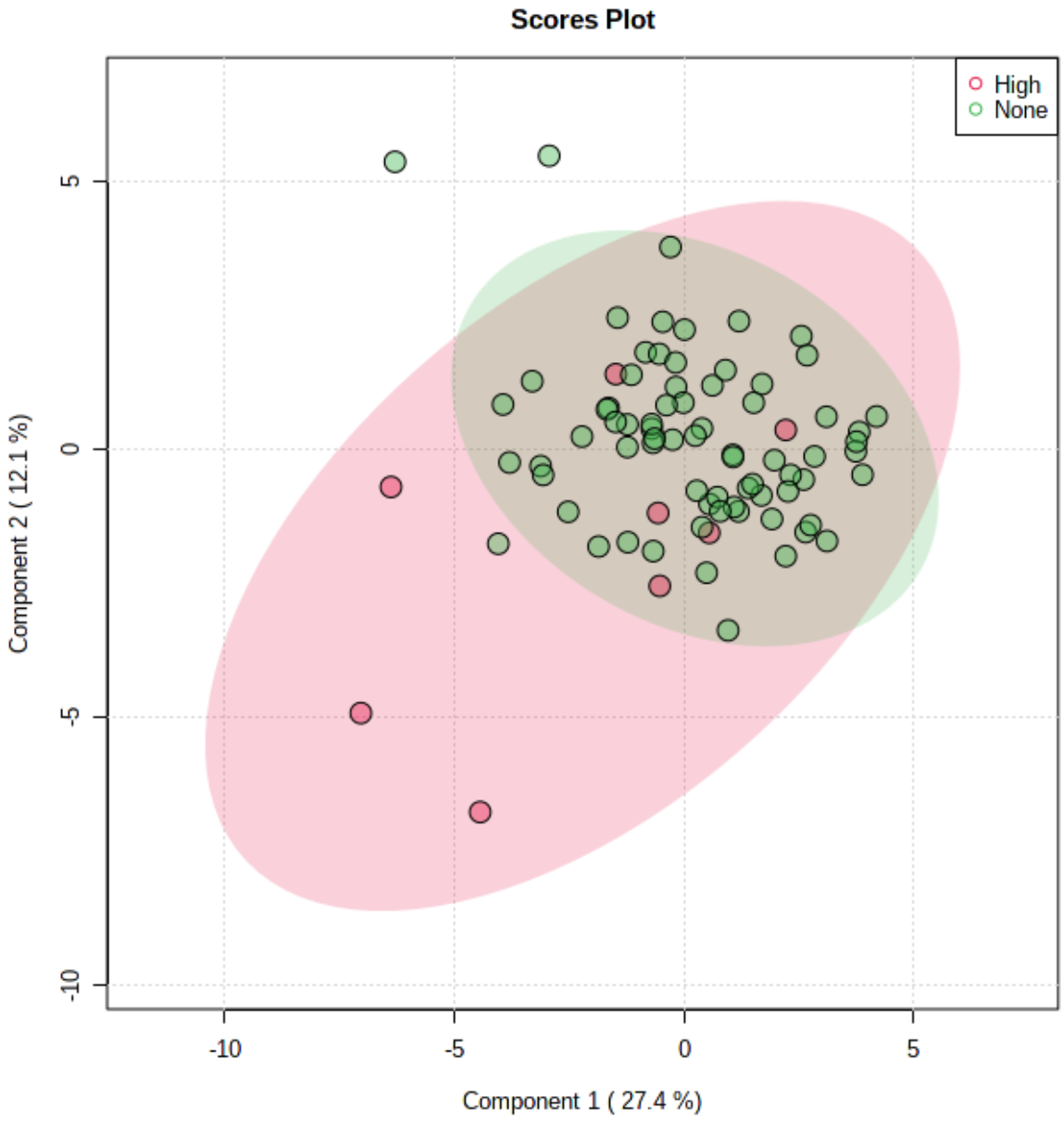

Figure 72. Significant features related to Cost High vs None identified by PLS-DA: Scores plot for cost high vs. low between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image there is no significant separation between the groups.

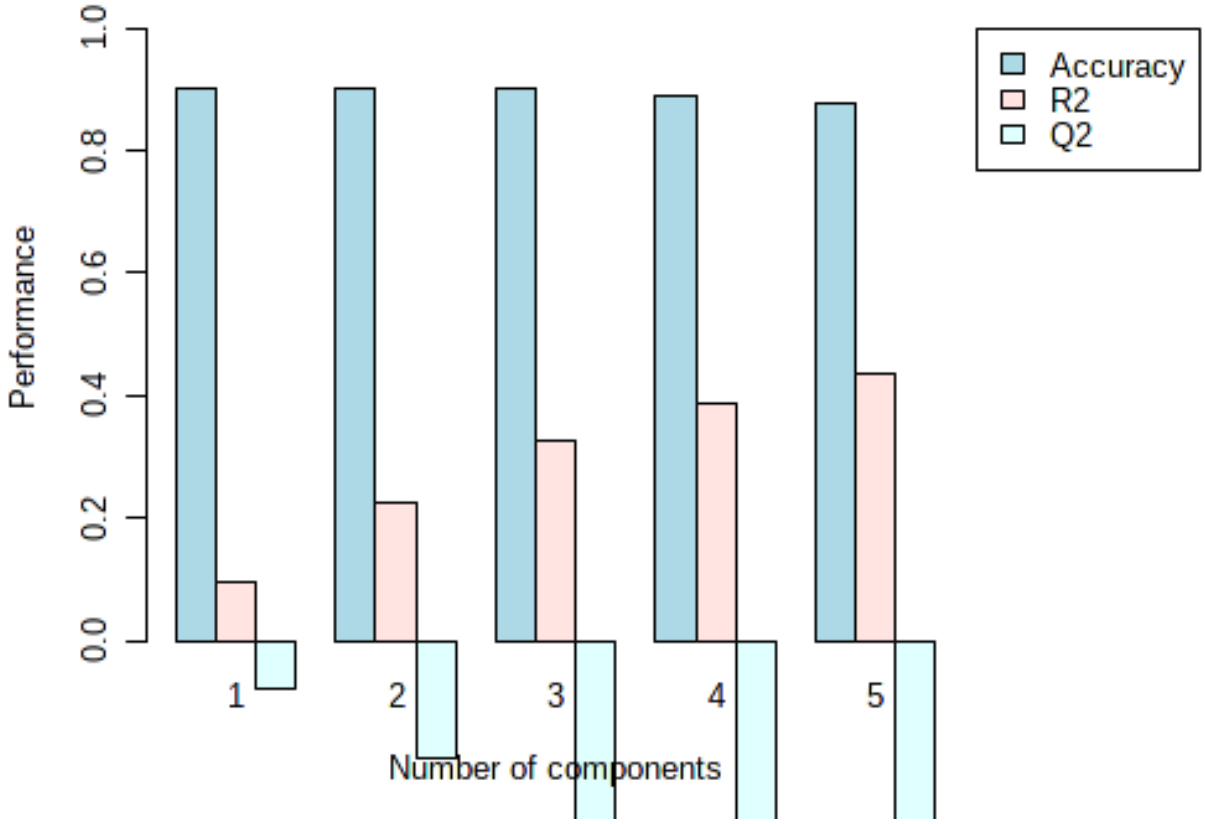

Figure 73. Significance related to Cost High vs None identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present, the negative Q2 indicates a lack of significance.

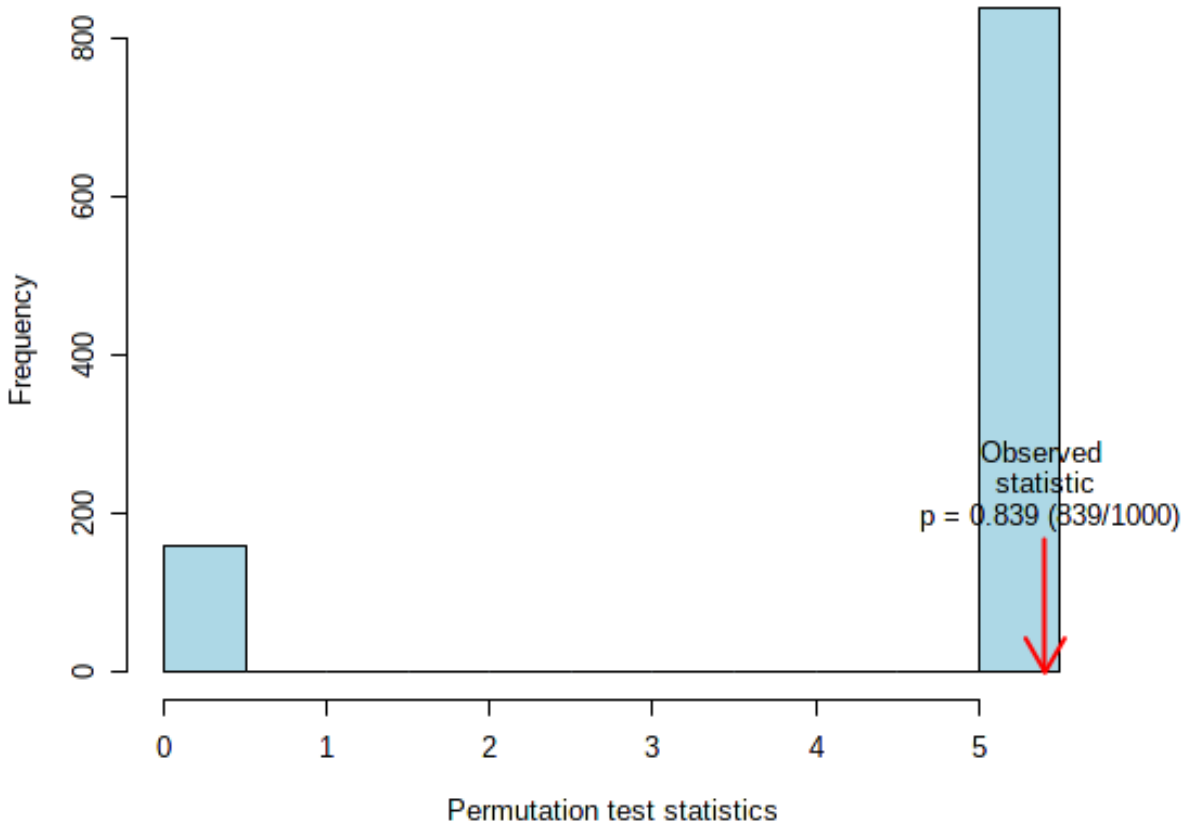

Figure 74. Significance related to Cost High vs None identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is $P = 0.839$ (839/1000). The *P*-value shows that there is no significant difference in the treatment cost classes. The reason for the spike on the right hand is this group has very low variation.

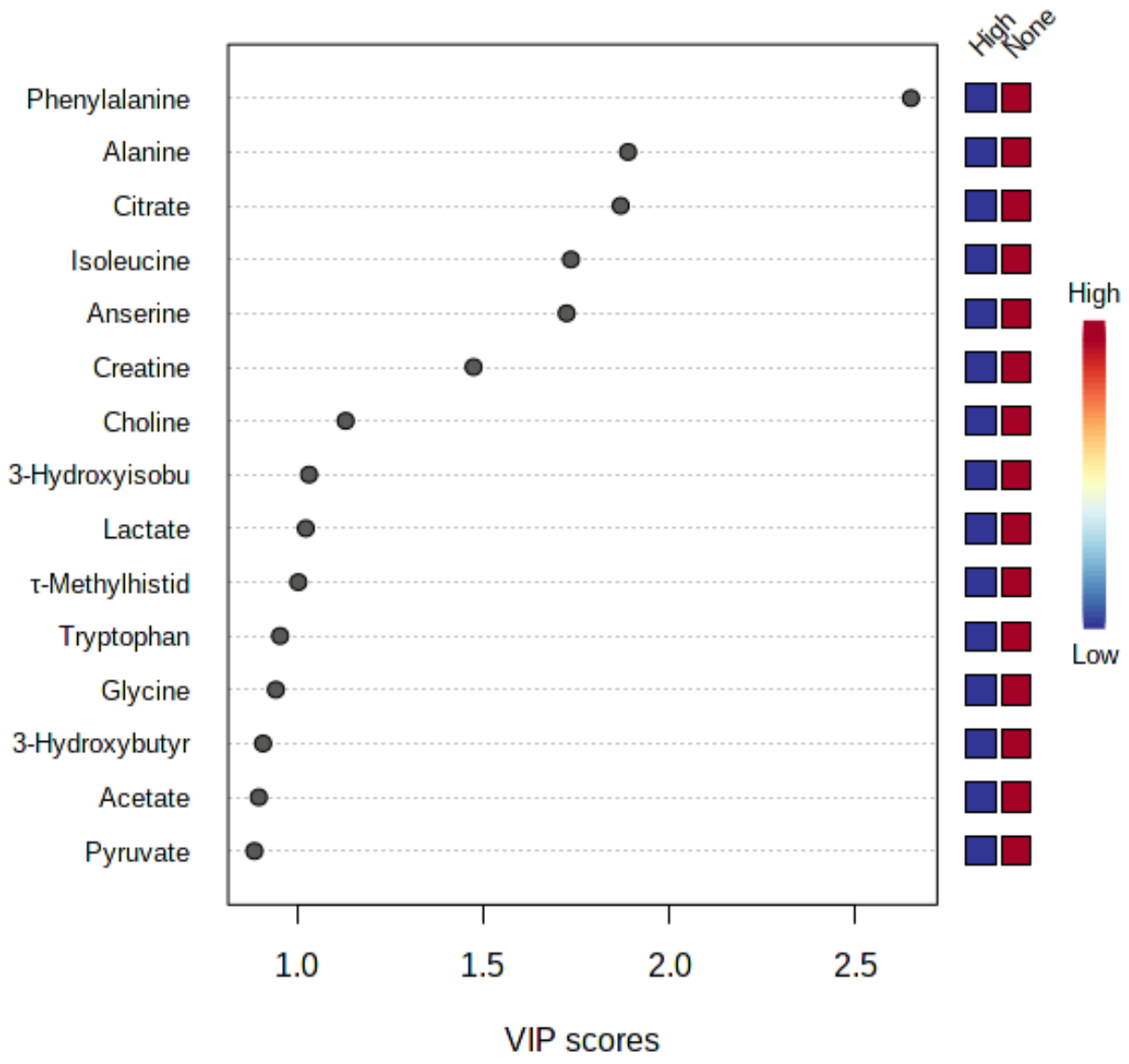

Figure 75. Significant Metabolites related to Cost High vs None identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each treatment cost class.

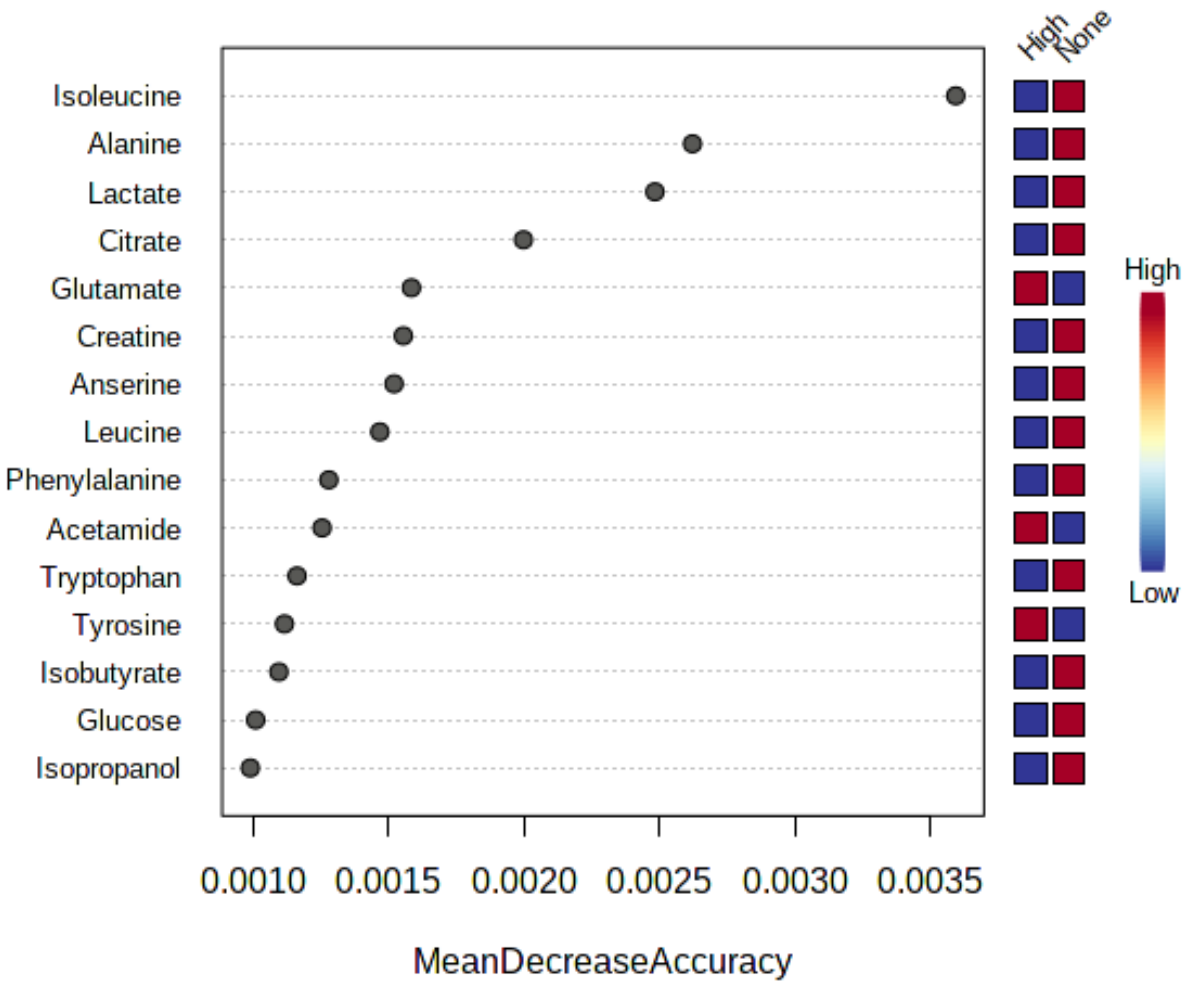

Figure 76. Significant Metabolites related to Cost High vs None identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

Treatment Costs Low vs. None NMR Data

Treatment Costs Low vs. None

This analysis compared animals that received a single treatment and had low treatment cost and those that were not treated while in the feedlot. The data normalization for treatment costs is shown in Appendix Figure 77. Data was normalized by sum using a row-wise procedure and then log transformed. PCA and PLSDA shown in Appendix Figure 78 and Appendix Figure 79 respectively, showed no separation between treatment costs of low vs. none classes. The PLSDA validation with permutation shown in Appendix Figure 81 generated a *P*-value based on permutation of $P = 0.722$ (722/1000). This shows that the model cannot differentiate between low and no treatment costs classes. Appendix Figure 80 looks at the number of components that would allow the best classifications. It indicates that there are no viable classifications based on metabolite features possible for this treatment cost comparisons. Appendix Figure 82 shows an ordered list of the most important features in discriminating between treatment costs low vs. none classs from the PLSDA analysis. Features with a VIP score above 1.5 are considered important in this analysis. The features above this threshold are tryptophan, glutamate, tyrosine, isobutyrate, and acetate. All these features exept for glutamate express a similar patter with higher concentrations in the low treatment cost class. Appendix Figure 83 shows a similar analysis using a random forest approach. The single most important feature identified using this method was isobutyrate.

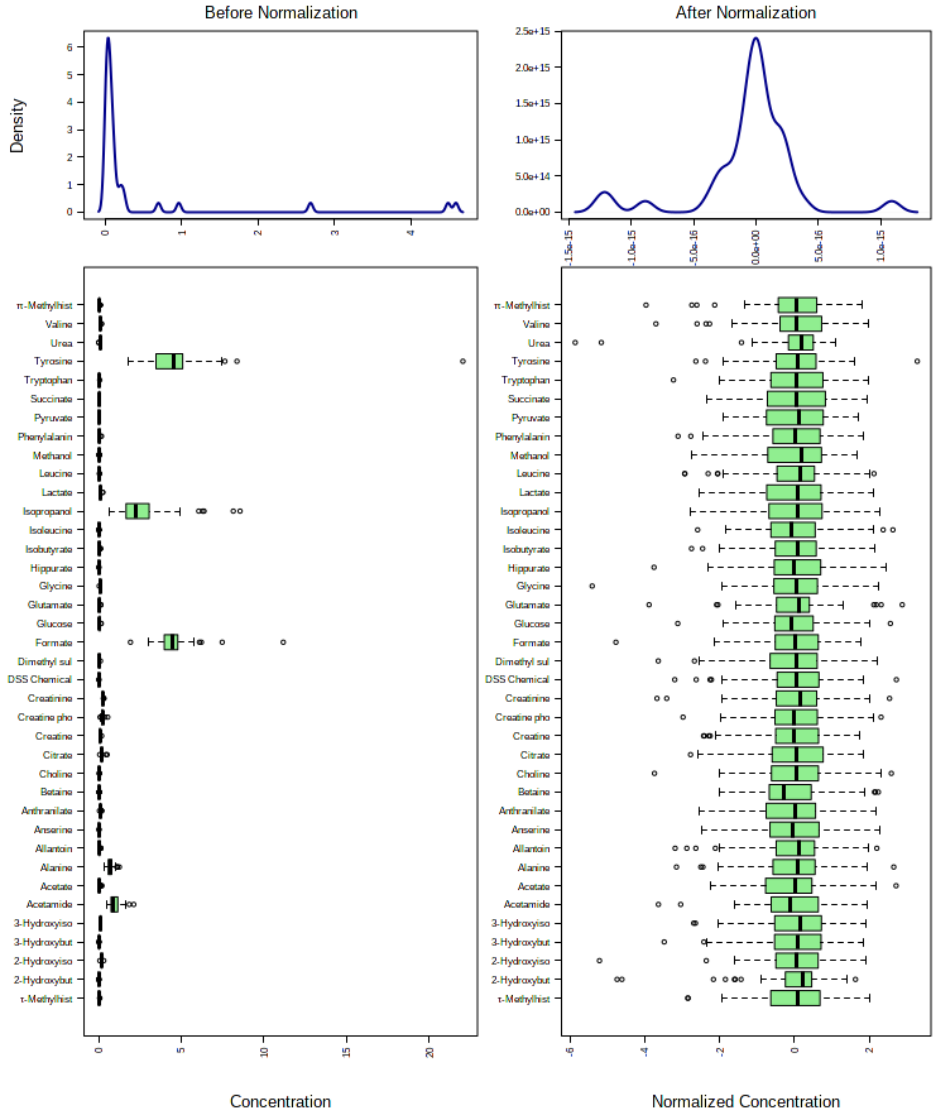

Figure 77. Cost Low vs None Data before and after Normalization Procedures: Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization to constant sum; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

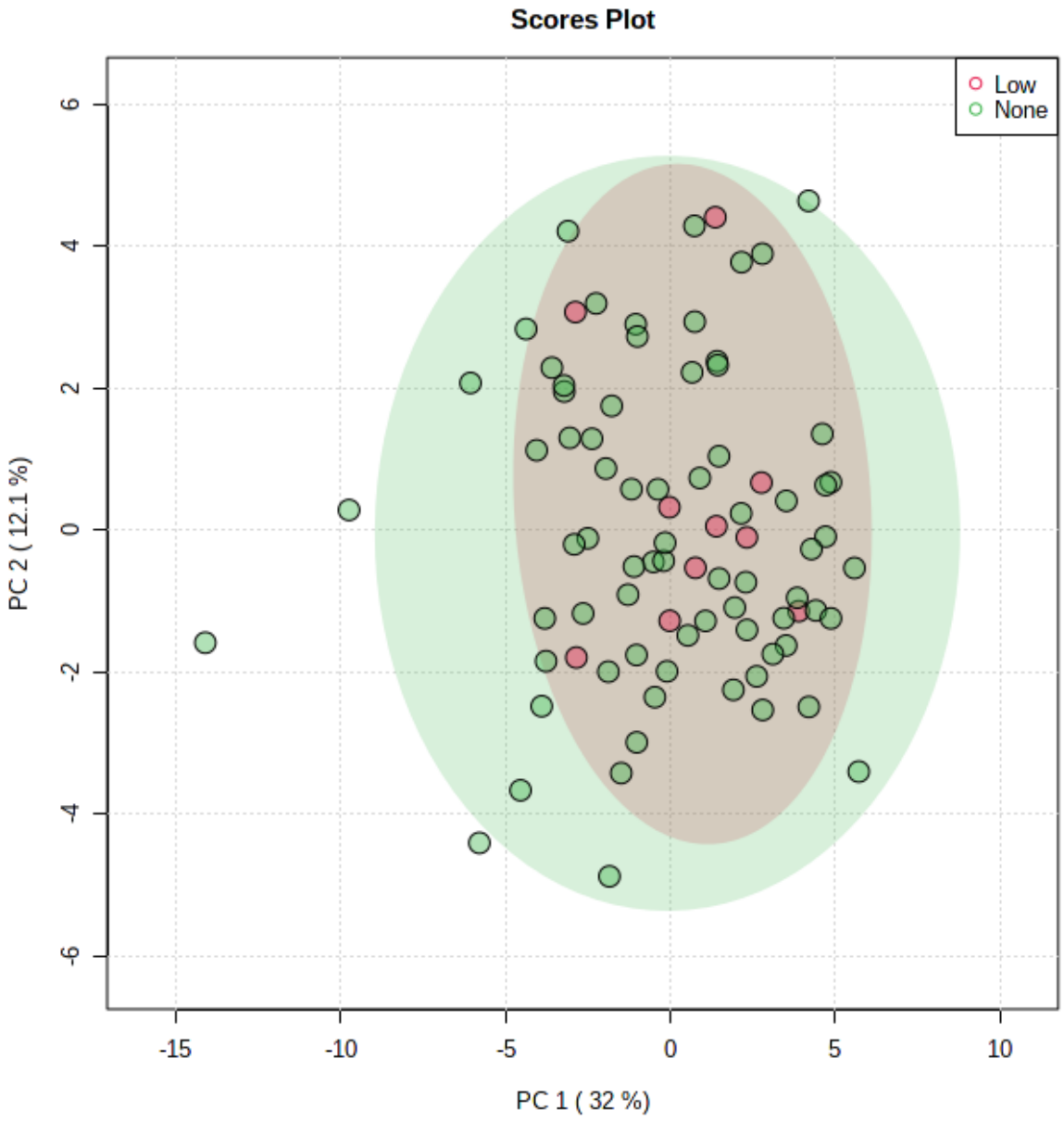

Figure 78. Significant features related to Costs Low vs None identified by PCA: Scores plot between the selected principal components or group classification for treatment costs. The variation explained by the first and second principal component is in parentheses.

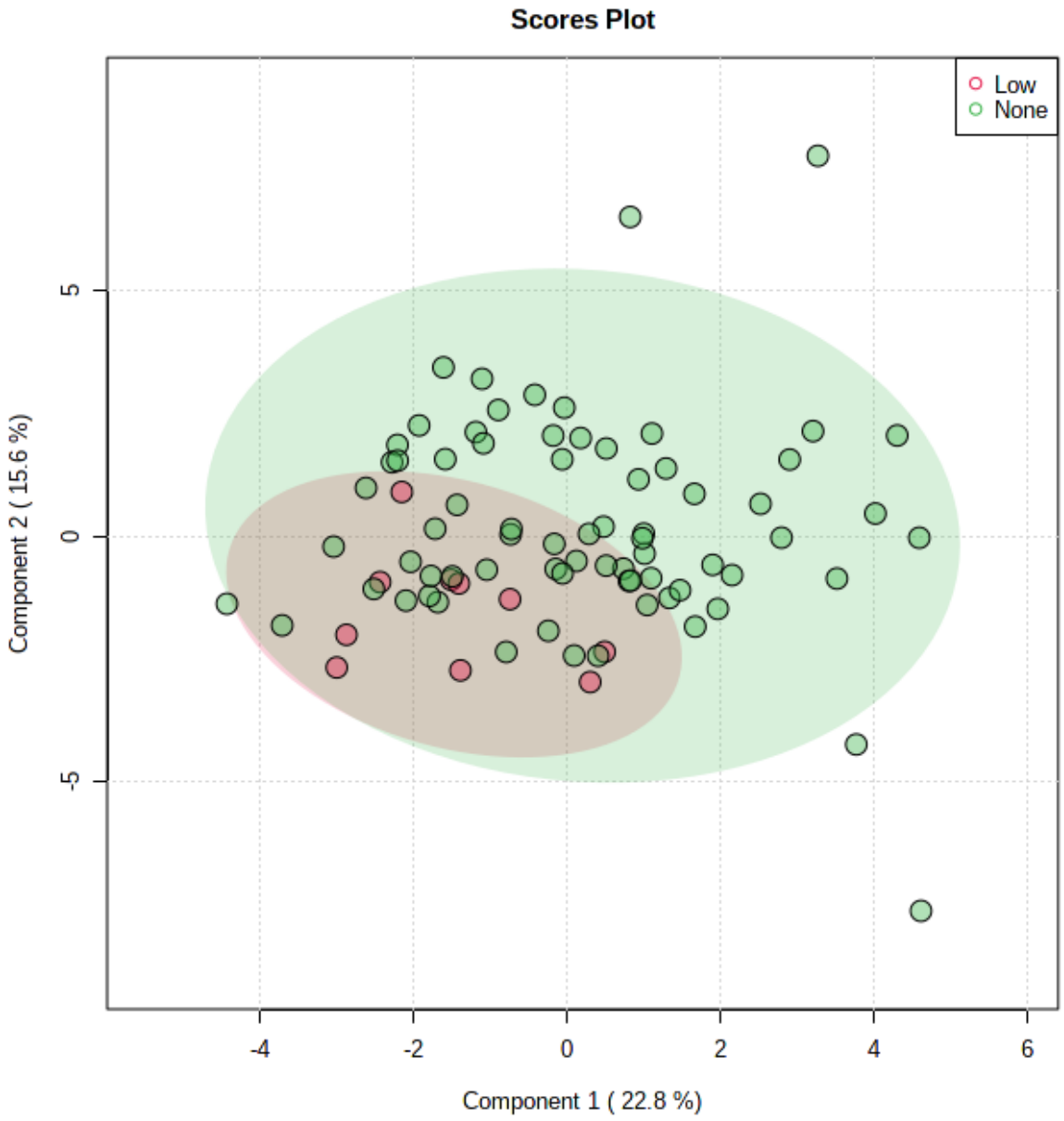

Figure 79. Significance features related to Cost Low vs None identified by PLS-DA: Scores plot for treatment cost low vs. none between the selected principal components. The variance explained by the two principal components are shown in parentheses. In this image no significant separation between treatment cost classes.

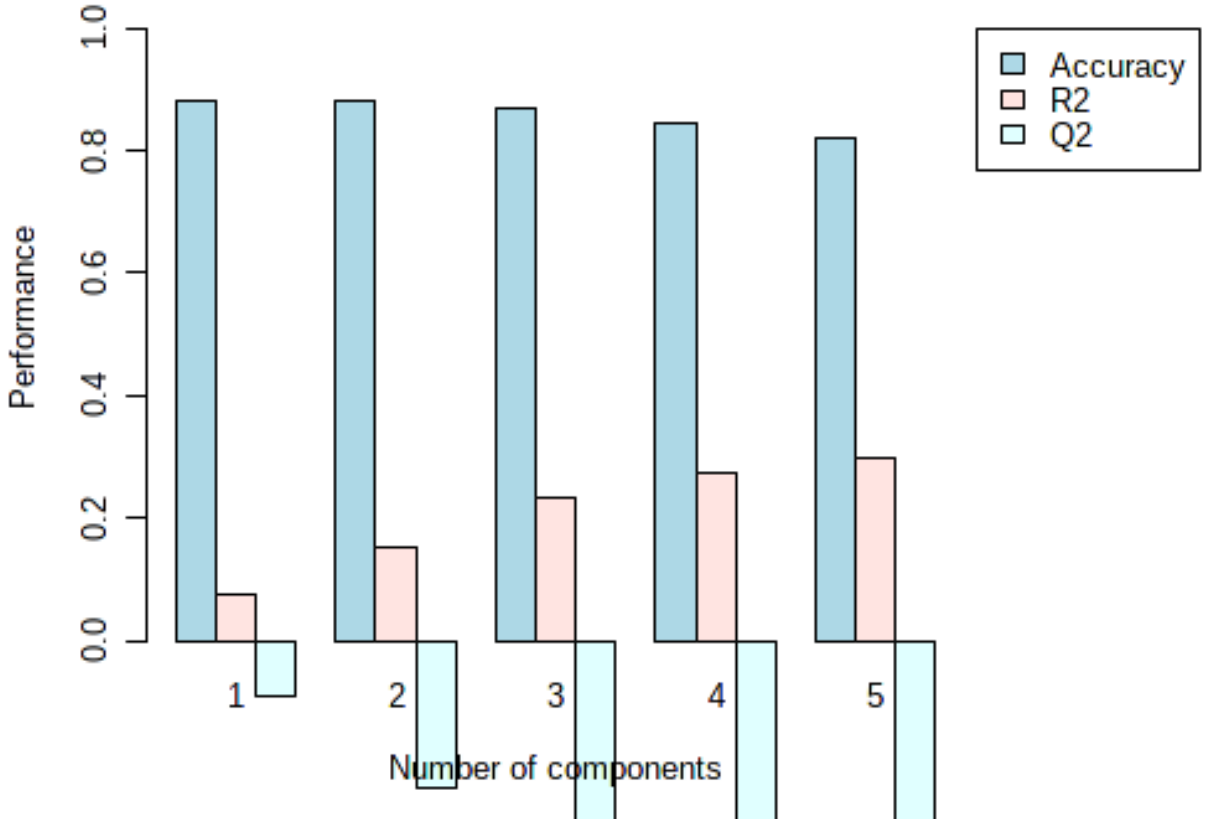

Figure 80. Significance related to Cost Low vs None identified by Components Test: PLS-DA classification using different number of components. The red star indicates the best classifier when present, the negative Q2 indicates a lack of significance.

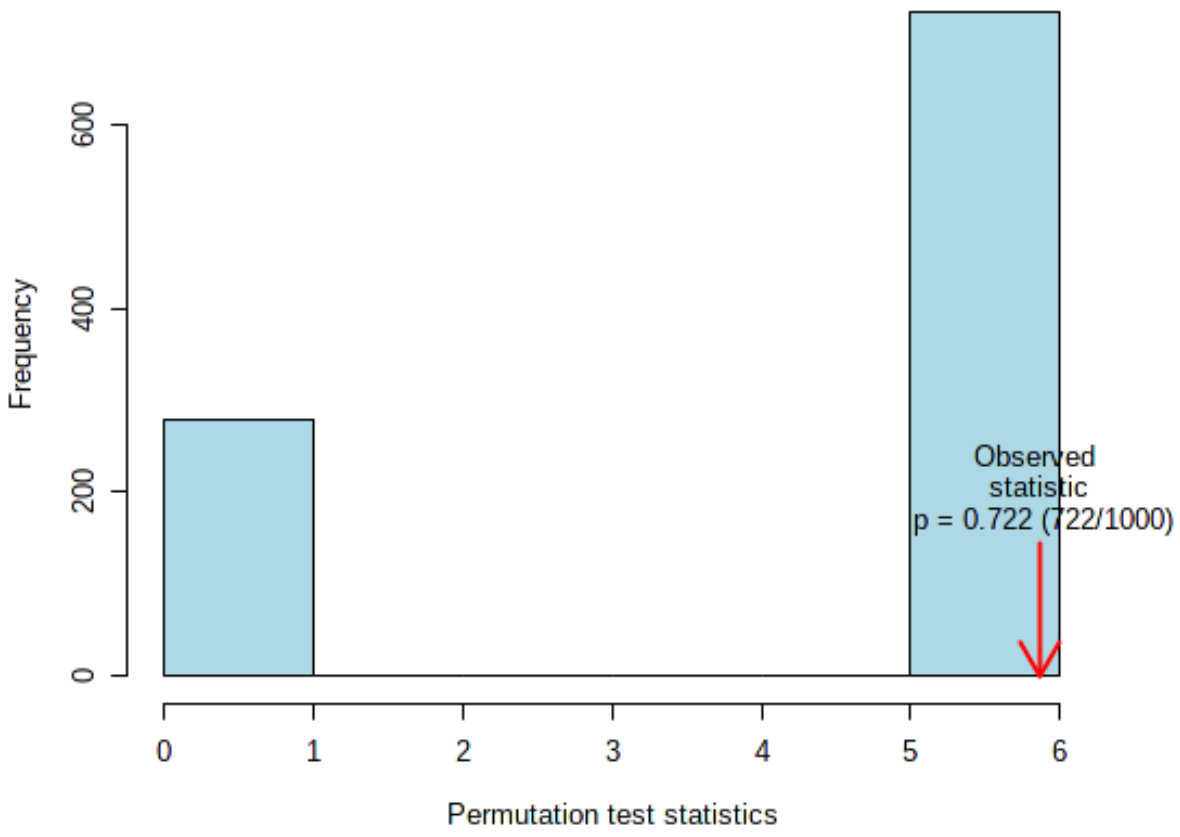

Figure 81. Significance related to Cost Low vs None identified by Permutation Test: PLS-DA model validation by permutation tests based on separation distance. The *P*-value based on permutation is *P* = 0.722 (722/1000). The *P*-value shows that there is no significant evidence between the data set and the model. The reason for the spike on the right hand is this group has low variability.

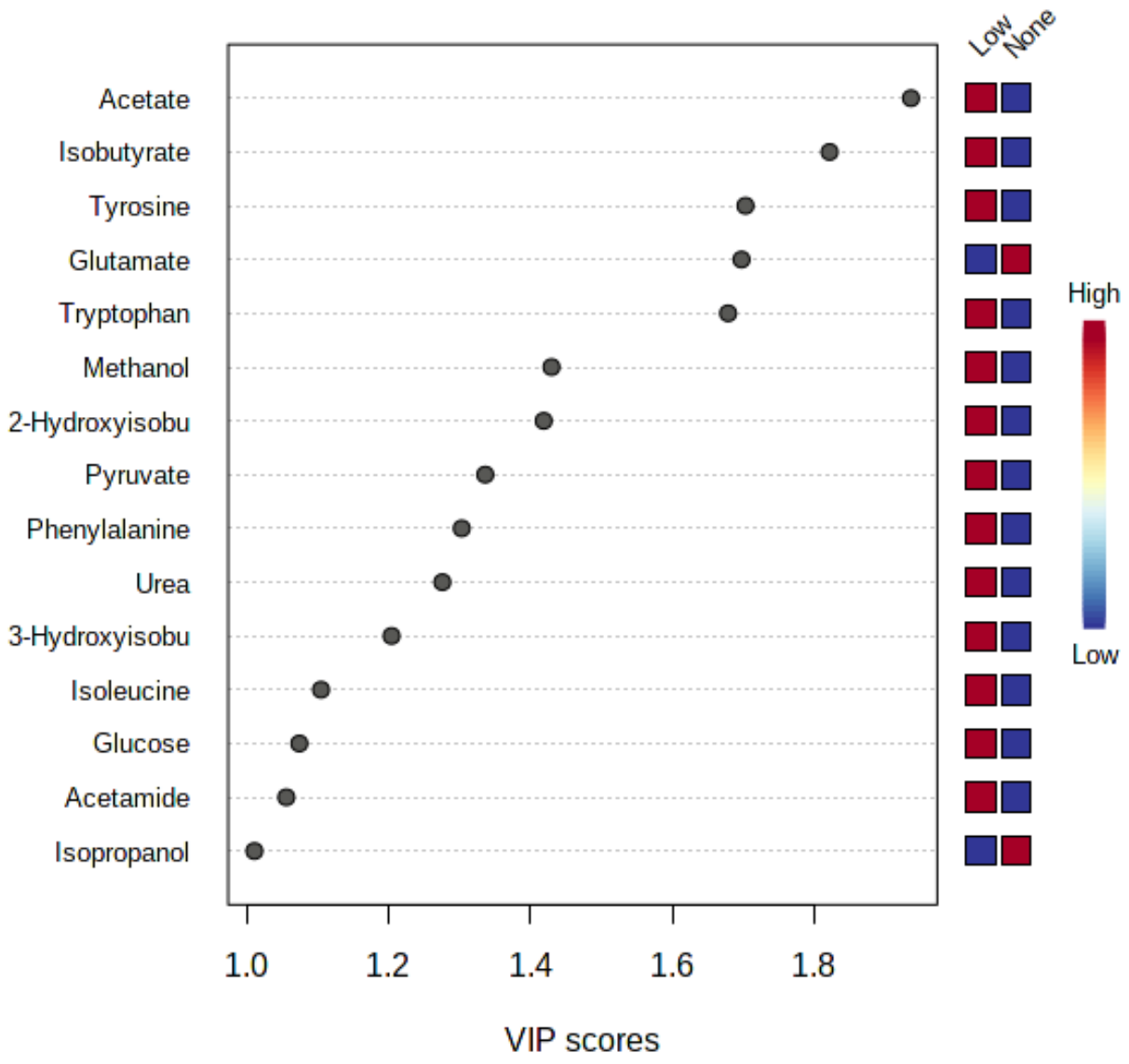

Figure 82. Significant Metabolites related to Cost Low vs None identified by VIP Scores: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each treatment cost class.

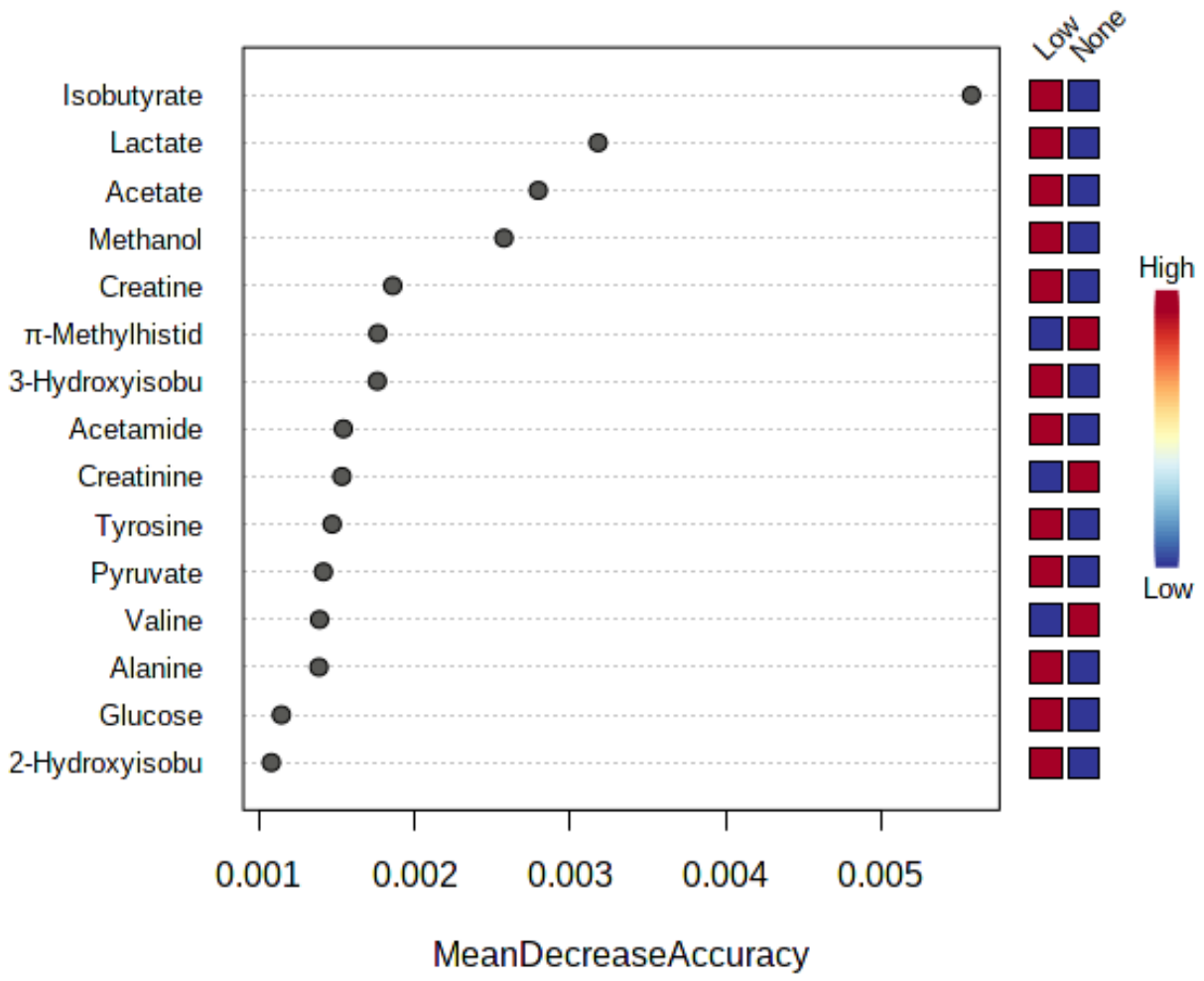

Figure 83. Significant Metabolites related to Cost Low vs None identified by Mean Accuracy: Significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted.

APPENDIX B:

IDENTIFICATION AND CHARCTERIZATION METABOITES IDENTIFIED BY NUCLEAR MAGNETIC RESONACE

Table 1: List of profiled metabolites. Acetone was removed for the purpose of this study due to its use in metabolite extraction.

Metabolite	Concentration (mM)	Maximum (mM)	Author
2-Hydroxyisbutyrate			Jesse Bouffiou
2-Hydroxybutyrate	0.0449	0.0563	Chenomx Inc.
3-Hydroxybutyrate	0.2037	0.1842	Chenomx Inc.
3-Hydroxyisbutyrate	0.0226	0.0178	Chenomx Inc.
Acetamide	0.1046	0.1039	Chenomx Inc.
Acetate	0.8575	0.8495	Chenomx Inc.
Alanine	0.085	0.0684	Chenomx Inc.
Allantoin			Jesse Bouffiou
Anserine			Jesse Bouffiou
Anthranilate	0.01881	0.0121	Chenomx Inc.
Betaine			Chenomx Inc.
Choline	0.0136	0.0136	Chenomx Inc.
Citrate	0.0136	0.0124	Chenomx Inc.
Creatine	0.0856	0.0857	Chenomx Inc.
Creatine Phosphate	0.0707	0.0993	Chenomx Inc.
Creatinine	0.708	0.1032	Chenomx Inc.
Dimethyl Sulfone	0.0075	0.0075	Chenomx Inc.
DSS	0.2328	0.1486	Chenomx Inc.

Formate	0.065	0.0651	Chenomx Inc.
Glucose	3.772	0.0168	Chenomx Inc.
Glutamate	0.0562	0.0606	Chenomx Inc.
Glycine	0.0271	0.1358	Chenomx Inc.
Hippurate	0.1007	0.0421	Chenomx Inc.
Isoleucine	0.0462	0.0462	Chenomx Inc.
Isopropanol	0.021	0.0138	Chenomx Inc.
Isobutyrate	0.0095	0.039	Chenomx Inc.
Lactate	1.0405	1.0395	Chenomx Inc.
Leucine	0.0692	0.0746	Chenomx Inc.
Methanol	0.0411	0.04	Chenomx Inc.
Phenylalanine	0.0395	0.0339	Chenomx Inc.
π-methylhistidine	0.01509	0.0921	Chenomx Inc.
Pyruvate	0.0485	0.0499	Chenomx Inc.
Succinate	0.0114	0.0251	Chenomx Inc.
T-methylhistidine	0.0334	0.0316	Chenomx Inc.
Tryptophan	0.0668	0.02	Chenomx Inc.
Tyrosine	0.0364	0.0036	Chenomx Inc.
Urea	4.7923	4.8266	Chenomx Inc.
Valine	0.094	0.0283	Chenomx Inc.
Acetone	0.0155	0.0153	Chenomx Inc.