DIAGNOSTIC APPLICATION OF FATTY ACID METHYL ESTER (FAME) ANALYSIS FOR THE IDENTIFICATION OF *MELOIDOGYNE* SPECIES

By

NICHOLAS SEKORA

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Dedicated to all of the aspiring Jedi Knights who look forward to the challenges life holds for them

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LIST OF ABBREVIATIONS

DGGE	Denaturing gradient gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay
FAME	Fatty acid methyl ester
I2	*Meloidogyne incognita* race 2
I3	*Meloidogyne incognita* race 3
J1	*Meloidogyne javanica* race 1
PCR	Polymerase chain reaction
QPCR	Real-time quantitative PCR
RA	Rapid FAME analysis
RE	Instant FAME extraction
RFLP	Restriction-fragment length polymorphism
SA	Standard FAME analysis using EUKARY method
SE	Standard FAME extraction method

Abstract of Dissertation Presented to the Graduate School
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Nicholas Sekora

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Previous studies have indicated that fatty acid methyl ester (FAME) analysis has the potential to be used as a diagnostic tool for diagnostic nematode identification. Four experiments were carried out from May 2010 to April 2012 to evaluate field-based applications, environmental influence, extraction and analysis methods, and detection limits on FAME analysis of *Meloidogyne* species. Utilizing *Solanum lycopersicum* root tissue samples infected with *M. arenaria*, *M. hapla*, *M. incognita*, or *M. javanica*, FAME analysis was able to separate the four nematode-infected tissues from each other and uninoculated tissue ($D^2 \geq 14.08$, $P \leq 0.0205$). To assess the effect of varying temperatures on nematode FAME profiles, populations of *M. incognita* and *M. javanica* were maintained on *S. lycopersicum* in three diurnal environments and two fixed-temperature environments. Temperature did not have a significant impact on FAME profiles of *M. incognita*-infected tissue ($D^2 \leq 5.69$, $P \geq 0.3192$) or uninoculated *S. lycopersicum* tissue ($D^2 \leq 3.13$, $P \geq 0.1006$) sustained in three diurnal environments, but did influence *M. javanica*-infected tissue ($D^2 \geq 27.64$, $P < 0.0001$). FAME profiles of *M. incognita*- and *M. javanica*-infected tissues maintained at 20°C and 26°C became more distinct over 135 days ($D^2 \geq 91.83$, $P \leq 0.0151$), but these infected tissues could not be

differentiated at 20°C (D^2 = 43.27, *P* = 0.2221). A comparison of FAME extraction and analysis methods determined Instant FAME extraction and Rapid analysis methods produced more robust and reliable FAME profiles ($D^2 \geq 25.08$, *P* < 0.0001) than standard methods (D^2 = 2.95, *P* = 0.9999) and reduced the sample size and time required for analysis. By assessing FAME analysis of *M. graminis* females at densities of 1, 2, and 5 individuals, single males, and single juveniles, it was possible to calculate the predicted response of a single nematode and establish a preliminary regression (*Response* = 13,019*(*Number*) – 10,827; R^2 = 0.5928, *P* < 0.0001) that could potentially be used to quantify nematodes in a sample. These experiments indicate that further development of FAME analysis for diagnostic identification of *Meloidogyne* species should be considered as an alternative to morphological or molecular methods.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

DIagnotic strategies for nematode manangement rely heavily on accurate identification of nematodes present either in soil or plant material. Management can vary greatly based on the genus, species, or, in some cases, host race present. For example, in the southern United States where cotton, peanut, and soybean are planted, identification of *Meloidogyne* species and host races is critical to make accurate recommendations for nematicide application, crop rotation, and other management practices (Kirkpatrick and Sasser, 1984; Rodríguez-Kábana *et al.*, 1992). In peanut growing regions of Florida, it is essential to identify the species of *Meloidogyne* species present in a field because both *M. arenaria* and *M. javanica* have been shown to infect peanut (Cetintas *et al*., 2003). Additionally, if *M. javanica* is present, it is important to determine the physiological race or races of the population since races 3 and 4 have demonstrated the ability to infected peanut while races 1 and 2 cannot (Lima *et al.*, 2002). To date no identification methods have been published that can identify species at the physiological race level consistently. Therefore, many of the molecular nematode identification methods developed are tageted toward species identificaition.

Identification of nematode species has been evolving rapidly over the last 20 years. Identification based strictly on morphological methods can range from easy to seemingly impossible depending on the identification resolution desired. For many nematodes, identification to genus (and some species) can be accomplished by visual inspection, such as with *Ironus* spp. and *Longidorus* spp. However, identification using morphological means requires intense training, the use of nematode extraction methods

specific to the habitat of the nematodes, and processes that can be time-consuming and sometimes labor-intensive. With the development of molecular identification methods, investigators are not required to recognize minute morphological characteristics of nematodes, identification is more accurate, and more in-depth studies of phylogeny and evolution can be performed. However, familiarity with the procedures used is still required. These methods may also be tedious and demand specialized equipment that is not always readily available to some laboratories.

This paper is a review of the molecular and biochemical methods most commonly used in the last 20 years to identify nematode species. These can be broken down into two primary categories: nucleic acid sequence analyses and methods utilizing specific biochemical components like protein electrophoresis and fatty acids. This list is not intended to be a complete listing of the studies done using these methods, but does represent the majority of procedures utilized today.

1.2 Nucleic Acid Sequence Analysis

Most of the recent work to identify methods for the identification of nematode species has focused on genetic differences, since these are the basis for the phenotypic expression of proteins and morphological features. DNA-based studies among nematode species began in the late 1980's (Curran *et al.*, 1986; Powers *et al.*, 1986; Curran and Webster, 1987; Bolla *et al.*, 1988; Kalinski and Huettel, 1988), and the increasing accessibility of the polymerase chain reaction (PCR) helped fuel studies on the genetic differences among nematode species. Currently, nearly every study of nematode phylogeny and genetic sequence comparisons utilizes PCR.

1.2.1 Polymerase Chain Reaction (PCR)

PCR was developed in 1983 by Dr. Kary Mullis (Saiki *et al.*, 1985). The method isolates specific areas of an organism's genome and amplifies the number of copies of the given sequence to levels that can be easily detected and used for further studies. Using PCR helps scientists observe the smallest differences among nematode species, making differentiation much easier than morphological studies of nearly identical species. However, finding genetic differences requires the amplification of the correct area of the organism's genome. Selection, amplification, and sequencing require the use of primers that are homologous to a desirable conserved sequence of nucleotides, gene, or locus within a genome that can be used to make comparisons among genotypes of a given group of organisms.

Primers used to begin sequencing of the selected areas can be very specific, selecting the sequence that will help to separate the designated species. Primer loci, such as the internally transcribed sequence 1 (ITS-1), small ribosomal subunit (SSU), and the D2 or D3 expansion segments are the most widely used primers in Nematoda, but new primers are being developed with more specificity to differentiate closely related species to a greater degree, such as the cytochrome oxidase II complex (Powers and Harris, 1993) and HSP 90 (Chitwood, 2003; Skantar and Carta, 2004). Often, the easily accessible primers cannot accurately separate certain closely-related species, requiring the development of a primer specific for the study. Primer development has become standard practice for most nematode genetic studies (Fullaondo *et al.*, 1999; Carta *et al.*, 2001; Floyd *et al.*, 2005).

The primary advantage of using PCR-based studies over other available methods is the potentially high degree of nematode species delineation. The smallest

differences among genomes can be observed, making identification, phylogeny, and evolutionary inference much easier. However, strictly using nucleotide sequences for phylogeny can lead to excessively splitting nematode species based on minute differences (Inserra *et al.*, 2007). Cryptic species are species that cannot be separated morphologically but differ genetically; there are many articles throughout biology discussing if these minute differences are really different species, just variation among individuals, or the beginnings of evolution within the species (Blouin, 2002; Gomez *et al.*, 2002; Herbert *et al.*, 2004; Sudhaus and Kiontke, 2007). However, Fonseca *et al*. (2008) proposed using the differences observed among multiple genes to better determine species delineation and better understand nematode species divergence. This approach, called integrative taxonomy, approaches species concepts as malleable for a given group of organisms based on morphological, molecular, ecological, and other definitive studies within that group that can be used to better define species and species concepts (Dayrat, 2005).

In addition to being potentially overly specific, the equipment required for sequencing the products of a PCR reaction are typically not present in most laboratories. Although sending amplicons to contract laboratories for Sanger sequencinghas become common practice, it still adds a step to identifying the amplified sequence, lengthening turnaround of sample identification. Even more specific and potentially expensive equipment is required for studies actually attempting to sequence a nematode's entire genome.

Despite the potential drawbacks mentioned above, the applicability of PCR has been widely accepted in nematological studies. Descriptions of new species require a

genetic analysis, typically using PCR, and publishing the unique sequence in a database (i.e. GenBank) for future comparisons among species (Kanzaki *et al.*, 2000; Rubtsova *et al.*, 2001; Karssen *et al.*, 2004). Studies of nematode genomes involving PCR appear to have a significant resolving and lasting power in nematology because of their numerous advantages over strictly morphological observations. To date, over 100 papers have been published using PCR for identification within several nematode groups (Table 1).

1.2.2 Methods Utilizing PCR

1.2.1.1 Restriction fragment length polymorphisms (RFLP)

RFLP comparisons, one widely used permutation of PCR, compares fragments of DNA homologues among species that have been broken down using restriction enzymes. Restriction enzymes cleave nucleotide sequences at specific points based on the conformation of the enzyme. For example, the enzyme EcoRI cleaves DNA sequences with the sequence 5'…GAATTC…3', and its complimentary strand 3'…CTTAAG…5', between the G-A phosphodiester bond on both strands, leaving a "sticky end" that can be used to anneal primers; other enzymes may create "blunt ends" that do not require a sequence match (Baum *et al.*, 1994). RFLPs are used to compare sequence lengths, as well as the sequence itself, among species to determine relatedness. RFLPs are the basis of all comparisons amplifying a specific region of an organism's genome using PCR (Gasser *et al.*, 1994; Gasser and Haoste, 1995; Reid *et al.*, 1997; Subbotin *et al.*, 1999; Madani *et al.*, 2004; Umehara *et al.*, 2006).

RFLPs are advantageous for identifying nematode species. Sequences can range in size with no real limitation on the length of the segment studied except the resolution of the equipment used for sequencing. They can also be used to track

changes in species genomes by determining at which locus a mutation occured, providing clues to evolutionary events and species divergence. The primary drawback of using RFLPs comes with the use of restriction enzymes. To properly compare species with RFLPs, the correct sequence must be selected in both species, requiring the correct restriction enzyme to cut the nucleotide sequence at the right position. Integral to this is the need to have some idea of the type of primer that will be used in the study; does the sequence of the primer match up with the sequence left by the restriction enzyme? Once these issues have been addressed, RFLPs can be utilized in many types of PCR applications (Subbotin *et al.*, 2000; Szalanski *et al.*, 1997).

A modification of RFLP-PCR, multiplex PCR, uses RFLP primers for multiple nematode species in the same reaction mixture. This allows a sample to be examined for several sequences simultaneously without having to use separate reaction mixtures. Multiplex PCR is used primarily where several similar species that are difficult to differentiate morphologically can be present in a sample (Stanton *et al.*, 1997; Zarlenga *et al.*, 2001; Oliveira *et al.*, 2005; Umehara *et al.*, 2008). However, there are applications where multiplex PCR can be used for detection of a specific nematode from samples that include numerous other species (Subbotin *et al.*, 2001).

1.2.1.2 Real-time quantitative PCR (qPCR)

Real-time quantitative PCR is a modification of the original PCR protocol that allows the amplified DNA to be measured in real time as the reaction proceeds. The volume of DNA replicates produced is quantified using the number of amplification reactions and the amount of fluorescent dye present in the reaction mixture. Using the results of the quantification process it is theoretically possible to determine the amount of DNA from the region amplified per organism, which could have future applications in

ecological studies and other studies where different levels of nematode populations are to be detected or quantified. The process is relatively new, but research has utilized this method for the detection and quantification of *Globodera* spp. and *Heterodera* spp. in soil (Madani *et al*., 2005; Quader *et al.*, 2008), determining levels of biological control agents in production areas (MacMillan *et al.*, 2006), and monitoring population levels of virus-transmitting nematode species (Holeva *et al.*, 2006; Sato *et al.*, 2007). The incorporation of real-time PCR equipment into diagnostic laboratories could help to increase the detection accuracy by reducing the extraction errors of nematode assay and allow diagnosticians to provide more complete recommendations to clients.

1.2.1.3 DNA barcoding using molecular operational taxonomix units (MOTUS)

The prospect of increasing our knowledge of species richness has been a goal of many scientists since taxonomy began. However, this task was immense and nearly impossible using traditional morphological characterization to separate and identify species within a sample (Floyd *et al.*, 2002; Powers, 2004; Blaxter *et al.*, 2005; De Ley *et al.*, 2005; Creer *et al.*, 2010). By applying PCR techniques to whole samples, many different species from nearly every corner of biology can be detected, making untargeted studies exciting but difficult to interpret. The purpose of DNA barcoding is to produce a primer set that can be used to successfully and consistently amplify sequences within a community (MOTUS) and distinguish the number of species present. Using primers specific for a selected group, such as nematodes, can help to reduce the PCR products by preventing the amplification of genomes from unwanted organisms. With the use of nematode-targeting primers in the PCR reaction, researchers can determine the nematode species richness of a sample as well as determine if any previously undescribed species are present in soil, forest, and marine

communities (Floyd *et al.*, 2005; Bhadury *et al.*, 2006a; Bhadury *et al.*, 2008; Porazinska *et al.*, 2009; Powers *et al.*, 2009; Derycke *et al.*, 2010; Porazinska *et al.*, 2010; Bucklin *et al.*, 2011). Although DNA barcoding shows great promise for ecological studies, it is still in its early stages and much work needs to be completed to ensure that certain groups are not overlooked or that other organisms from another phylum are not included erroneously (Blaxter *et al.*, 2005).

1.2.1.4 Denaturing gradient gel electrophoresis (DGGE)

Another method of using PCR products similar to DNA barcoding is subjecting the products to gradient electrophoresis that separates DNA segments based on the points at which they denature during migration within the gel. As opposed to using temperature gradient gel electrophoresis (TGGE), DGGE utilizes a chemical gradient to cause denaturation of the DNA molecules. However, using a chemical gradient can be problematic since it is difficult to consitently replicate the exact mixture required for accurately separating DNA, reproduction of the chemical mixture for future runs may be difficult, and the ionic interactions can cause artificial recombination of DNA strands. Regardless of these pitfalls, DGGE has been included in several studies attempting to describe nematode communities from marine (Cook *et al.*, 2005; Bhadury *et al.*, 2006; Derycke *et al.*, 2007), soil (Foucher and Wilson, 2002; Waite *et al.*, 2003; Foucher *et al.*, 2004; Fujii *et al.*, 2004; Griffiths *et al.*, 2006; Donn *et al.*, 2007; Okada and Oba, 2008; Wang *et al.*, 2008; Chen *et al.*, 2010), and Antarctic environments (Christner *et al.*, 2003; Yergeau *et al.*, 2006). Based on these studies, DGGE may be applicable to nematode community studies in the future, but it is currently overshadowed by DNA barcoding and multiplex PCR.

1.3 Biochemical Assays

1.3.1 Proteins

Protein studies of nematode species began in the 1950's (Allen, 1952; Holt, 1958) as a novel observation in nematodes. Several papers followed these first observations, finding esterases in many nematode species (Rhode, 1960; Lee, 1964; Geraert, 1965; Bird, 1966). Based on the presence of these enzymes in so many nematode species, a method was developed to identify nematodes based on electrophoresis (Dickson *et al.*, 1970; Dickson *et al.*, 1971). This method requires the proteins within a nematode migrating through a polyacrylamide gel matrix that separates the proteins by size and charge (Davis, 1964; Ornstein, 1964). After the proteins have separated on the gel, stains are applied specific to the desired enzymes to be observed. Today the enzyme profiles to be observed are primarily esterases and malate dehydrogenase in the genus *Meloidogyne* (Esbenshade and Triantaphyllou, 1985; Fargette, 1987; Fargette and Braaksma, 1990; Navas *et al.*, 2002; Cetintas *et al.*, 2003; Oka *et al.*, 2003; Handoo *et al.*, 2004; Karssen *et al.*, 2004; Flores-Romero and Navas, 2005; Castagnone-Sereno, 2006; Brito *et al.*, 2008), but can be various other allozymes for other nematode groups (Chilton *et al.*, 1992, 1992a; La Rossa *et al.*, 1992; Chilton *et al.*, 1993; N'zobadila *et al.*, 1993; George-Nascimento and Llanos, 1995; Beveridge, 1998; Noel and Liu, 1998; Andrews and Chilton, 1999).

For nematode identification, isozyme and allozyme analysis can be very useful. Most of these enzymes, with a few exceptions, are species specific in their electrophoretic pattern. However, resolution beyond the species level (biotypes, races, pathotypes, etc.) is not reliable with protein studies (Carneiro *et al.*, 2000). Like genetic

studies, these protein assays can be time consuming and relatively expensive due to the specific stains and equipment required for the analyses.

1.3.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) use antibody labeling to identify an organism based on very specific binding to the proteins assayed. As in isozyme and allozyme studies, nematode proteins can be run through a polyacrylamide gel to separate them and then transferred to cellulose paper for the assay, or the whole nematodes can be placed in a well-plate to conduct the assay. The proteins present are then detected by either using a conformation change of the antibody caused by enzyme binding to release a dye, or more commonly by adding an enzyme that breaks down to an indicator dye. ELISA assays are widely used in the sciences, but have had limited application with nematodes (Davies and Lander, 1992; Lawler *et al.*, 1993; Ibrahim *et al.*, 1996; Kennedy *et al.*, 1997; Ding *et al.*, 1998; Ibrahim *et al.*, 2001; Abrantes, *et al.*, 2004; Lima *et al.*, 2005). Although they have not been developed yet, a movement toward ELISA-based identification strips could allow for field identification of nematode species. These so-called immunostrips have already been developed for many applications in medical and phytopathological sciences with great success (Fernández-Sánchez *et al.*, 2005; Liebenberg *et al*., 2009), so a nematode identification form could be just as widely accepted.

1.3.3 Other Methods

1.3.3.1 Glycoproteins

Methods using glycoproteins showed promise as a means to identify nematodes below the species level (McClure and Stynes, 1988), but many of these methods have been eclipsed by PCR-based methods. An alternative method using fatty acids to

identify nematodes has also been proposed (Sekora *et al.*, 2009, 2010), but the actual application has yet to be determined. Currently, the most widely accepted means of nematode identification are PCR-based methods (qPCR and RFLP) and protein assays (isozymes). These methods are reproducible and reliable, making them ideal candidates for nematode assays in diagnostic and regulatory fields as well as in scientific studies.

1.3.3.2 Fatty acid methyl ester (FAME) analysis

Nematode fatty acids have been studied since 1964 (Beames and Fisher, 1964), and many studies compared the fatty acid composition of several nematode genera and species (Sivapalan and Jenkins, 1966; Krusberg, 1967; Krusberg, 1972; Krusberg *et al.*, 1973; Orcutt *et al.*, 1978; Chitwood and Krusberg, 1981; Chitwood and Krusberg, 1981a; Hutzell and Krusberg, 1982). With the introduction of DNA-based methods, fatty acid studies have gone out of favour and have not been conducted as extensively as in previous decades. However, Sekora *et al.* (2008a) began studies to adapt the FAME (fatty acid methyl ester) system, developed for bacterial identification (Sasser, 1990), for diagnostic nematode identification. These studies found that plant-parasitic nematode genera and species could be identified from juveniles in soil (Sekora *et al*., 2009) and that it may be possible to increase the sensitivity of the FAME system to identify individuals within a sample (Sekora *et al.*, 2008). Although the limits of the FAME system have not yet been established, there may be the potential for using it as a means for diagnostic identification in extension and regulatory labs.

1.4 Conclusions

Of the numerous methods mentioned, nearly all of them are used by select labs around the world. Many of these methods are used in conjunction with one another to

provide confirmation for results developed using morphological observations. Although still second to morphology, DNA-based methods currently appear to be the most widely used means for identification of many nematode species and could potentially overshadow morphology in recognition of novel species (Blaxter *et al.*, 2005).

Although many of these methods are incorporated into nematology labs, diagnostics of nematode soil samples is usually still carried out using morphology supplemented by these advanced methods in select circumstances (*Meloidogyne* species identification, for example). Using these methods can require certain life stages (juveniles, males, females), some of which are not easily obtained from soil samples (isozyme analysis of female *Meloidogyne*) and are not always in the best condition or without accompanying plant material. An additional step in processing to extract the desired compound for analysis (extracting nematode DNA from plant tissue) may be required before the actual analysis can be performed.

Given these restrictions, most of the advanced identification methods covered in this chapter would not be applicable for rapid diagnosis of nematode samples. In these instances a method is needed that can isolate the necessary information from the plant tissue without adding cost or an additional, and sometimes lengthy, step. Considering these limitations and requirements, the following dissertation focuses on adapting FAME analysis for use as a diagnostic tool for these special circumstances.

Table 1-1. Selected publications utilizing molecular sequences for nematode identification.

Nematode Group	Genus	Reference
Animal Parasites	Multiple	Gasser and Monti, 1997
	Marshallagia and *Ostertagia*	Dallas *et al.*, 2000
	Anisakis	Pontes, *et al.*, 2005
	Dirofilaria and *Acanthocheilonema*	Casiraghi *et al.*, 2006
	Toxocara and *Toxascaris*	Li *et al.*, 2007
Entomopathogenic	*Heterorhabditis* and *Steinernema*	Liu and Berry, 1995
	Heterorhabditis and *Steinernema*	Pamjav *et al.*, 1999
	Heterorhabditis	Nguyen *et al.*, 2004
	Teratorhabditis	Kanzaki *et al.*, 2008
Free-living	Multiple	van der Knaap *et al.*, 1993
	Panagrolaimus	Abebe and Blaxter, 2003
Marine Nematodes	Multiple	Pereira *et al.*, 2010
	Multiple	Thomas *et al.*, 1997
	Pseudoterranova	Zhu *et al.*, 2002
	Multiple	Floyd *et al.*, 2005
	Terschellingia	Bhadury *et al.*, 2008
	Multiple	Donn *et al.*, 2011
Plant Parasites	*Meloidogyne*	Harris *et al.*, 1990
	Meloidogyne	Powers and Harris, 1993
	Heterodera	Szalanski *et al.*, 1997
	Pratylenchus	Uehara *et al.*, 1998
	Heterodera and Meloidogyne	Clapp *et al.*, 2000
	Heterodera	Amiri *et al.*, 2002
	Heterodera	Maafi *et al.*, 2003
	Xiphinema	Hübschen *et al.*, 2004
	Bursaphelenchus	Kang *et al.*, 2004
	Rotylenchulus	Agudelo *et al.*, 2005
	Meloidogyne	Powers *et al.*, 2005
	Bursaphelenchus	Takeuchi *et al.*, 2005
	Meloidogyne	Tigano *et al.*, 2005
	Belonolaimus	Gozel *et al.*, 2006
	Meloidogyne	Adam *et al.*, 2007

Table 1-1. Continued.

	Hirschmanniella	De Ley *et al*., 2007
	Fergusobia	Ye *et al*., 2007
	Buraphelenchus	Ye *et al.*, 2007a
	Meloidogyne	Hu *et al.*, 2011

CHAPTER 2
FATTY ACID METHYL ESTER ANALYSIS USED TO IDENTIFY *MELOIDOGYNE* SPECIES IN *SOLANUM LYCOPERSICUM* ROOT TISSUE

2.1 Introduction

Many methods have been developed to identify *Meloidogyne* species, but most of these techniques require isolation of individuals from soil or roots (Barker *et al*., 1985; Powers and Harris, 1993). This isolation step can be a limiting factor when the quality of the sample is crucial for the type of analysis to be performed. For example, isozyme analysis requires healthy females that have begun to produce egg masses (Esbenshade and Triantaphyllou, 1985). If the sample is old, dried, or if the females are damaged by fungi, dehydration, or other means, it can be nearly impossible to get accurate results.

In an attempt to bypass some of the possible difficulties encountered with the most widely used methods, Sekora *et al*. (2010) were able to use fatty acid methyl ester (FAME) analysis to identify several *Meloidogyne* species. Even though these previous studies were performed using juveniles, it is likely that this method could be used on other life stages such as mature females. It is also possible that the FAME method could be used to identify *Meloidogyne* spp. within root tissue, eliminating the need for isolation of mature females.

If nematode species identification using infected root tissue is possible, methods for preparing tissue for analysis need to be evaluated. For example, root tissue dried prior to FAME extraction may produce different profiles than fresh tissue immediately submitted to extraction. This could also be true for tissue that is homogenized before extraction since the extraction method may be limited in its ability to penetrate the root tissue. Based on these concerns, the following objectives were developed: 1)

determine the ability of the FAME method to identify *Meloidogyne* species from infected root tissue, 2) conclude if homogenization produces a more reliable FAME profile than whole tissue and 3) evaluate the effect, if any, of fresh versus dried root tissue in conjunction with standard and concentrated extractions on FAME profiles.

2.2 Materials and Methods

Based on the stated objectives, a series of tests were set up to determine the best preparation of infected root tissue for FAME analysis. These tests included comparisons of *Meloidogyne*-infected roots to uninoculated root tissue, homogenized roots to whole roots, and fresh roots to dried roots combined with standard (dilute) and concentrated FAME preparations.

2.2.1 Fatty Acid Methyl Ester (FAME) Analysis

Root tissue samples weighing 40 mg were used for FAME extraction in all experiments. Extraction of fatty acids was conducted using the method described by Sasser (1990) and involved the four steps of saponification, methylation, extraction, and washing (Figure 2-1). Samples were analyzed using an Agilent 6890N Gas Chromatography System (Agilent Technologies, Santa Clara, CA). For each analysis, 2.0 µL of sample solution was injected into an Ultra 2 Cross-linked 5% Phenyl Methyl Siloxane column (Agilent Technologies, Santa Clara, CA) linked to a flame-ionization detector and analyzed using the EUKARY method of the Sherlock Analysis Software® (MIDI, Newark, DE). Sample profiles included total response of the sample (mV), responses for each fatty acid observed (mV), and the calculated proportion of each fatty acid response as a percentage of the total response. All profiles were exported to a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA) for further analysis.

2.2.2 Experimental Procedures

2.2.2.1 FAME evaluation of root tissue infected with *Meloidogyne* spp.

Confirmed single-egg mass populations of *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, and *M. javanica* were maintained on *Solanum lycopersicum* 'Rutgers' under greenhouse conditions at the University of Florida IFAS Greenhouse Complex in Gainesville, FL. In July, 2010, samples were selected from root tissue infected with each of the four *Meloidogyne* species and uninfected root tissue (negative control). Root samples of each *Meloidogyne* species were selected by the presence of galls or egg masses; tissue samples of uninoculated *S. lycopersicum* were randomly selected throughout the root system. Replications were based on the availability of tissue for analysis and ranged from 24 (*M. hapla*) to 78 (*M. arenaria*); 260 samples were analyzed in total (Table 2-1).

2.2.2.2 Tissue homogenization

Root tissue of *S. lycopersicum* 'Rutgers' was used to compare homogenized tissue to whole tissue without homogenation. Two samples, one for each preparation, were selected at random from a single root system; a total of 20 replicates were prepared per treatment for a total of 40 samples. Tissue homogenization was achieved by using a modified steel spatula attached to a Dremel (Robert Bosch Tool, Farmington Hills, MI) while the root sample was submerged in the first FAME reagent (3.75 M NaOH in 50% CH_3OH; Figure 2-1).

2.2.2.3 Fresh versus dried tissue and standard versus concentrated samples

Samples of *S. lycopersicum* tissue infected with *Meloidogyne javanica* were selected for analysis as described previously. Half of the 48 40-mg samples were subjected to drying in an incubator at 50°C for 2 days followed by FAME extraction,

whereas the remaining half immediately underwent FAME extraction. From each 24-sample set, 12 samples of 1.25 mL extract were analyzed directly while the remaining 12 were evaporated under a fume hood and reconstituted in 75 µL of 50/50 methyl tertiary-butyl ether/hexane. Therefore, the experimental design included four treatments with 12 replicates each and was repeated, for a total of 96 experimental units. Resulting FAME profiles for each of the samples were analyzed jointly to determine the most desirable combination. Independent runs were combined for a larger data set since runs were not a significant factor in the analysis ($P = 0.7948$).

2.2.3 Statistical Analysis

Exported profiles were imported into SAS (SAS Institute, Cary, NC) for further analysis. Mean profiles for each character or categorical "class" (homogenized tissue, *M. arenaria* infected tissue, etc.) were calculated with PROC MEANS which provided the average response for each fatty acid in all samples for the given class. Additional statistical tests were performed using with PROC STEPDISC in combination with PROC CANDISC following the method of Sekora *et al*. (2010a). PROC STEPDISC was used to determine which fatty acids were significant for discrimination among classes using a series of stepwise analysis of variance (ANOVA) tests that evaluate the *F*-value of each fatty acid before and after inclusion (Johnson, 1998). After analysis of each fatty acid, fatty acids significant for delineation ($P < 0.15$) were used for canonical discriminant analysis (CDA) with PROC CANDISC.

CDA produces class means based on sample variance within each compared class and then represents relationships among classes in dimensional space. The dimensional space is represented by canonical variates (CAN1, CAN2, up to class *n*-1) that demonstrate class separation in graphical representation and can be assigned to *x*,

y, or *z* axes depending on the desired class comparisons. Canonical variates are also used to describe the total multivariance within a test, and the number of variates in these tests was reduced to the fewest that could define at least 75% cumulative proportion of the total multivariance (*n*CAN $\leq$ 3). Separation among classes is defined by the degree of "between canonical structure" correlation (-1 to 1) of a given fatty acid along the chosen canonical variate. Absolute values approaching |1.000| indicate a high degree of correlation and help to separate classes on the specified dimension. The greater the value of correlation, the greater the spatial distance (Mahalanobis distance or D^2) among means graphically along a given canonical variate (Johnson, 1998). For the experiments described in this dissertation, high canonical correlation was described by correlations greater than |0.750| and significant means separation was achieved with D^2 having a *P*-value less than 0.05.

Additional information provided by CDA is the canonical correlation and eigenvalue of each canonical variate. Canonical correlation values range from 0 to 1 and are an indicator of the importance of each canonical variate to the separation of classes. Canonical correlation values approaching 1 are considered more informative for describing the majority of multivariance within a given analysis. The eigenvalue is another statistic similar to canonical correlation that is used to rank canonical variates based on the multivariance explained by the selected variate. As with canonical correlations, higher values indicate a greater degree of explained multivariance in an analysis for the given canonical variate (Johnson, 1998).

In total, 396 samples were prepared for FAME analysis. However, samples with FAME profiles of a single fatty acid assigned the observed fatty acid a percentage

abundance of 100%. Therefore, samples producing one fatty acid were excluded from analysis to prevent data skewing. As a result, 356 samples total were analyzed among the three experiments.

2.3 Results

2.3.1 FAME Evaluation of Root Tissue Infected with *Meloidogyne* Species

Fatty acid chain lengths ranged from 10 carbons (capric acid) to 25 carbons (pentacosylic N alcohol). Several fatty acids that have yet to be fully identified by chain length and structural configuration were detected during analysis. Commonly encountered unidentified peaks in the EUKARY database are designated by their retention time (i.e. unknown 20.588). Several fatty acid peaks were observed during these experiments that were not in the EUKARY database and were therefore named based on their respective retention times (unknown 8.281, unknown 13.671, etc.). Some of these unknown fatty acids made up at least 33% of the total fatty acids observed among all tissue treatments.

One of these unknown fatty acids, unknown 21.808, was the most prevalent in *S. lycopersicum* (31.01%), *M. arenaria* infected tissue (24.79%), and tissue containing *M. incognita* (40.73%); root tissue infected with *M. hapla* or *M. javanica* also contained 27.00% and 19.95%, respectively, of this fatty acid (Table 2-2). Elaidic acid (18:1ω9t) was the most commonly occurring fatty acid in *M. hapla* and *M. javanica* infected tissue, (48.58% and 24.94%, respectively), the second most common in tissue containing *M. incognita* (20.02%), and the third highest for tissue infected with *M. arenaria* (16.44%), but was not observed in uninoculated *S. lycopersicum*. Palmitic acid (16:0), the third most predominant fatty acid, was found in both infected and uninfected root tissue and

ranged in concentration from 11.49% in *M. incognita*-containing tissue to 21.10% in *M. arenaria*-infected root tissue.

Because *S. lycopersicum* tissue was the most predominant tissue in samples, FAME profiles of uninoculated root tissue were similar to those of infected tissue. However, there were enough differences in fatty acid expression to differentiate infected root tissue from uninfected, and to discriminate among infection by the different *Meloidogyne* species using CDA ($P \leq 0.0026$; Table 2-3; Figure 2-2). The first canonical variate (CAN1) described 46.8% of the total variation among root treatments. Along CAN1, four fatty acids were responsible for separating *S. lycopersicum* root tissue from tissue infected with *M. hapla*, elaidic acid, unknowns 11.981 and 8.281, and sebacic acid, with absolute values of canonical coefficients |0.934|, |0.824|, |0.811|, and |0.765|, respectively (Table 2-4). Four other fatty acids - unknown 22.682 (|0.845|), pentacosylic N alcohol (|0.813|), unknown 20.588 (|0.786|), and unknown 23.670 (|0.770|) - were responsible for separating *M. hapla*-infected tissue and uninoculated *S. lycopersicum* from tissue infected with *M. javanica* along CAN2 and accounted for 28.1% of the total variation. All four of these fatty acids were found in tissue containing either *M. hapla* or *M. incognita* as well as uninoculated *S. lycopersicum*, but were not present in tissue infected with either *M. arenaria* or *M. javanica*. Although the four significant fatty acids in CAN2 did not separate root tissue containing *M. arenaria* from *M. incognita*-infected tissue, palmitic acid was significant for their separation along CAN3 (|0.951|) and accounted for 13.5% of the variation described by CDA (Table 2-4; Figure 2-2).

2.3.2 Root Tissue Preparation

2.3.2.1 Tissue homogenation

A total of 11 fatty acids were observed, five in homogenized tissue and eight in whole tissue (Table 2-5). Of these fatty acids, palmitic acid and unknown 8.281 were the only two found in both tissue preparations. Palmitic acid was the most abundant fatty acid in homogenized tissue samples, accounting for 65% of fatty acids observed. Whole tissue preparations contained six unknown fatty acid peaks that accounted for 86% of the total fatty acid content. Palmitic acid (3.1%) and a nonadecylic N alcohol (9.9%) were the only named fatty acid peaks contributing to the whole tissue profile.

CDA of homogenized and whole tissue revealed a D^2 separation of 1087 ($P < 0.0001$). Due to the large number of fatty acids appearing exclusively in either tissue preparation, the eight fatty acids selected by STEPDISC were perfectly correlated with the first canonical variate (CAN1=1 or -1; Table 2-6). Palmitic acid, stearic acid, and unknown 8.281 were aligned with a canonical correlation value of 1 while nonadecylic N alcohol and four unknown fatty acids (13.671, 20.588, 21.808, and 22.682) aligned along CAN1 at -1 (Table 2-6).

2.3.2.2 Fresh versus dried tissue and standard versus concentrated samples

Sixty of the 96 total samples analyzed produced usable FAME profiles, of which 44 were from samples dried before extraction. In addition, tissue dried before FAME extraction produced more fatty acids in both standard (17) and concentrated (12) samples than fresh tissue (3 and 8 fatty acids, respectively; Table 2-7). Although fatty acid chain lengths of 16 and 18 carbons were the most abundant in all samples, profiles from fresh samples were restricted to these chain lengths while dried samples produced profiles with a wider range of fatty acids (13 to 21 carbons).

Observable differences among profiles were more apparent using CDA, where each type of preparation (fresh/dried and standard/concentrated) was separated by a canonical axis; CAN1 separated standard and concentrated samples while CAN2 divided fresh and dry tissue (Figure 2-3). Values of D^2 were greatest when comparing standard to concentrated extractions ($D^2 \geq 14.92$, $P < 0.0001$), but were also significant between fresh and dried tissue that was not concentrated before analysis ($D^2 = 11.89$, $P = 0.0205$; Table 2-8). Among combinations of tissue and extraction preparation, standard extractions of fresh and dried tissue were significantly different from each other, but no statistical difference could be seen between fresh or dried tissue extractions that were concentrated before analysis.

2.4 Discussion

These results indicate that identification of *Meloidogyne* species may be possible using infected root tissue. Separation among species was clear using 40-mg of infected tissue, but future research may determine if smaller amounts of tissue could provide a more accurate depiction of the infecting species and reduce background noise from plant tissue, endophytic fungi, and bacteria. Reducing the amount of tissue per sample while increasing the number of samples analyzed could also help detect mixed populations by preventing a more prevalent species from masking a less common species (Goodell and Ferris, 1981). By coupling a reduced amount of tissue with techniques like homogenation and sample concentration before analysis, it may be possible to be as accurate in identification as with a larger sample, but without a reduction in sensitivity.

Some fatty acids may also serve as potential indicators for an individual species based on the amount present in a sample. Previous studies have indicated that fatty

acids such as arachidonic acid could serve as a genus indicator for *Heterodera glycines* (Sekora *et al.*, 2009a), so it is possible that specific fatty acids found within the *Meloidogyne* group could vary in their incidence among species. In this study we observed that myristic acid, and the overall abundance of several 18-carbon fatty acids could be used as indicators for the presence of specific species in plant tissue. Some evidence of this was observed by Sekora *et al.* (2010) when comparing extracted juveniles of four *Meloidogyne* species using FAME analysis. In their study, it was possible to separate the four species and to tentatively discriminate among three host races of *M. incognita*. By incorporating the techniques presented in this paper, it may be possible to duplicate the results of Sekora *et al.* (2010) with infected plant tissue containing fewer individuals.

Future studies will focus on improving these techniques to improve sensitivity as well as to determine the amount of infected plant tissue required for accurate identification. At this point in development, it will be necessary to include uninfected tissue to act as a baseline for studies comparing *Meloidogyne* species, but it may be possible to create an identification library that focuses only on fatty acids present in *Meloidogyne* species regardless of host. The effect of different plant hosts, and cultivars of the same host, should also be considered to determine the applicability of this method when comparing nematode species from different plant hosts.

Based on the studies presented in this chapter, it may be possible to use FAME analysis of root tissue infected with *Meloidogyne* species as a means for identification in conjunction with traditional methods. Homogenizing tissue and concentrating the final extraction product can help increase the sensitivity of samples processed from infected

root tissue without a need to select fresh or dried tissue. Further development of these methods could lead to a rapid alternative for diagnostic identification of *Meloidogyne* species without the need for isolation of specific life stages or acquiring samples in pristine condition for analysis.

Table 2-1. Number of replicates used of *Meloidogyne*-infected *Solanum lycopersicum* root tissue and controls for FAME analysis.

Treatment	Replications
M. arenaria infected tissue	78
M. hapla infected tissue	24
M. incognita infected tissue	43
M. javanica infected tissue	49
S. lycopersicum alone	44

Table 2-2. Mean FAME concentrations (percentage of total response) of root tissue containing *Meloidogyne arenaria*, *M*. *hapla*, *M*. *incognita*, and *M*. *javanica* versus uninoculated *Solanum lycopersicum* tissue.

Fatty Acid	Uninoculated *S. lycopersicum*	*M. arenaria*	*M. hapla*	*M. incognita*	*M. javanica*
iso-undecylic 3OH	--†	0.80	1.16	--	--
iso-tridecylic 3OH	0.46	0.06	--	--	0.26
Myristic acid	--	0.58	0.62	--	1.05
iso-pentadecylic	--	0.23	0.76	--	0.43
15:1 *iso* F	0.37	0.09	--	--	0.32
Palmitic acid	17.21	21.10	13.69	11.49	14.43
Palmitoleic acid	--	0.88	0.86	--	1.40
Stearic acid	7.07	8.46	6.88	4.94	7.20
18:1 ω8*t*	--	5.41	8.63	--	8.56
Oleic acid	0.33	0.91	--	0.10	1.68
Elaidic acid	--	16.44	48.58	20.02	24.94
Linoleic acid	3.91	3.97	4.63	2.13	3.49
Nonadecylic N Alcohol	3.74	--	1.61	0.92	--
20:1 ω7*c*/20:1 ω9*t*	--	0.27	1.04	--	0.47
Heneicosylic acid	0.15	--	--	--	0.33
Pentacosylic N Alcohol	0.24	--	0.75	0.12	--
Sebacic acid	2.58	--	--	--	0.19
Unknown 8.281	20.29	8.72	6.00	8.65	7.64
Unknown 11.981	0.45	2.08	4.31	1.93	--
Unknown 13.671	5.68	4.97	6.25	9.28	3.82
Unknown 19.276	--	--	--	--	0.48
Unknown 20.588	3.42	--	0.94	0.16	--
Unknown 21.808	31.01	24.79	27.00	40.73	19.95
Unknown 22.682	3.52	--	1.54	0.79	--
Unknown 23.670	3.44	--	0.81	0.64	--
n	44	78	24	43	49

†=Not detected.

Table 2-3. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis of root tissue treatments from FAME analysis.

		From Species				
To Species		SL†	MA	MH	MI	MJ
SL	D^2	0	5.22	14.08	5.01	6.11
	P	1	<0.0001	0.0205	<0.0001	<0.0001
MA	D^2	5.22	0	7.45	1.73	2.19
	P	<0.0001	1	<0.0001	0.0026	<0.0001
MH	D^2	14.08	7.45	0	7.37	10.34
	P	0.0205	<0.0001	1	<0.0001	<0.0001
MI	D^2	5.01	1.73	7.37	0	2.27
	P	<0.0001	0.0026	<0.0001	1	0.0001
MJ	D^2	6.11	2.19	10.34	2.27	0
	P	<0.0001	<0.0001	<0.0001	0.0001	1

†= Abbreviations as follows: SL=*Solanum lycopersicum* 'Rutgers' uninoculated control, MA=*Meloidogyne arenaria*, MH=*M. hapla*, MI=*M. incognita*, and MJ=*M. javanica.*

Table 2-4. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, CAN3) separating uninoculated *Solanum lycopersicum* from tissue infected with *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3
iso-undecylic 3OH	0.714	0.246	0.636
iso-tridecylic 3OH	-0.745	0.264	0.047
Myristic acid	0.392	-0.522	0.320
iso-pentadecylic	0.747	-0.015	0.122
Palmitic acid	-0.055	0.077	**0.951**
Palmitoleic acid	0.406	-0.519	0.388
18:1 ω8*t*	0.583	-0.330	0.325
Elaidic acid	**0.934**	-0.088	-0.240
Linoleic acid	0.185	0.335	0.673
Heneicosylic acid	-0.383	-0.316	-0.193
Pentacosylic N Alcohol	0.470	**0.813**	-0.235
Sebacic acid	**-0.765**	0.571	-0.135
Unknown 8.281	**-0.811**	0.509	-0.094
Unknown 11.981	**0.820**	0.414	0.208
Unknown 13.671	0.072	0.208	-0.612
Unknown 19.276	-0.057	-0.615	-0.208
Unknown 20.588	-0.568	**0.786**	-0.025
Unknown 21.808	-0.149	0.175	-0.620
Unknown 23.670	-0.594	**0.770**	-0.089
Unknown 22.682	-0.462	**0.845**	-0.130
Canonical Correlation	0.686	0.590	0.452
Eigenvalue	0.889	0.533	0.257
Cumulative Proportion	0.468	0.748	0.884

Table 2-5. Mean FAME concentrations (percentage of total response) of homogenized and whole *Solanum lycopersicum* 'Rutgers' root tissue.

Fatty Acid	Homogenized tissue	Whole tissue
iso-tridecylic 3OH	1.70	--
Palmitic acid	34.29	3.05
Stearic acid	10.62	--
Linoleic acid	14.34	--
Nonadecylic N Alcohol	--†	9.92
Unknown 8.281	14.99	5.00
Unknown 13.671	--	5.10
Unknown 20.588	--	10.02
Unknown 21.808	--	39.63
Unknown 22.682	--	10.12
Unknown 23.670	--	9.88
n	12	13

†=Not detected.

Table 2-6. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first canonical variate separating *Solanum lycopersicum* 'Rutgers' homogenized and whole root tissue.

Fatty Acid	CAN1
Palmitic acid	1
Stearic acid	1
Nonadecylic N Alcohol	-1
Unknown 8.281	1
Unknown 13.671	-1
Unknown 20.588	-1
Unknown 21.808	-1
Unknown 22.682	-1
Canonical Correlation	0.998
Eigenvalue	294.97
Cumulative Proportion	1

Table 2-7. Mean FAME concentrations (percentage of total response) of root tissue infected with *Meloidogyne javanica,* prepared using a combination of fresh/dried root material and standard/concentrated FAME samples.

Fatty Acid	*Meloidogyne javanica* Fresh Standard	*M. javanica* Fresh Concentrated	*M. javanica* Dried Standard	*M. javanica* Dried Concentrated
iso-tridecylic 3OH	--†	--	0.54	--
Myristic acid	--	--	1.81	0.22
iso-pentadecylic	--	--	0.68	0.35
15:1 *iso* F	--	--	0.46	--
Palmitic acid	29.64	16.84	14.37	16.26
Palmitoleic acid	--	--	2.63	0.28
Stearic acid	27.73	16.68	12.60	15.26
18:1 ω8*t*	--	--	17.47	--
Oleic acid	--	0.87	3.25	2.32
Elaidic acid	--	40.77	19.30	35.51
iso-elaidic	42.63	13.94	12.27	16.51
Linoleic acid	--	0.43	4.03	1.82
20:1 ω7*c*/20:1 ω9*t*	--	0.89	0.88	1.80
Eicosapentaenoic acid	--	--	0.31	0.09
Heneicosylic acid	--	--	0.67	--
Unknown 8.281	--	7.18	1.46	6.77
Unknown 19.276	--	--	0.98	--
n	3	13	24	20

†=Not detected.

Table 2-8. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis of root tissue preparations analyzed by FAME analysis.

		From Species			
To Species		MJRD†	MJRDC	MJRF	MJRFC
MJRD	D^2	0	15.70	11.89	14.92
	P	1	<0.0001	0.0205	<0.0001
MJRDC	D^2	15.70	0	29.52	0.82
	P	<0.0001	1	<0.0001	0.9049
MJRF	D^2	11.89	29.52	0	26.21
	P	0.0205	<0.0001	1	<0.0001
MJRFC	D^2	14.92	0.82	26.21	0
	P	<0.0001	0.9049	<0.0001	1

†= Abbreviations as follows: MJRD= *Meloidogyne javanica*-infected roots dried, MJRDC=*M. javanica*-infected roots dried and concentrated, MJRF=*M. javanica*-infected roots fresh, and MJRFC=*M. javanica*-infected roots fresh and concentrated.

Table 2-9. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, CAN3) separating fresh (standard/concentrated) and dried (standard/concentrated) root tissue infected with *Meloidogyne javanica*. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3
15:1 *iso* F	**-0.876**	0.477	-0.071
Palmitic acid	0.006	**-0.990**	0.140
Palmitoleic acid	**-0.855**	0.518	0.006
Stearic acid	0.170	**-0.985**	0.041
18:1 ω8*t*	**-0.876**	0.477	-0.071
Oleic acid	-0.494	**0.785**	0.373
Elaidic acid	**0.928**	0.266	-0.260
Linoleic acid	-0.714	0.663	0.225
20:1 ω7*c*/20:1 ω9*t*	0.699	0.454	0.552
Heneicosylic acid	**-0.876**	0.477	-0.071
Unknown 8.281	**0.995**	0.034	-0.092
Canonical Correlation	0.897	0.604	0.289
Eigenvalue	4.112	0.575	0.091
Cumulative Proportion	0.861	0.981	1

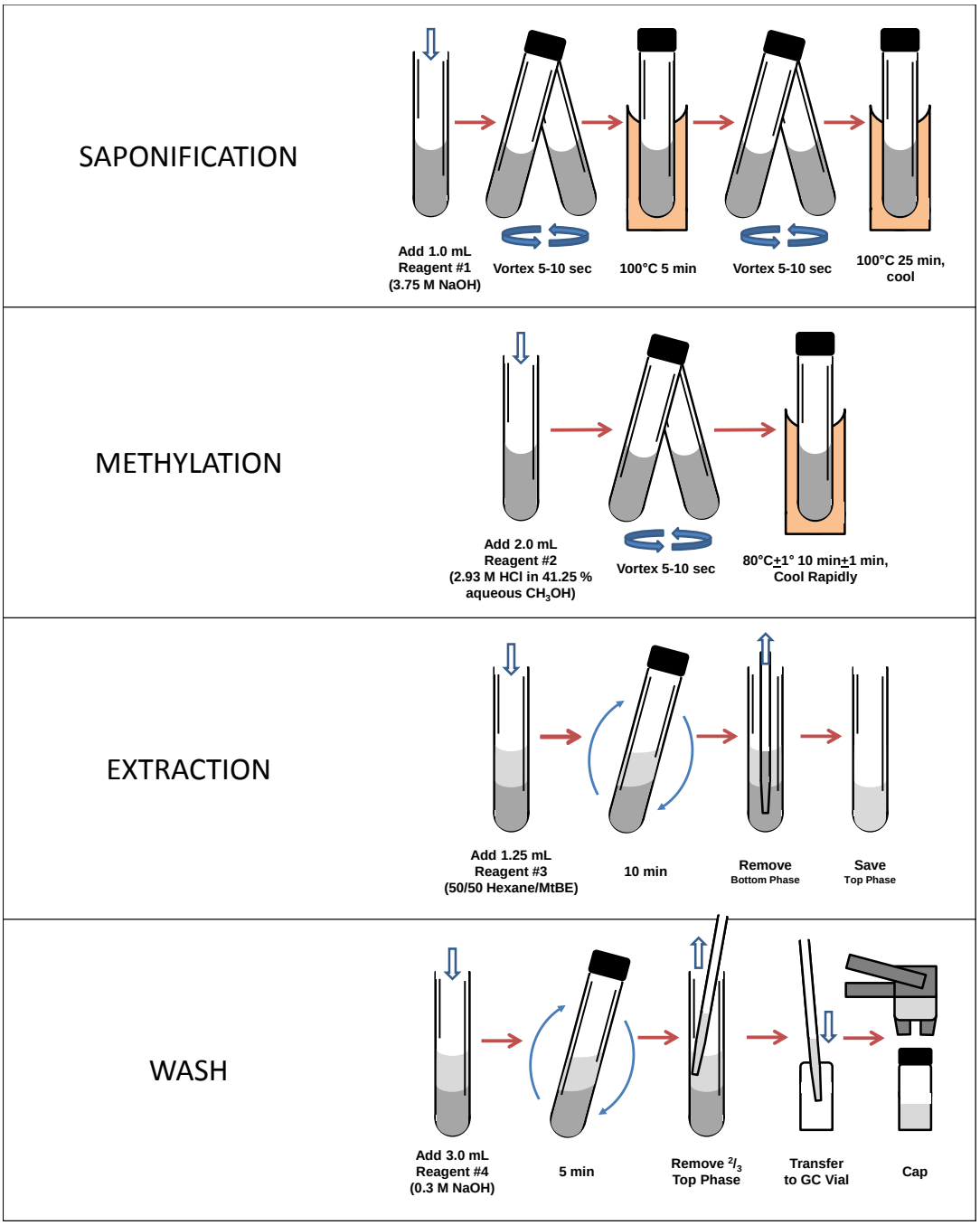


Figure 2-1. Fatty acid methyl ester (FAME) extraction method described by Sasser (1990).

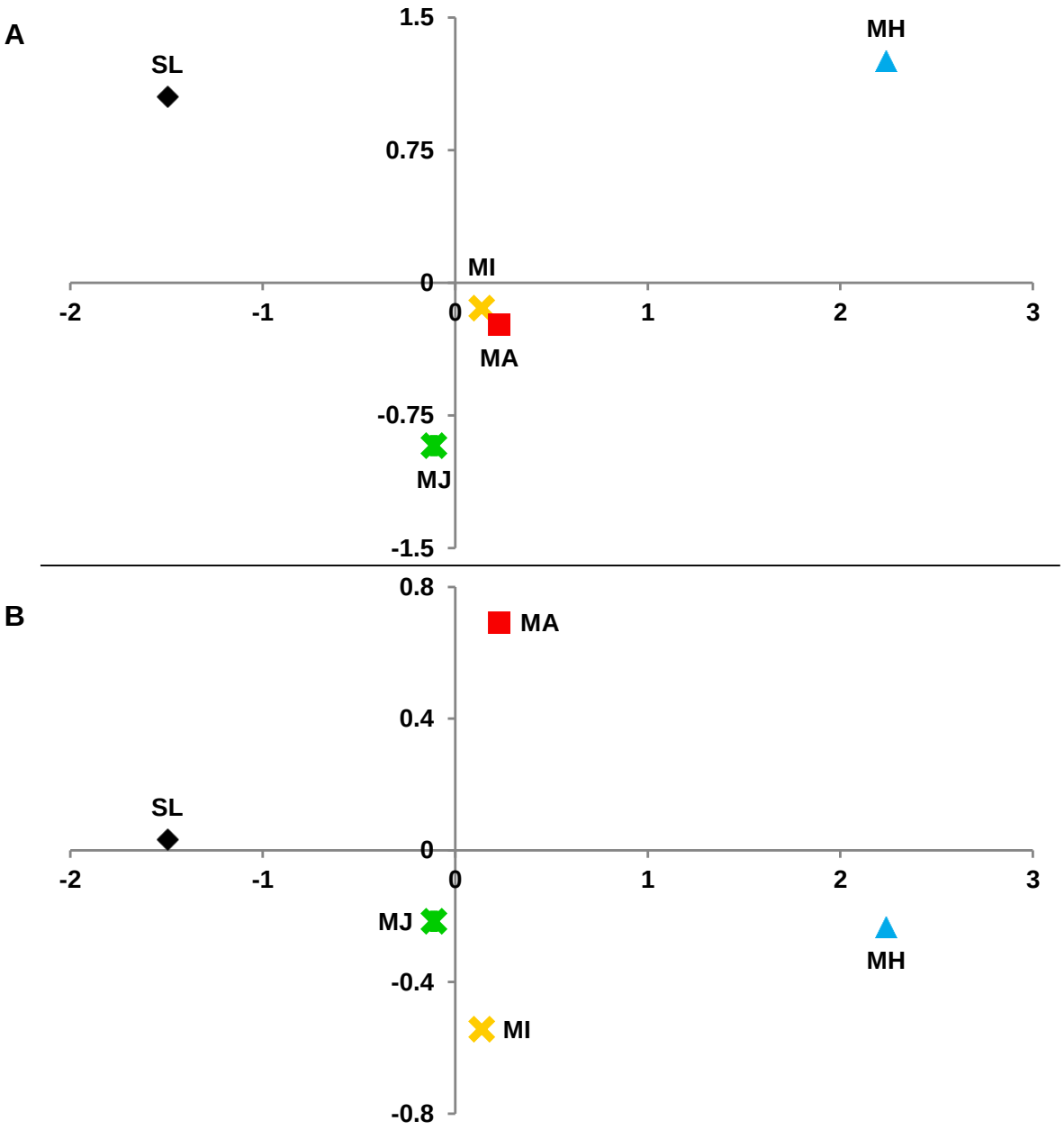


Figure 2-2. Canonical distribution of root FAME profile means subjected to one of five nematode treatments, *Meloidogyne arenaria* (MA), *M. hapla* (MH), *M. incognita* (MI), *M. javanica* (MJ), and uninoculated *Solanum lycopersicum* (SL). D^2 values are greater than 1.73 and significant at $P < 0.0026$ (Table 2-3). A) CAN1 (*x*-axis) versus CAN2 (*y*-axis) and B) CAN1 (*x*-axis) versus CAN3 (*y*-axis).

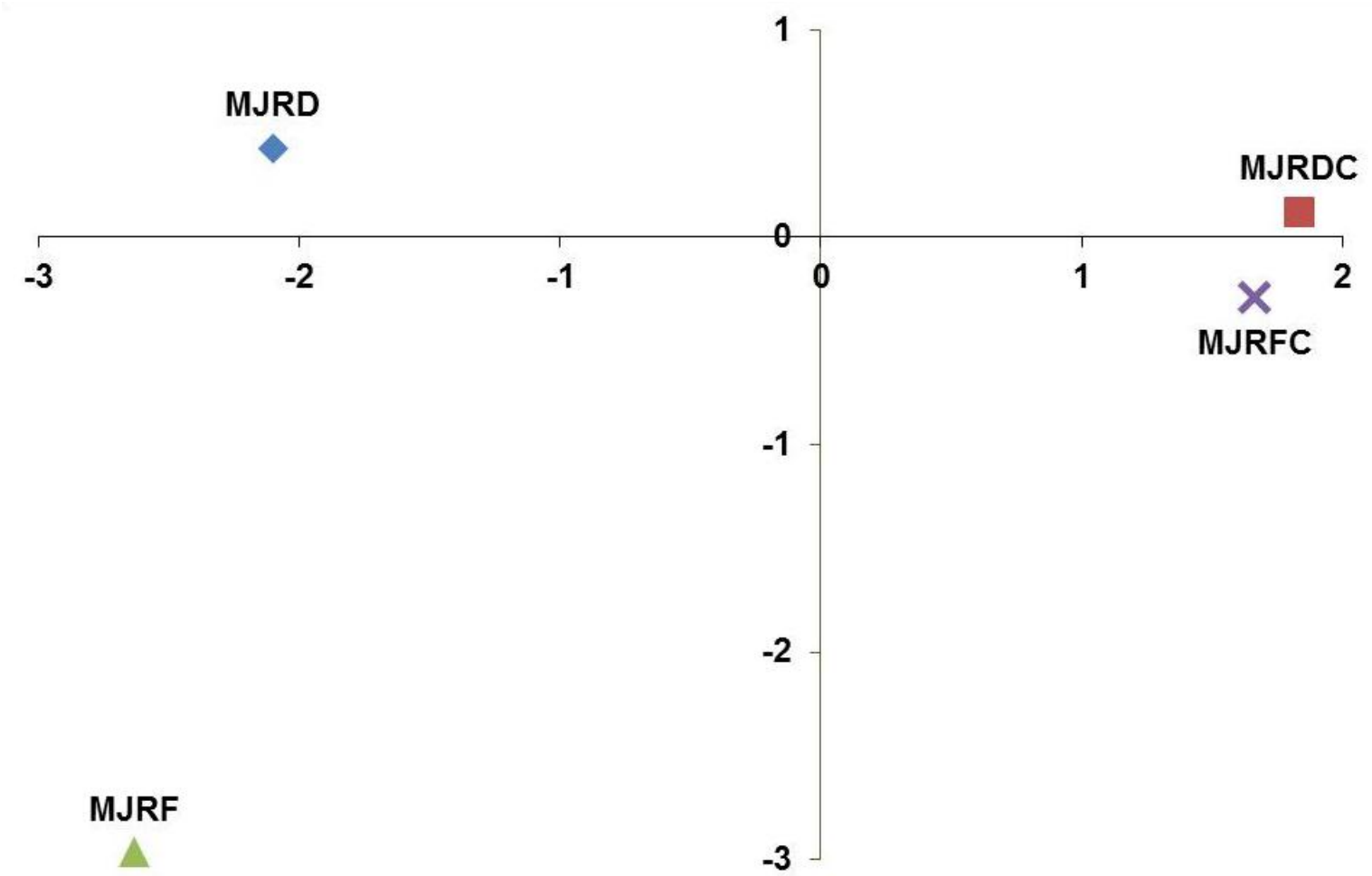


Figure 2-3. Canonical distribution of root FAME profile means subjected to one of four preparations, *Meloidogyne javanica*-infected dried roots, standard preparation (MJRD), *M. javanica*-infected dried roots concentrated preparation (MJRDC), *M. javanica*-infected fresh roots, standard preparation (MJRF), and *M. javanica*-infected fresh roots, concentrated preparation (MJRFC); CAN1 (*x*-axis) versus CAN2 (*y*-axis). D^2 values are listed in Table 2-8 and are significant ($P \leq 0.0205$) for all comparisons except MJRDC to MJRFC ($D^2 = 0.82$, $P = 0.9049$).

CHAPTER 3
TEMPERATURE EFFECTS ON FATTY ACID METHYL ESTER PROFILES OF *MELOIDOGYNE INCOGNITA* AND *M. JAVANICA*

3.1 Introduction

Fatty acids are the primary component of biological membranes and help to regulate the physiological functionality of those membranes. Membranes contain a balance of saturated fatty acids, straight-chain fatty acids that have only C-C single bonds along their carbon chains, and unsaturated fatty acids, "kinked" fatty acids that possess C-C double bonds along their carbon chain. Regulation of the proportions of these two types of fatty acids is the primary mechanism for maintaining membrane fluidity at varying temperatures. Due to the increased molecular movement at higher temperatures, membranes in these environments contain more saturated fatty acids to maintain rigidity through the ability of the straight carbon chains to form more van der Waals interactions with other fatty acid chains. Biological membranes containing more unsaturated fatty acids are more fluid due to the "kinking" of the fatty acid chain which prevents the fatty acid chains from packing together as tightly as straight chains and are utilized by organisms in cooler climates where molecular movement is reduced. Based on these chemical properties, an organism from a warm environment (>30°C) would have a higher percentage of saturated fatty acids while an organism from a cool environment (<18°C) would have a higher proportion of unsaturated fatty acids, but the membrane fluidity in these two organisms would be identical (Horton *et al.*, 2001).

Organisms have the ability to change the composition of their membranes as their environment warms or cools and these changes can be monitored using various techniques of fatty acid analysis. Studies following the effect of cold acclimation and heat stress of turfgrasses on their fatty acid composition have tracked the shift from

saturated to unsaturated fatty acids and indicate that there are primarily four fatty acids that undergo the most changes. In a study of physiological changes of bermudagrass (*Cynodon dactylon* x *C. transvaalensis*) cultivars 'Midiron' and 'U3,' the percentage of palmitic acid (16:0), stearic acid (18:0), and linoleic acid (18:2) decreased as the length of time at cooler temperatures increased, whereas the percentage of linolenic acid (18:3) increased (Samala *et al.*, 1998). Similar results were found by Cyril *et al.* (2002) comparing three seashore paspalum (*Paspalum vaginatum*) germplasms, 'SeaIsle1,' 'Adalayd,' and 'PI 299042,' the former being cold-tolerant and the latter two being cold susceptible. However, in these experiments stearic acid increased over the period of the cold treatment for all three cultivars. Heat stress and tolerance studies on the fatty acid composition of creeping bentgrass (*Agrostis stolonifera*) cultivars 'L-93,' 'Penncross,' and 'Crenshaw,' found that the percentages of palmitic acid and stearic acid increased as those of oleic acid (18:1), linolenic acid, and linoleic acid decreased in leaf tissue (Larkindale and Huang, 2004). Root tissue did not exhibit any significant change in fatty acid composition during the heat treatment.

In addition to influencing the fatty acid composition of membranes, temperature can also impact the survival, development, and growth habit of organisms. Bergeson (1959) observed that eggs and juveniles of *Meloidogyne incognita acrita* had the greatest survival rate at 10°C in soil without a plant host present. Survival was decreased as temperature was both increased and decreased. Egg hatching was found to be constant through 22 days at 16°C. Hatching rates increased at 21°C, 27°C, and 32°C and were arrested at 4°C and 10°C. In addition, studies by Bird and Wallace (1965) found that the optimum hatching rates and mobility occurred at 20°C and 25°C

for *M. hapla* and 25°C and 30°C for *M. javanica*. Sex determination is also influenced by temperature, as described for *M. graminis* by Laughlin *et al.* (1969). The proportion of males in populations reproducing on bermudagrass increased to nearly 85% at 32°C from less than 10% at lower temperatures.

It is possible to differentiate and identify nematode species and life stages based on their fatty acid compositions. Krusberg *et al.* (1973) observed that there were differences in the fatty acids expressed by *Meloidogyne* species at different life stages and that those differences could be used as a means for differentiation. By using fatty acid methyl ester (FAME) analysis, Sekora *et al.* (2010) confirmed that it was indeed possible to use fatty acids for the identification of *Meloidogyne* species. These analyses also indicated that the fatty acid profiles developed could be used for further identification of nematode species based on host and also for studying physiological changes in nematodes. By using the methods developed by Sekora *et al.* (2010a), it is likely that the effect of temperature on the fatty acid profiles of *Meloidogyne* species, if any, can be resolved. The objectives of this study were to 1) determine if nematode-infected tissue varies in its expression of fatty acids when maintained in diurnal environments with diverse temperature ranges and means, 2) evaluate fixed-temperatures over time on the FAME profiles of *Meloidogyne*-infected tissue, and 3) determine if the effects of temperature on FAME profiles of *M. incognita*- and *M. javanica*-infected tissue, if any, hinder differentiation of these two organisms.

3.2 Materials and Methods

Two experiments were conducted to determine the influence of differing temperatures on the FAME profiles of *Meloidogyne incognita* race 3 and *M. javanica* race 1. The first experiment compared three different temperature environments, but

these environments exhibited real-world diurnal temperature fluctuations and were weather-dependent for their maximum and minimum temperatures. The second experiment utilized two constant temperature environments and evaluated changes in FAME profiles over time to determine what deviations were induced over long-term exposure to a fixed temperature.

3.2.1 Diurnal Experiment

To evaluate the effect of fluctuating day/night cycle temperatures on the FAME profiles of *M. incognita* and *M. javanica*, *S. lycopersicum* 'Rutgers' plants inoculated with either species were maintained in three environments exhibiting diurnal temperature fluctuations. The experiment utilized a 3×3 factorial design (nematode × temperature) with 20 replications, for a total of 180 experimental units. Pots containing a single 'Rutgers' plant in autoclaved medium containing five parts field soil (Candler sand), three parts USGA greens mix sand, and one part commercial potting medium (ProMix®, Premier Tech Horticulture, Québec, Canada) and inoculated with their respective nematode treatment (*M. incognita*, *M. javanica*, or uninoculated). Sixty experimental units were maintained in each of three diurnal environments with a 12-hour photoperiod: a greenhouse with a 29.5°C average temperature (34°C day/25°C night), a growth room with a mean temperature of 26°C (28°C/24°C), and a shadehouse with an average temperature of 17.5°C (24°C/11°C). Pots were grouped by nematode treatment with 0.5 m between treatments to prevent contamination during the 60 day period of the experiment from 1 September to 31 October 2011. Pots were irrigated twice a day using a custom irrigation system and fertilized as needed to maintain healthy plant growth.

3.2.1.1 Sample preparation

Root tissue samples were collected by removing two 0.1-g root samples from washed roots within each pot after 60 days. Roots samples from uninoculated plants were randomly selected while roots samples from inoculated plants were selected based on the presence of visible galls or females. Immediately before FAME extraction, roots were ground to release all tissues present within the roots as described in Chapter 2. Root evaluation by FAME analysis included 360 samples (2 per experimental unit).

3.2.1.2 FAME analysis

Extraction of fatty acids was conducted using the method described by Sekora *et al.* (2010a) and involved the four steps of saponification, methylation, extraction, and washing. Samples were analyzed using an HP 6890N Gas Chromatography System (Agilent Technologies, Santa Clara, CA). For each analysis, 2.0 µL of sample solution was injected into an Ultra 2 Cross-linked 5% Phenyl Methyl Siloxane column (Agilent Technologies, Santa Clara, CA) linked to a flame-ionization detector and analyzed using the EUKARY method of the Sherlock Analysis Software (MIDI, Newark, DE). Sample profiles included total response of the sample (mV), responses for each fatty acid observed (mV), and the calculated proportion of each fatty acid response as a percentage of the total response. All profiles were exported to a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA) for further analysis.

3.2.2 Constant Temperature Experiment

Meloidogyne incognita and *M. javanica* were evaluated at two fixed temperatures (20°C and 26°C, $\pm$ 2°C between day and night cycles) on *S. lycopersicum* 'Tiny Tim' plants in plant growth chambers. Root samples were collected for evaluation of direct identification of *Meloidogyne* species in tissue samples. A set of uninoculated tomato

plants, serving as controls for uninfected root tissue, were grown at each temperature and evaluated in conjunction with inoculated plants at each extraction point. Extraction times were 45, 90, and 135 days after inoculation, based on multiples of the approximate time required for the completion of a single life cycle at 20°C (45 days) to determine if any changes in fatty acid composition of the nematodes were immediate or progressive over consecutive life cycles.

The experiment was conducted following a 3-way factorial design (nematode × temperature × evaluation time) with six replications for a total of 108 experimental units. Each pot (experimental unit) contained a single plant grown in the same growth medium used in the previous experiment. Each plant was inoculated with 500 eggs of *M. incognita*, *M. javanica*, or remained uninoculated. Six replications of each nematode treatment were placed in a growth chamber set at a given temperature (20°C or 26°C). Chambers contained four dual-bulb ballasts fitted with bulbs emitting growth-promoting wavelengths and were set for a photoperiod of 14 hours.

Within a given chamber, each of the 18 pots was assigned to one of three evaluation times (45, 90, or 135 days after inoculation). Pots were arranged in a completely randomized design within the chamber that was re-randomized every nine days to reduce growth habits induced by potential microclimates within a chamber. Pots were checked daily for water loss and watered as needed to prevent excessive drying.

3.2.2.1 Sample preparation

Root tissue samples were collected at each evaluation period as described in the diurnal experiment. A total of 216 root tissue samples were selected for FAME analysis (2 per experimental unit).

3.2.2.2 FAME analysis

Fatty acid extraction and analysis for the 45-day evaluation was performed using the same methods described for the diurnal experiment. In an effort to increase FAME profile resolution and the number of usable samples, root tissue samples from the 90- and 135-day evaluations were subjected to the new Instant FAME extraction and Rapid analysis methods developed by MIDI (Figure 3-1) as described in Chapter 4. Root tissue samples collected for these new methods were 3 mg as opposed to the 0.1 g used for the diurnal experiment. Instant FAME extraction does not utilize water bath heating for fatty acid extraction, but substitutes the addition of a series of solvents with vortexing between steps (Figure 3-1). The Rapid analysis method is comparable to standard analysis methods for the fatty acids. It is able to detect between 9 and 20 carbons in chain length and uses the same column for analysis (MIDI, 2011).

3.2.3 Statistical Analysis

Five comparisons were made using the fatty acid analyses of nematodes and root tissues from both experiments 1) among the three nematode treatments at a given temperature, and 2) a given nematode treatment across temperatures. FAME profiles were imported into SAS (SAS Institute, Cary, NC) for further analysis. Mean profiles for each character or categorical “class” (18°C, *M. incognita* J2, etc.) were calculated with PROC MEANS which provided the average response for each fatty acid in all samples for the given class. Additional statistical tests were performed using PROC STEPDISC in combination with PROC CANDISC following the method of Sekora *et al*. (2010). Stepwise discriminant analysis (SDA) by PROC STEPDISC was used to determine which fatty acids were significant for discrimination among classes using a series of stepwise analysis of variance (ANOVA) tests that evaluate the *F*-value of each fatty acid

before and after inclusion (Johnson, 1998). After analysis of each fatty acid, those significant for delineation ($P < 0.15$) were used for canonical discriminant analysis (CDA) with PROC CANDISC.

Canonical discriminant analysis produces class means based on sample variance within each compared class and then represents relationships among classes in dimensional space. The dimensional space is represented by canonical variates (CAN1, CAN2, up to class n-1) that demonstrate class separation in graphical representation and can be assigned to x, y, or z axes depending on the desired class comparisons. Canonical variates are also used to describe the total multivariance within a test, and the number of variates in these tests was reduced to the fewest that could define at least 75% cumulative proportion of the total multivariance (nCAN $\leq$ 3). Separation among classes is defined by the degree of "between canonical structure" correlation (-1 to 1) of a given fatty acid along the chosen canonical variate. Absolute values approaching |1.000| indicate a high degree of correlation and help to separate classes on the specified dimension. The greater the value of correlation, the greater the spatial distance (Mahalanobis distance or D^2) among means graphically along a given canonical variate (Johnson, 1998). For the experiments described in this paper, high canonical correlation was described by correlations greater than |0.750|, and significant mean separation was achieved with D^2 having $P < 0.05$.

Additional information provided by CDA is the canonical correlation and eigenvalue of each canonical variate. Canonical correlation values range from 0 to |1.000| and are indicators of the importance of each canonical variate to the separation of classes. Canonical correlation values approaching |1.000| are considered more

informative for describing the majority of multivariance within a given analysis. The eigenvalue is another statistic similar to canonical correlation that is used to rank canonical variates based on the multivariance explained by the selected variate. As with canonical correlations, higher values indicate a greater degree of explained multivariance in an analysis for the given canonical variate (Johnson, 1998).

3.3 Results

3.3.1 Diurnal Experiment

A total of 122 samples, only a third of those prepared, produced usable FAME profiles. Visible trends appeared in the mean FAME profiles for each nematode treatment as the mean environmental temperature decreased, but none of these differences were significant (Table 3-1). For example, the unnamed peak designated unknown 8.281 increased slightly in *M. incognita*-infected tissue as mean temperature decreased, but decreased in uninoculated root tissue.

The most dramatic differences across the three mean temperatures occurred in *M. javanica*-infected tissue. Both palmitic acid and elaidic acid increased from 12.12% and 23.04%, respectively, in infected tissue maintained in the greenhouse environment to 19.21% and 57.41%, respectively, in tissue maintained in the shadehouse environment (Table 3-1). *Meloidogyne javanica*-infected root tissue maintained in the growth room exhibited a mean FAME profile different from all others, with almost 90% comprised of unknown peaks 8.281, 13.671, and 21.808 (based on retention time during analysis).

Canonical discriminant analysis was able to separate each nematode treatment within any of the three temperature environments (Table 3-2). The only overlap among nematode treatments across the three environments was between *M. incognita*-infected plant tissue maintained in the shadehouse and *M. javanica*-infected plant tissue from

the growth room (D^2 = 5.368, *P* = 0.1835). *Meloidogyne incognita*-infected plant tissue and uninoculated plant tissue were clearly separated within each of the three temperatures, but each formed a group of similar FAME profiles ($D^2 \leq 3.126$, $P \geq 0.1006$) across temperatures (Figure 3-2). *Meloidogyne javanica*-infected tissue was distinctly separated from other treatments within the three temperatures aside from *M. incognita*-infected tissue from the shadehouse ($D^2 \geq 13.526$, $P \leq 0.0049$), but did not group together as observed for *M. incognita*-infected and uninoculated root tissues.

Nine fatty acids were responsible for separating the nine tissue types along the first two canonical variates, seven along CAN1, and two along CAN2 (Table 3-3). The first canonical axis defined 51.7% of the total multivariance among the classes and primarily separated the three uninoculated *S. lycopersicum* tissues from *M. javanica*-infected tissue maintained in the greenhouse (Figure 3-2). Of the fatty acids responsible for this separation, only unknown 8.281 was correlated at greater than |0.900| (|0.912|). The fatty acids responsible for the separation of uninoculated *S. lycopersicum* tissue and greenhouse-grown *M. javanica*-infected tissue along CAN1 were split into three groups, those found only in *M. javanica*-infected tissue (*iso*-pentadecylic acid, myristic acid, and palmitoleic acid), fatty acids at higher mean concentrations in uninoculated *S. lycopersicum* tissue than either *M. incognita* or *M. javanica*-infected tissue (unknown 8.281, unknown 20.588, and sebacic C10 decarboxylase), and elaidic acid that is found in *Meloidogyne*-infected root tissue. The two fatty acids significant along CAN2 (oleic acid and unknown 13.671) were differentially expressed within each nematode treatment/environment combination and described an additional 34.4% of the total multivariance (Table 3-3).

3.3.2 Constant Temperature Experiment

3.3.2.1 FAME profiles at 45 days

After 45 days, FAME profiles of uninoculated and *Meloidogyne*-infected root tissue did not demonstrate significant separation from each other between temperatures, but this is likely because only 31 of the 72 samples prepared produced FAME profiles that could be analyzed statistically (Table 3-4). Even with the low replication, it is possible to see that uninoculated tissue does not contain either oleic or elaidic acids, but they are found in *Meloidogyne*-infected tissue. The two unknown peaks 13.671 and 21.808 were also found in all tissue types but uninoculated tissue maintained at 20°C.

Canonical discriminant analysis of the usable FAME profiles produced some differences among the six classes (Table 3-5). The FAME profile of *M. incognita*-infected root tissue maintained at 26°C was different from all other classes along CAN1 ($D^2 \geq 27.57$, $P \leq 32.12$) as a result of the high concentration of elaidic acid (Table 3-6). Although the remaining five classes were distributed along CAN2 (Figure 3-3A), no fatty acids were significant for this separation. However, both unknown peaks (13.671 and 21.808) were significant along CAN3 and separated uninoculated tissue maintained at 20°C from tissue infected by either *Meloidogyne* species at the same temperature (Figure 3-3B).

3.3.2.2 FAME profiles at 90 days

More differences among FAME profiles were visible after 90 days (Table 3-7). Using the Instant FAME extraction and rapid analysis methods, all 72 samples produced usable FAME profiles with a greater number of fatty acids (n = 49) than the standard methods. Because of this increased resolution, more patterns were visible among the six classes. For example, pelargonic acid was not found in uninoculated *S.*

lycopersicum tissue at either 20°C or 26°C and arachidic acid, the longest detectable saturated fatty acid, was observed at higher mean concentrations in 20°C tissues than 26°C tissues. However, most of the differences in FAME profiles subjected to either 20°C or 26°C were variations in the concentrations of selected fatty acids (γ-linoleic and arachidonic acids). In tissues infected with either *M. incognita* or *M. javanica*, the proportion of unsaturated fatty acids to saturated fatty acids increased with temperature from 2.04 to 3.50 in *M. incognita*-infected tissue and 1.87 to 3.94 in *M. javanica*-infected tissue; uninoculated tissue only increased by 0.03.

Canonical discriminant analysis of the FAME profiles generated after 90 days indicated that the degree of separation between the two temperatures had increased ($D^2 \geq 34.74$, $P < 0.0001$; Table 3-8), as well as the discrimination among the three nematode treatments at 26°C ($D^2 \geq 8.78$, $P \leq 0.0005$). However, it was statistically unlikely to separate the uninoculated *S. lycopersicum* tissue from either of the *Meloidogyne*-infected tissues at 20°C ($D^2 \leq 3.84$, $P \geq 0.6569$). Differences among the three 20°C profiles and either 26°C *Meloidogyne*-infected tissue profile were explained by 21 fatty acids significantly correlated along CAN1, 13 of which were correlated at greater than |0.900| (Table 3-9). These fatty acids accounted for 79.5% of the total multivariance among classes and had mean concentrations in *Meloidogyne*-infected tissues at 26°C either both greater than or both less than those of the three 20°C tissues (Table 3-7). Uninoculated *S. lycopersicum* tissue was separated from the other five tissues along CAN2 (15.0% of total multivariance) by two fatty acids with mean concentrations greater than those of the other classes (*anteiso*-margaric and 17:1 ω8*c*) and eicosenoic acid, which was not detected in uninoculated tissue at 26°C but was

present in the other five classes. Although none of the detected fatty acids were significant along CAN3, separation of *M. incognita*-infected and *M. javanica*-infected tissues at 26°C was still observed based on differential expression in their respective profiles (Figure 3-4).

3.3.2.3 FAME profiles at 135 days

At the conclusion of the experiment, only 30 of the remaining 36 plants were still alive and able to be sampled, but all 60 samples produced statistically usable FAME profiles. Uninoculated *S. lycopersicum* tissue from both temperatures did not contain 16:1 ω5*c* or 20:1 ω7*c*, and 16:1 ω11*c* was not detected in *M. incognita*-infected tissue from either 20°C or 26°C (Table 3-10). Interestingly, oleic acid was not identified in tissue infected with either *M. incognita* or *M. javanica* at 20°C but was present at 26°C in tissue infected with either species. Saturated fatty acids of 14 carbons and shorter were generally more abundant in tissues maintained at 26°C than 20°C, while arachidic acid was found at higher mean concentrations in 20°C tissues. Proportions of unsaturated fatty acids to saturated fatty acids again increased with temperature after 145 days in all three tissue types (1.77 to 1.94 in uninoculated tissue, 2.03 to 2.97 in *M. incognita*-infected tissue, and 2.02 to 3.32 in *M. javanica*-infected tissue).

Using the combination of SDA and CDA, it was possible to separate all six classes from one another ($D^2 \geq 91.83$, $P \leq 0.0151$) except *M. incognita*-infected and *M. javanica*-infected tissues from 20°C ($D^2 = 43.27$, $P = 0.2221$; Table 3-11). The greatest proportion of multivariance (83.39%) separated uninoculated *S. lycopersicum* tissues from *Meloidogyne*-infected tissues at 26°C (Figure 3-5). The 12 fatty acids responsible for the separation consisted of two groups, those with mean concentrations in 26°C *Meloidogyne*-infected tissues greater than uninoculated tissues ($n = 10$) and those fatty

acids with a lower mean concentration (*n*=2; Table 3-10). The separation of uninoculated *S. lycopersicum* and *M. incognita*-infected tissues at 26°C from *M. javanica*-infected tissues (CAN2, 9.0% of multivariance) was due to six fatty acids, four with mean concentrations lower in *M. javanica*-infected tissues and two with higher concentrations in *M. javanica* tissues. While the separation of uninoculated tissues along CAN3 was significant at $P < 0.0001$, no fatty acids were significantly correlated with this canonical variate (Table 3-12).

3.4 Discussion

Temperature does not appear to have a significant degree of influence on the fatty acids in uninoculated *S. lycopersicum* root tissue or *M. incognita*-infected *S. lycopersicum* tissue when plants are maintained in environments with diurnal temperature fluctuations (Table 3-2), but does greatly influence these tissues when they are sustained in fixed-temperature environments over a period of time (Tables 3-8 and 3-12). The lack of influence in diurnal environments may be due to the need for these tissues to maintain a mixture of long-chain, short-chain, and unsaturated fatty acids to prevent membrane instability when temperatures rise and fall each cycle.

While it appears that the temperature ranges used for the diurnal experiment presented were not enough to prevent FAME profile variation that would hinder identification, comparing the effects from drastically different environments with larger ranges in diurnal temperatures or a greater difference in day/night average temperature could indicate how stable these profiles are across a wide variety of environments. This could be accomplished by comparing tissues from areas maintained with diurnal temperature cycles similar to those found in desert climates (with up to 26°C difference

between day and night) and by contrasting tissues from cool climates such as Nova Scotia or Britain to tissues from tropical regions similar to Mexico or Indonesia.

The influence of the target nematode species may also have a significant impact on the fatty acid variation observed. Although the results from the diurnal experiment are unclear, fixed-temperature experiments indicated that *M. javanic*a-infected tissue had a larger degree of change in the proportion of saturated to unsaturated fatty acids between 20°C and 26°C than *M. incognita*-infected tissue (Tables 3-7 and 3-10). These changes may reflect the adaptation of a given nematode species from the climate of its origin. Similar results could possibly be obtained by comparing a tropical *Meloidogyne* species, such as *M. enterolobii*, to a cool-weather species, like *M. hapla*. The changes observed could indicate the adaptive capabilities of each species to a given temperature range, but a limit may also be detected once the temperature deviates beyond what is commonly encountered by that nematode.

Meloidogyne-infected tissues increased in their degree of separation over the progress of the three evaluation periods more than uninoculated tissue. The changes over time may reflect the effect of each nematode life cycle acclimatizing to their environment while uninoculated *S. lycopersicum* is unable to change as quickly in a single life cycle. It is also possible that the differences observed in infected tissue are side effects of nematode infection causing stress on the host or even by changing the chemistry within the giant cells over time. Changing the temperature of the environment may help to better understand if these changes are acquired through subsequent life cycles of the nematodes or as the result of increased populations on the host plants. For example, by maintaining tissue at 26°C for 90 days and then dropping the

temperature to 20°C for another 45 to 90 days, it may be possible to discern if the changes progress regardless of temperature when compared to tissue maintained at a constant temperature for the entire course of the experiment.

The results of these two studies indicate that although fatty acid profiles of *Meloidogyne*-infected root tissues change when submitted to fixed temperatures for an extended period of time, the changes are less pronounced when the tissues are maintained in environments with diurnal temperature fluctuation. By not hindering identification of the same *Meloidogyne* species from two different environments, the possibility of using FAME analysis for diagnostic purposes is more likely. However, the impact of temperature on other nematode species, including others within *Meloidogyne*, should be pursued to determine if these results are consistent. Widening the range of temperatures studied and introducing temperature changes after a period of acclimatization will help to evaluate the full impact of temperature on nematode species and determine if the real-world application of FAME analysis for nematode-infected root tissue is prospective.

Table 3-1. Mean FAME concentrations (percentage of total response) of two nematode species (*Meloidogyne incognita* race 3 [Mi], *M. javanica* race 1 [Mj]) infecting *Solanum lycopersicum* 'Rutgers' root tissue and an uninoculated control (C) maintained in one of three diurnal temperature environments for 60 days.

	Greenhouse (29°C)			Growth room (26°C)			Shadehouse (18°C)		
Fatty Acid	Mi	Mj	C	Mi	Mj	C	Mi	Mj	C
iso-tridecylic 3OH	--†	0.72	0.29	--	--	2.02	--	--	0.49
Myristic acid	--	2.41	--	--	--	--	--	1.35	--
iso-pentadecylic	--	0.91	--	--	--	--	--	0.76	--
15:1 ISO F	--	0.61	0.28	--	--	1.46	--	--	0.92
Palmitic acid	15.82	12.12	11.24	9.93	3.72	17.16	14.77	19.21	18.96
Palmitoleic acid	--	3.50	--	--	--	--	--	0.94	--
Stearic acid	5.56	10.69	3.08	5.97	--	7.02	6.00	11.33	5.09
18:1 ω8*t*	--	23.30	--	--	--	--	--	--	--
Oleic acid	--	4.34	0.07	--	--	1.22	0.53	0.69	0.52
Elaidic acid	28.27	23.04	--	34.64	5.29	--	8.42	57.41	--
Linoleic acid	5.38	4.67	5.56	4.75	1.15	7.48	1.31	5.22	9.30
Nonadecylic N Alcohol	0.47	--	5.60	1.14	--	2.97	--	--	2.27
20:1 ω7*c*/20:1 ω9*t*	--	1.17	--	--	--	--	--	0.35	--
Heneicosylic acid	--	0.89	0.32	--	--	--	--	--	0.40
Pentacosylic N Alcohol	0.31	--	--	0.75	--	0.67	--	--	--
Sebacic C10 Decarbox	--	--	4.43	--	0.85	3.56	--	--	2.07
Unknown 8.281	3.56	1.51	21.34	4.21	14.09	16.16	6.18	2.73	13.20
Unknown 11.981	3.91	--	0.51	4.79	--	--	3.44	--	0.72
Unknown 13.671	12.24	--	4.44	13.28	9.12	2.73	15.98	--	2.67
Unknown 19.276	--	1.31	--	--	--	--	--	--	--
Unknown 20.588	0.41	--	5.08	0.99	--	3.57	--	--	4.70
Unknown 21.808	29.26	--	29.82	30.40	65.77	13.24	45.86	--	23.91
Unknown 22.682	0.35	--	5.14	0.85	--	4.32	--	--	3.47
Unknown 23.670	0.33	--	5.05	0.81	--	4.40	--	--	2.81
n	17	18	20	7	11	16	8	6	19

†=Not detected

Table 3-2. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis comparing FAME profiles of root tissue of *Solanum lycopersicum* 'Rutgers' infected with either *Meloidogyne incognita* race 3 (Mi), *M. javanica* race 1 (Mj), or an uninoculated control (Rut) maintained with diurnal temperature conditions in either a greenhouse (G), growth room (R), or shade house (S) for 60 days.

		From Enviro								
To Enviro		MiG	MiR	MiS	MjG	MjR	MjS	RutG	RutR	RutS
MiG	D^2	0	0.905	2.555	35.422	10.723	13.544	20.714	24.706	20.210
	P	1	0.9987	0.7319	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	<0.0001
MiR	D^2	0.905	0	5.691	38.622	16.244	13.526	28.428	32.195	28.069
	P	0.9987	1	0.3192	<0.0001	<0.0001	0.0049	<0.0001	<0.0001	<0.0001
MiS	D^2	2.555	5.691	0	35.808	5.368	20.834	14.444	18.142	13.846
	P	0.7319	0.3192	1	<0.0001	0.1835	<0.0001	<0.0001	<0.0001	<0.0001
MjG	D^2	35.422	38.622	35.808	0	39.046	39.261	42.567	41.509	40.814
	P	<0.0001	<0.0001	<0.0001	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MjR	D^2	10.723	16.244	5.368	39.046	0	27.635	10.279	14.384	10.143
	P	<0.0001	<0.0001	0.1835	<0.0001	1	<0.0001	<0.0001	<0.0001	<0.0001
MjS	D^2	13.544	13.526	20.835	39.261	27.635	0	38.704	43.217	38.270
	P	0.0002	0.0049	<0.0001	<0.0001	<0.0001	1	<0.0001	<0.0001	<0.0001
RutG	D^2	20.714	28.428	14.444	42.567	10.279	38.704	0	3.126	0.550
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	1	0.1006	0.9958
RutR	D^2	24.706	32.195	18.142	41.509	14.384	43.217	3.126	0	2.802
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.1006	1	0.1997
RutS	D^2	20.210	28.069	13.846	40.814	10.143	38.270	0.550	2.802	0
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.9958	0.1997	1

Table 3-3. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, CAN3) for separating FAME profiles of *Solanum lycopersicum* 'Rutgers' root tissue infected with either *Meloidogyne incognita* race 3, *M. javanica* race 1, or an uninoculated control maintained with diurnal temperature conditions in either a greenhouse (29°C), growth room (26°C), or shade house (18°C) for 60 days. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3
iso-tridecylic 3OH	-0.283	0.588	0.196
Myristic acid	**0.793**	0.570	0.154
iso-pentadecylic	**0.795**	0.497	0.268
Palmitoleic acid	**0.768**	0.636	0.021
Stearic acid	0.670	0.337	0.504
18:1 ω8*t*	0.723	0.680	-0.105
Oleic acid	0.631	**0.755**	0.004
Elaidic acid	**0.811**	-0.420	0.392
Pentacosylic N Alcohol	-0.093	-0.237	0.110
Sebacic C10 Decarbox	**-0.833**	0.346	0.201
Unknown 8.281	**-0.912**	0.193	-0.002
Unknown 11.981	0.227	-0.747	-0.217
Unknown 13.671	0.024	**-0.803**	-0.518
Unknown 19.276	0.723	0.680	-0.105
Unknown 20.588	**-0.840**	0.308	0.267
Unknown 21.808	-0.389	-0.539	-0.661
Canonical Correlation	0.918	0.884	0.629
Eigenvalue	5.375	3.570	0.654
Cumulative Proportion	0.517	0.861	0.924

Table 3-4. Mean FAME concentrations (percentage of total response) of two nematode species infecting *Solanum lycopersicum* 'Tiny Tim' root tissue (*Meloidogyne incognita* race 3 [Mi], *M. javanica* race 1 [Mj]) and an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 45 days.

Fatty Acid	C20	Mi20	Mj20	C26	Mi26	Mj26
Palmitic acid	48.59	24.78	27.68	37.58	27.28	42.63
Stearic acid	51.41	27.83	27.26	17.55	17.64	26.77
Oleic acid	--[†]	4.38	4.67	--	1.89	5.15
Elaidic acid	--	--	--	--	30.52	1.03
Linoleic acid	--	--	--	8.92	10.11	12.31
Unknown 13.671	--	13.65	10.56	11.22	1.56	2.22
Unknown 21.808	--	29.36	22.78	17.14	2.60	4.24
n	1	8	4	6	3	9

[†]=Not detected

Table 3-5. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis comparing FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3 (Mi), *M. javanica* race 1 (Mj), or an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 45 days.

		From Species					
To Species		C20	C26	Mi20	Mi26	Mj20	Mj26
C20	D^2	0	6.87	3.29	32.12	2.71	2.70
	P	1	0.4280	0.8138	0.0053	0.8817	0.8375
C26	D^2	6.87	0	9.40	30.37	7.86	2.19
	P	0.4280	1	0.0083	<0.0001	0.0392	0.2143
Mi20	D^2	3.29	9.40	0	36.28	0.21	4.68
	P	0.8138	0.0083	1	<0.0001	0.9974	0.0957
Mi26	D^2	32.12	30.37	36.28	0	34.92	27.57
	P	0.0053	<0.0001	<0.0001	1	<0.0001	<0.0001
Mj20	D^2	2.71	7.86	0.21	34.92	0	3.26
	P	0.8817	0.0392	0.9974	<0.0001	1	0.3294
Mj26	D^2	2.70	2.19	4.68	27.57	3.26	0
	P	0.8375	0.2143	0.0957	<0.0001	0.3294	1

Table 3-6. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, CAN3) for separating FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3, *M. javanica* race 1, or an uninoculated control maintained at either 20°C or 26°C for 45 days. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3
Palmitic acid	-0.411	0.581	0.673
Stearic acid	-0.450	-0.541	0.507
Elaidic acid	**0.996**	-0.087	-0.023
Unknown 13.671	-0.547	-0.055	**-0.833**
Unknown 21.808	-0.550	-0.306	**-0.770**
Canonical Correlation	0.923	0.752	0.396
Eigenvalue	5.729	1.305	0.186
Cumulative Proportion	0.786	0.965	0.991

Table 3-7. Mean FAME concentrations (percentage of total response) of two nematode species infecting *Solanum lycopersicum* 'Tiny Tim' root tissue (*Meloidogyne incognita* race 3 [Mi], *M. javanica* race 1 [Mj]) and an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 90 days.

Fatty Acid	C20	Mi20	Mj20	C26	Mi26	Mj26
Pelargonic acid	--†	0.44	0.19	--	0.24	0.03
iso-undecylic	0.13	0.12	0.16	0.22	0.27	0.07
Lauric acid	0.10	0.23	0.16	0.31	0.15	0.22
anteiso-tridecylic	0.22	0.03	--	0.09	--	0.09
iso-tridecylic	0.13	0.12	--	--	--	0.07
Myristic acid	0.75	0.52	0.44	0.70	1.67	1.37
anteiso-myristic	--	0.15	--	0.04	0.10	0.14
iso-myristic	--	0.29	--	0.12	0.68	0.64
iso-pentadecylic	0.46	0.60	0.27	3.97	3.04	2.82
15:1 iso F	0.08	0.04	--	0.18	--	0.09
15:1 iso G	--	--	0.03	--	0.12	0.09
15:1 iso H/13:0 3OH	0.03	0.05	--	0.04	0.20	0.10
15:1 ω5*c*	0.03	0.05	0.04	0.07	--	--
15:1 ω6*c*	0.05	0.05	--	0.10	--	--
Palmitic acid	16.96	17.03	18.25	17.47	9.44	8.23
Palmitic 10-*methyl*	0.05	0.02	0.07	--	0.14	0.09
Palmitic N Alcohol	0.11	0.02	--	--	--	0.02
anteiso-palmitic	0.18	0.09	0.17	0.69	0.20	0.14
iso-palmitic	0.45	0.26	0.11	2.03	1.83	1.92
16:1 ω5*c*	0.02	0.03	--	--	1.90	1.84
Palmitoleic alcohol	0.03	0.40	0.28	0.08	--	0.13
16:1 ω7*c*/16:1 ω6*c*	0.83	0.90	0.69	1.90	4.20	3.03
16:1 ω9*c*	0.15	--	0.08	--	--	0.07
Margaric acid	0.53	0.45	0.79	0.78	0.45	0.67
anteiso-margaric	0.35	0.21	0.18	0.99	0.60	1.06

Table 3-7. Continued.

Fatty Acid	C20	Mi20	Mj20	C26	Mi26	Mj26
cyclo-margaric	0.11	0.23	--	1.36	1.15	1.01
iso-margaric	3.64	3.67	3.68	2.10	1.33	1.53
iso-margaric 3OH	0.01	0.16	0.12	--	--	0.03
17:1 *iso* ω9*c*	0.02	0.04	--	--	0.13	0.20
17:1 ω7*c*	--	0.23	--	--	0.03	0.13
17:1 ω8*c*	0.14	0.12	0.07	0.46	0.18	0.17
Stearic acid	4.22	5.05	4.99	5.38	5.09	4.85
Stearic 2OH	4.84	4.32	4.04	10.81	16.54	23.67
iso-stearic	0.26	0.14	0.25	0.05	0.34	0.15
18:1 ω5*c*	0.18	0.24	0.30	0.74	1.96	0.96
18:1 ω6*c*	0.04	0.09	0.07	0.11	0.06	--
cis-vaccenic acid	8.95	9.02	8.26	7.07	15.65	13.48
cis-vaccenic 11-*methyl*	0.13	0.02	0.11	0.44	0.51	0.82
Oleic acid	--	0.29	--	1.82	2.01	3.23
Linoleic/*anteiso*-stearic	35.24	32.78	34.18	20.38	8.28	5.58
γ-linolenic acid	2.23	2.67	2.53	2.21	2.06	1.42
Nonadecylic acid	0.10	0.10	0.04	--	0.04	0.03
cyclo-nonadecylic C10	1.29	1.99	1.85	3.47	5.00	5.02
cyclo-nonadecylic C8	0.39	0.31	0.33	0.91	1.26	1.37
Arachidic acid	8.47	8.66	9.64	5.45	4.79	4.34
iso-arachidic	5.78	6.14	6.24	2.67	3.79	3.50
20:1 ω7*c*	--	0.08	--	0.06	1.19	1.01
Eicosenoic acid	0.14	0.08	0.06	--	0.07	0.09
Arachidonic acid	0.05	0.15	0.23	0.39	1.73	1.99
n	12	12	12	12	12	12

†=Not detected

Table 3-8. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis comparing FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3 (I), *M. javanica* race 1 (J), or an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 90 days.

		From Species					
To Species		C20	C26	Mi20	Mi26	Mj20	Mj26
C20	D^2	0	41.88	3.84	93.14	3.83	93.76
	P	1	<0.0001	0.6569	<0.0001	0.6602	<0.0001
C26	D^2	41.88	0	34.74	53.24	35.45	54.53
	P	<0.0001	1	<0.0001	<0.0001	<0.0001	<0.0001
Mi20	D^2	3.84	34.74	0	82.92	2.29	84.48
	P	0.6569	<0.0001	1	<0.0001	0.9656	<0.0001
Mi26	D^2	93.14	53.24	82.92	0	91.78	8.78
	P	<0.0001	<0.0001	<0.0001	1	<0.0001	0.0050
Mj20	D^2	3.83	35.45	2.29	91.78	0	93.10
	P	0.6602	<0.0001	0.9656	<0.0001	1	<0.0001
Mj26	D^2	93.76	54.53	84.48	8.78	93.10	0
	P	<0.0001	<0.0001	<0.0001	0.0050	<0.0001	1

Table 3-9. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, CAN3) for separating FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3, *M. javanica* race 1, or an uninoculated control maintained at either 20°C or 26°C for 90 days. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3	Fatty Acid	CAN1	CAN2	CAN3
anteiso-tridecylic	-0.199	0.019	-0.406	17:1 iso ω9*c*	**0.868**	-0.422	-0.228
Myristic acid	**0.932**	-0.233	0.184	17:1 ω7*c*	0.037	-0.363	-0.235
anteiso-myristic	0.523	-0.266	-0.131	17:1 ω8*c*	0.243	**0.933**	-0.002
iso-myristic	**0.899**	-0.293	0.070	Stearic acid	0.254	0.641	0.251
iso-pentadecylic	**0.759**	0.640	0.029	*iso*-stearic	0.062	-0.691	0.549
15:1 *iso* G	**0.866**	-0.379	0.176	18:1 ω5*c*	**0.875**	0.019	0.477
15:1 ω5*c*	-0.678	0.708	-0.007	*cis*-vaccenic 11-*methyl*	**0.928**	0.151	-0.314
Palmitic acid	**-0.933**	0.349	0.073	Oleic acid	**0.938**	0.198	-0.277
Palmitic N Alcohol	-0.350	-0.374	-0.226	Linoleic/*anteiso*-stearic	**-0.995**	-0.076	0.055
anteiso-palmitic	0.064	**0.978**	0.050	*cyclo*-nonadecylic C10	**0.988**	0.108	0.010
iso-palmitic	**0.859**	0.482	-0.048	*cyclo*-nonadecylic C8	**0.984**	0.135	-0.080
16:1 ω5*c*	**0.937**	-0.345	0.030	Arachidic acid	**-0.937**	-0.286	0.069
16:1 ω7*c*/16:1 ω6*c*	**0.963**	-0.012	0.264	*iso*-arachidic	-0.762	-0.623	0.078
16:1 ω9*c*	-0.327	-0.441	-0.411	20:1 ω7*c*	**0.942**	-0.314	0.120
Margaric acid	-0.084	0.567	-0.445	Eicosenoic acid	-0.112	-0.877	-0.151
cyclo-margaric	**0.794**	0.586	0.062	Arachidonic acid	**0.966**	-0.224	-0.079
iso-margaric	**-0.968**	-0.238	-0.051	Pelargonic acid	-0.247	-0.394	0.434
iso-margaric 3OH	-0.589	-0.354	-0.062				
				Canonical Correlation	0.973	0.877	0.650
				Eigenvalue	17.69	3.33	0.73
				Cumulative Proportion	0.795	0.945	0.977

Table 3-10. Mean FAME concentrations (percentage of total response) of two nematode species infecting *Solanum lycopersicum* 'Tiny Tim' root tissue (*Meloidogyne incognita* race 3 [Mi], *M. javanica* race 1 [Mj]) and an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 135 days.

Fatty Acid	C20	Mi20	Mj20	C26	Mi26	Mj26
Pelargonic acid	--†	--	--	0.38	0.23	0.05
iso-undecylic 3OH	--	0.13	0.07	0.25	--	0.07
Lauric acid	0.12	0.15	0.29	0.15	0.14	0.31
Lauric 2OH	0.18	0.07	0.06	--	0.25	--
Tridecylic acid	0.16	0.04	0.04	0.26	--	0.08
anteiso-tridecylic	0.27	--	0.23	1.38	0.30	0.43
13:1 at 12-13	--	0.08	0.04	0.34	0.51	0.05
Myristic acid	1.00	0.86	0.98	1.57	1.53	1.29
Myristic 2OH	0.06	0.12	--	0.34	0.00	0.33
anteiso-myristic	--	0.13	0.06	0.17	0.14	0.12
iso-myristic	0.19	0.15	0.21	0.05	0.27	0.63
iso-myristic 3OH	0.07	0.11	0.06	--	0.19	--
anteiso-pentadecylic	0.37	0.22	0.53	1.08	1.12	1.23
iso-pentadecylic	0.76	0.45	0.87	0.66	3.19	2.43
15:1 *iso* G	0.11	0.07	--	--	0.36	0.17
15:1 *iso* H/13:0 3OH	0.05	0.07	0.06	0.24	--	0.24
15:1 *iso* ω9*c*	--	--	0.09	0.34	--	0.04
15:1 ω6*c*	0.16	0.09	0.10	0.37	--	0.25
Palmitic acid	19.09	18.77	15.86	19.84	10.39	9.65
Palmitic 2OH	0.11	0.40	0.15	0.24	0.06	0.32
Palmitic N alcohol	0.16	--	0.16	0.20	0.09	0.03
anteiso-palmitic	0.19	0.21	0.08	0.96	--	--
iso-palmitic	0.44	0.28	0.45	1.12	1.29	1.16
iso-palmitic 3OH	0.11	0.09	0.17	0.10	0.10	0.19
16:1 2OH	--	0.06	--	0.15	0.08	0.25
16:1 ω11*c*	0.06	--	0.08	0.42	--	0.18

Table 3-10. Continued.

Fatty Acid	C20	Mi20	Mj20	C26	Mi26	Mj26
16:1 ω5*c*	--	0.10	0.27	--	2.86	1.15
Palmitoleic Alcohol	0.32	0.13	0.49	0.37	0.10	0.45
16:1 ω7*c*/16:1 ω6*c*	0.62	0.69	1.52	1.94	2.84	2.64
Margaric acid	1.10	0.45	0.89	0.53	1.07	0.89
Margaric 10-*methyl*	0.11	0.13	0.05	0.09	--	--
anteiso-margaric	0.51	0.49	0.52	1.13	0.52	0.91
cyclo-margaric	0.11	0.15	0.20	0.17	0.43	1.04
iso-margaric	4.26	2.51	2.90	2.81	1.22	1.34
iso-margaric 3OH	--	0.07	0.57	1.10	0.41	0.39
17:1 iso ω9*c*	0.06	0.15	0.05	0.15	0.19	0.28
17:1 ω7*c*	0.22	0.14	0.16	0.05	0.29	0.16
17:1 ω8*c*	0.05	0.17	0.17	0.14	0.29	0.38
Stearic acid	5.01	5.05	5.07	6.03	6.41	5.39
Stearic 2OH	3.59	2.74	3.08	4.91	8.13	12.04
iso-stearic	0.59	2.41	1.18	0.55	0.54	0.16
18:1 2OH	0.20	--	0.11	0.12	0.08	0.10
18:1 ω5*c*	0.56	0.94	1.10	0.76	1.14	0.74
18:1 ω6*c*	0.43	0.04	0.09	0.33	--	0.08
cis-vaccenic acid	6.48	11.60	15.66	4.16	16.80	16.76
cis-vaccenic 11-*methyl*	--	0.23	0.04	0.13	0.54	0.71
Oleic acid	--	--	--	--	5.59	4.73
Linoleic/*anteiso*-stearic	29.37	30.31	24.00	25.73	10.14	9.97
γ-linolenic acid	2.49	2.27	2.11	0.87	1.27	2.15
Nonadecylic acid	0.10	0.04	0.06	0.27	--	--
cyclo-nonadecylic C10	1.29	1.60	1.32	2.46	2.76	3.99
cyclo-nonadecylic C8	0.26	0.31	0.45	0.40	1.30	1.27
19:1 ω11*c*/19:1 ω9*c*	0.07	0.14	--	0.32	--	--
Arachidic acid	8.21	7.08	9.15	3.19	4.16	4.84

Table 3-10. Continued.

Fatty Acid	C20	Mi20	Mj20	C26	Mi26	Mj26
iso-arachidic	6.67	5.19	4.87	2.53	2.37	3.06
20:1 ω7*c*	--	0.39	0.91	--	1.55	1.38
Eicosenoic acid	0.32	0.16	0.10	--	--	0.09
Eicosadienoic acid	0.07	0.24	0.19	2.60	0.60	--
Arachidonic acid	--	0.04	0.08	0.32	1.31	1.19
n	10	12	10	8	11	9

†=Not detected

Table 3-11. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis comparing FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3 (I), *M. javanica* race 1 (J), or an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 135 days.

		From Species					
To Species		C20	C26	Mi20	Mi26	Mj20	Mj26
C20	D^2	0	142.96	91.83	949.23	110.92	1142.00
	P	1	0.0013	0.0151	<0.0001	0.0056	<0.0001
C26	D^2	142.96	0	139.11	922.61	170.96	1209.00
	P	0.0013	1	0.0009	<0.0001	0.0002	<0.0001
Mi20	D^2	91.83	139.11	0	873.26	43.27	1024.00
	P	0.0151	0.0009	1	<0.0001	0.2221	<0.0001
Mi26	D^2	949.23	922.61	873.26	0	746.14	158.90
	P	<0.0001	<0.0001	<0.0001	1	<0.0001	0.0007
Mj20	D^2	110.92	170.96	43.27	746.14	0	851.47
	P	0.0056	0.0002	0.2221	<0.0001	1	<0.0001
Mj26	D^2	1142.00	1209.00	1024.00	158.90	851.47	0
	P	<0.0001	<0.0001	<0.0001	0.0007	<0.0001	1

Table 3-12. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first two canonical variates (CAN1 and CAN2) for separating FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3, *M. javanica* race 1, or an uninoculated control maintained at either 20°C or 26°C for 135 days. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	Fatty Acid	CAN1	CAN2
iso-undecylic 3OH	-0.450	0.374	*cyclo*-margaric	**0.887**	-0.216
Lauric acid	0.416	-0.619	*iso*-margaric	**-0.853**	-0.119
Lauric 2OH	0.144	0.257	*iso*-margaric 3OH	-0.081	0.589
Tridecylic acid	-0.471	0.474	17:1 ω7*c*	0.476	-0.063
anteiso-tridecylic	-0.158	0.707	17:1 ω8*c*	**0.931**	-0.101
13:1 at 12-13	0.261	**0.900**	Stearic acid	0.408	**0.892**
Myristic acid	0.432	**0.837**	*iso*-stearic	-0.523	-0.373
Myristic 2OH	0.153	0.194	18:1 ω5*c*	0.203	0.011
anteiso-myristic	0.256	0.542	18:1 ω6*c*	-0.556	0.289
iso-myristic 3OH	0.147	0.154	*cis*-vaccenic acid	0.739	-0.447
anteiso-pentadecylic	0.705	0.515	*cis*-vaccenic 11-*methyl*	**0.944**	0.012
iso-pentadecylic	**0.951**	0.214	Oleic acid	**0.979**	0.165
15:1 *iso* G	**0.770**	0.272	Linoleic/*anteiso*-stearic	**-0.973**	-0.137
15:1 *iso* H/13:0 3OH	0.128	0.122	γ-linolenic acid	-0.058	**-0.907**
15:1 *iso* ω9*c*	-0.357	0.578	Nonadecylic acid	-0.628	0.579
15:1 ω6*c*	-0.181	0.279	*cyclo*-nonadecylic C10	**0.838**	0.177
Palmitic N Alcohol	-0.467	0.469	*cyclo*-nonadecylic C8	**0.990**	0.125
anteiso-palmitic	-0.562	0.624	19:1 ω11*c*/19:1 ω9*c*	-0.597	0.566
iso-palmitic	0.697	0.637	Arachidic acid	-0.422	**-0.756**
iso-palmitic 3OH	0.458	-0.570	*iso*-arachidic	-0.578	-0.613
16:1 2OH	0.619	0.147	Eicosenoic acid	-0.382	-0.558
16:1 ω11*c*	-0.150	0.486	Arachidonic acid	**0.960**	0.277
16:1 ω5*c*	0.832	0.327	Pelargonic acid	0.027	**0.936**
Margaric acid	0.459	-0.062			
			Canonical Correlation	0.998	0.979
			Eigenvalue	216.10	23.07
			Cumulative Proportion	0.834	0.923

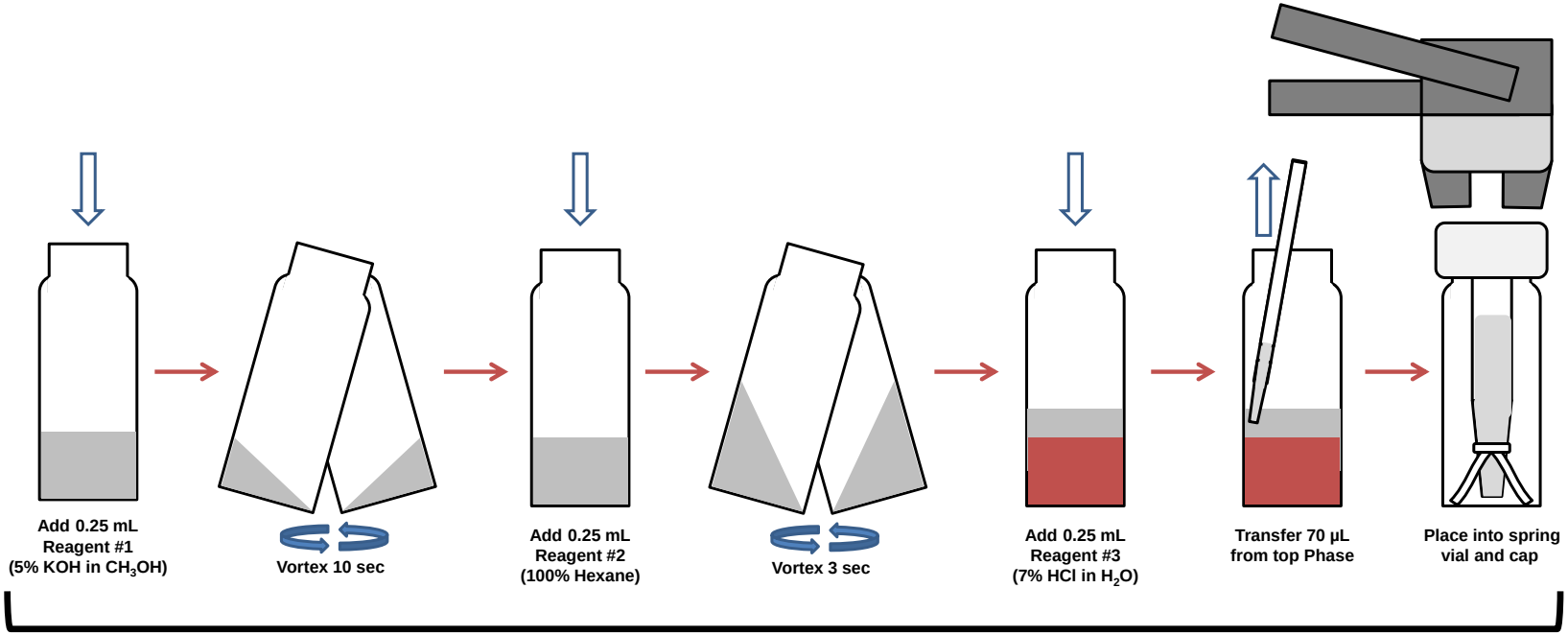


Figure 3-1. Instant FAME extraction and Rapid analysis method developed by MIDI (Newark, DE).

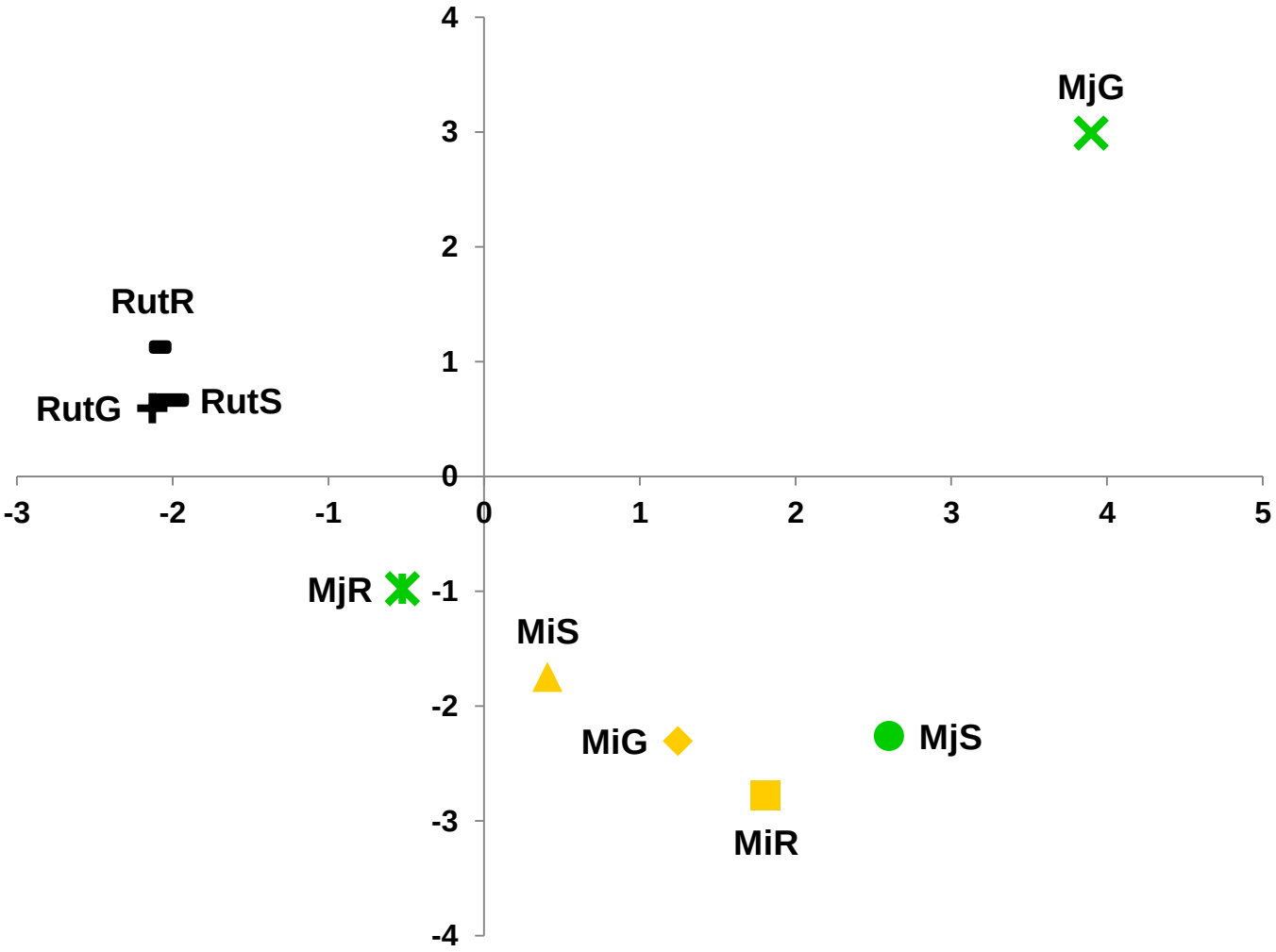


Figure 3-2. Canonical discriminant analysis after stepwise discriminant analysis comparing FAME profiles of *Solanum lycopersicum* 'Rutgers' root tissue infected with either *Meloidogyne incognita* race 3 (Mi), *M. javanica* race 1 (Mj), or an uninoculated control (Rut) maintained with diurnal temperature conditions in either a greenhouse (G), growth room (R), or shade house (S) for 60 days.

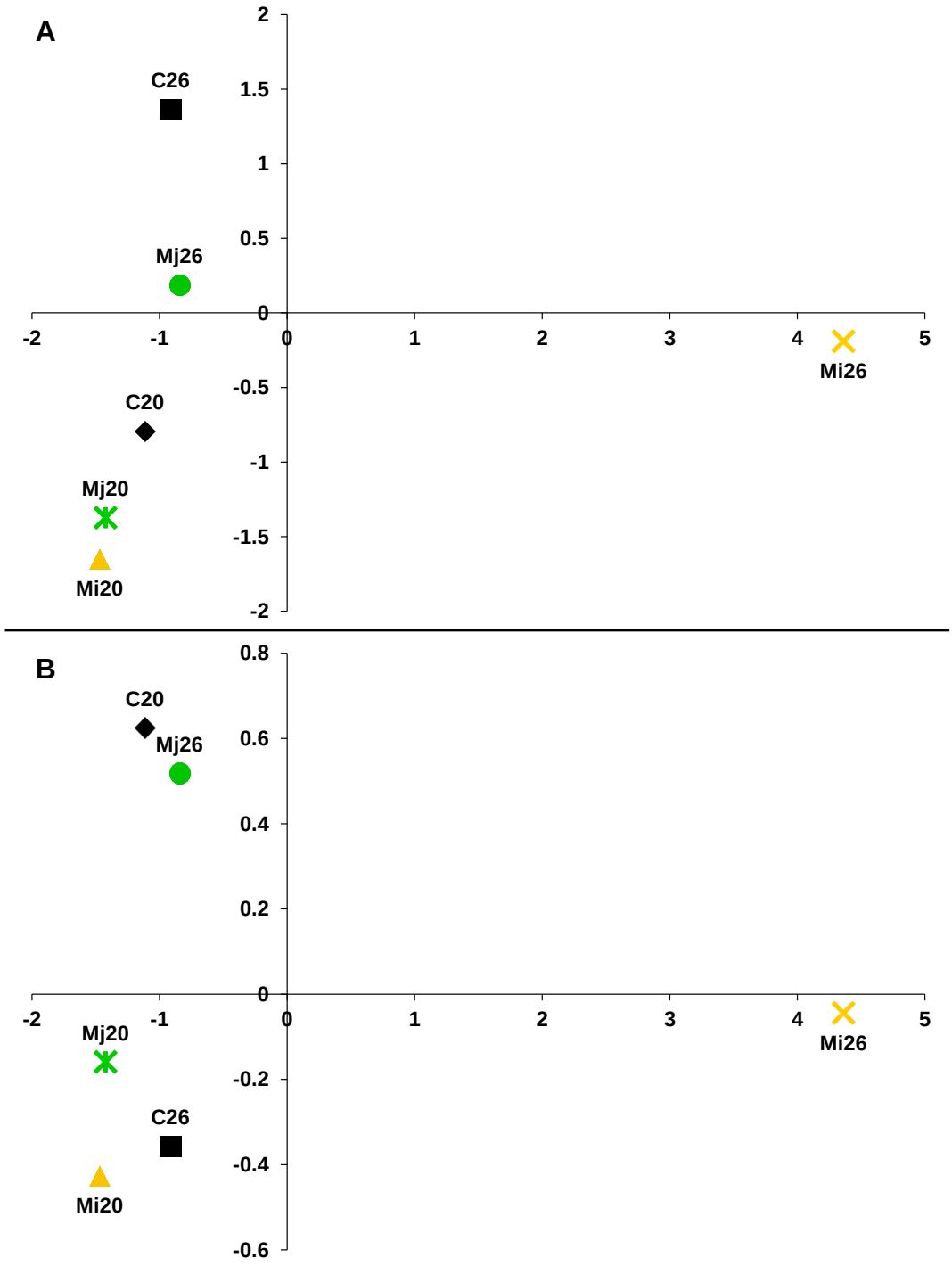


Figure 3-3. Canonical discriminant analysis after stepwise discriminant analysis comparing FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3 (Mi), *M. javanica* race 1 (Mj), or an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 45 days. A) CAN1 (*x*-axis) versus CAN2 (*y*-axis) and B) CAN1 (*x*-axis) versus CAN3 (*y*-axis).

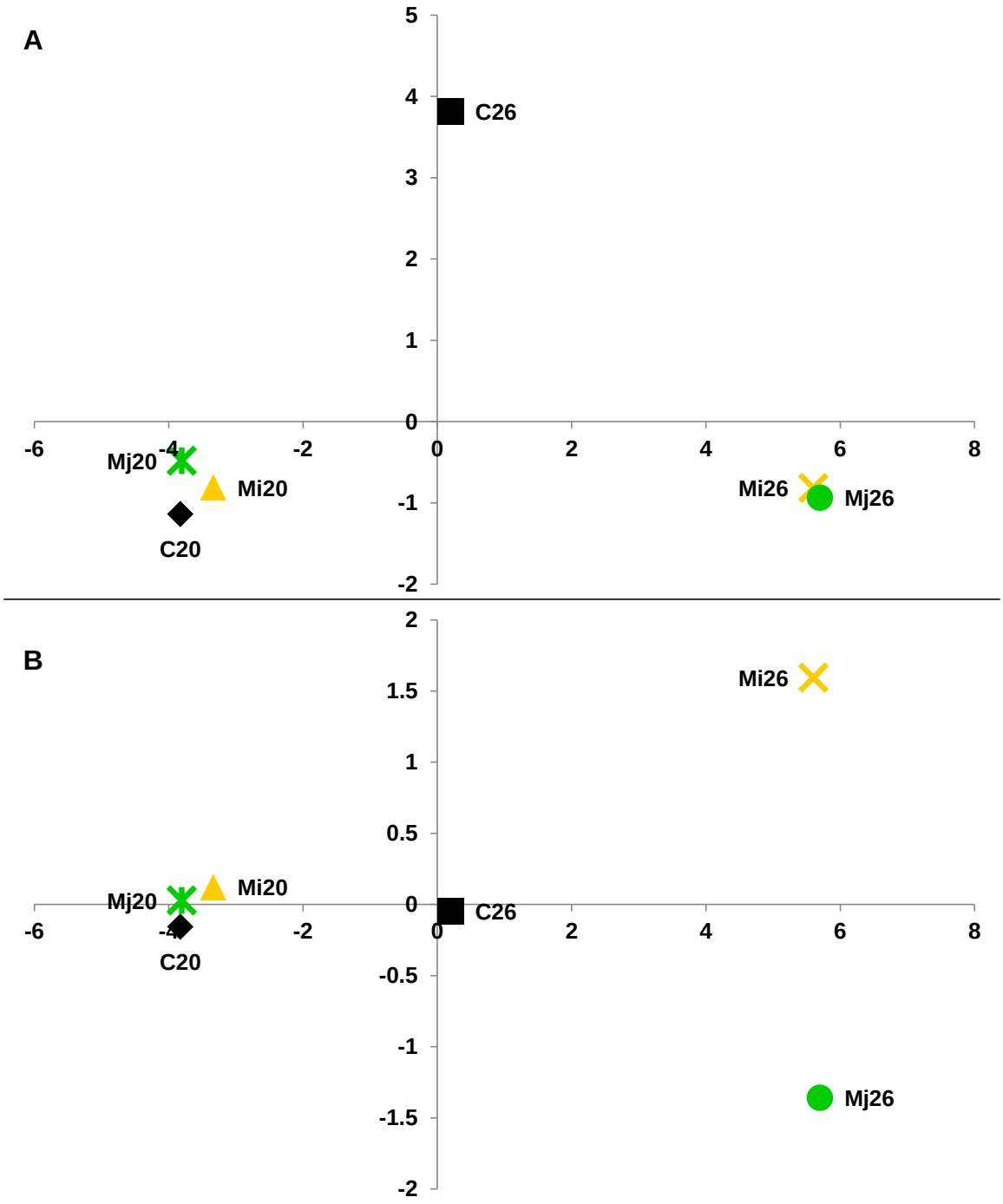


Figure 3-4. Canonical discriminant analysis after stepwise discriminant analysis comparing FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3 (Mi), *M. javanica* race 1 (Mj), or an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 90 days. A) CAN1 (*x*-axis) versus CAN2 (*y*-axis) and B) CAN1 (*x*-axis) versus CAN3 (*y*-axis).

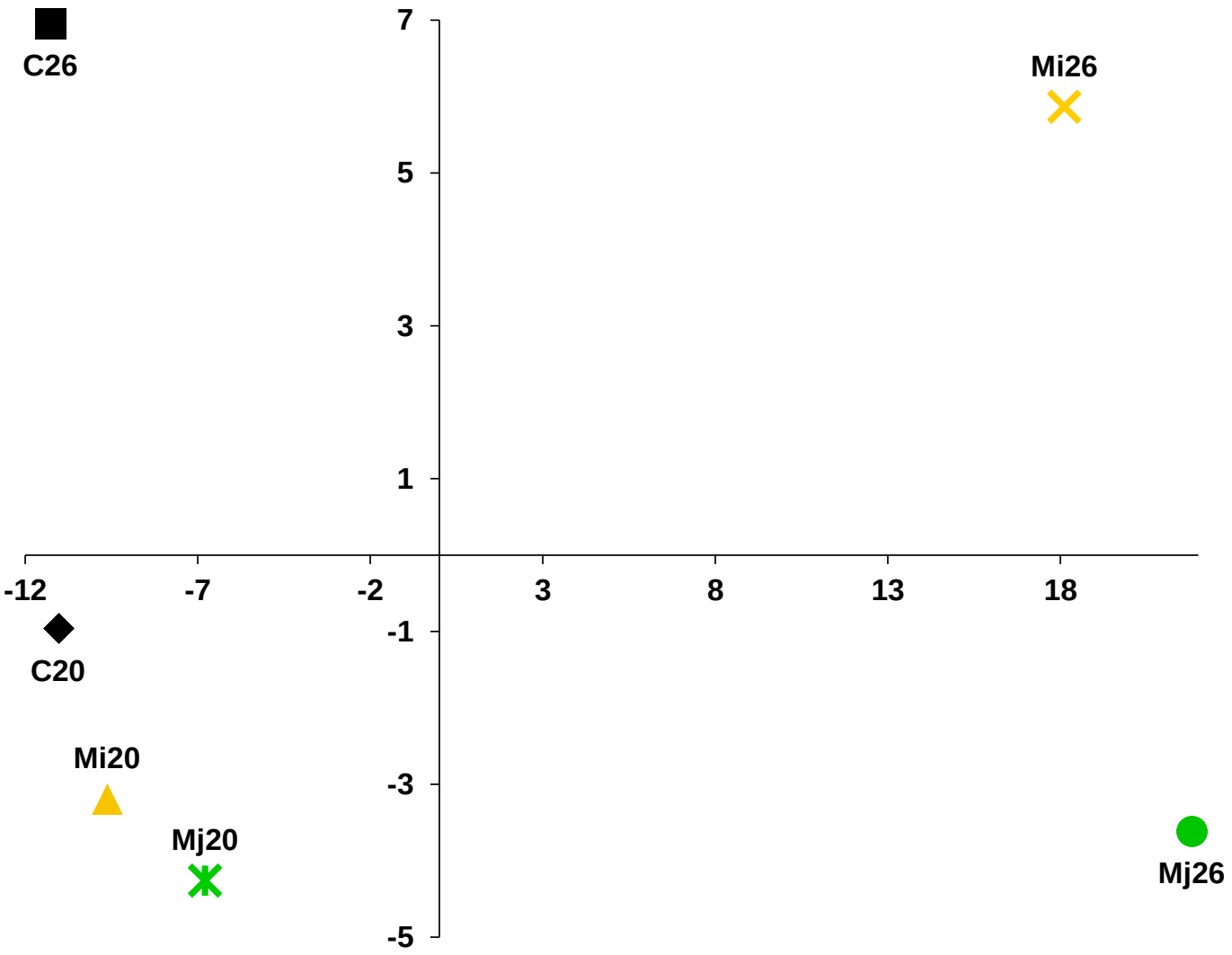


Figure 3-5. Canonical discriminant analysis after stepwise discriminant analysis comparing FAME profiles of *Solanum lycopersicum* 'Tiny Tim' root tissue infected with either *Meloidogyne incognita* race 3 (Mi), *M. javanica* race 1 (Mj), or an uninoculated control (C) maintained at either 20°C (20) or 26°C (26) for 135 days.

CHAPTER 4
APPLICATION OF INSTANT FAME FOR IDENTIFICATION OF *MELOIDOGYNE* SPP.

4.1 Introduction

Studies have indicated that *Meloidogyne* species can be identified within root tissue using the standard fatty acid methyl ester (FAME) analysis (Sekora *et al.*, 2010a). This identification procedure involves extracting nematode fatty acids from root tissue and then analyzing the proportions of each fatty acid within an extraction. The standard extraction method for FAME analysis of 1 to 72 samples requires a process that takes approximately 3.5 hours to complete using at least 40 mg of tissue (Kunitsky *et al.*, 2005; Sasser, 1990). The subsequent analysis using gas chromatography can take 20 to 45 minutes depending on the type of analysis performed and the chain length of the target fatty acids. Therefore, to extract and analyze 30 samples using the standard methods would take 14 to 27 hours and be limited by the speed of sample analysis.

To alleviate these time limitations, MIDI developed their Instant FAME extraction and Rapid analysis method to completely extract and analyze a sample in 15 minutes. According to MIDI, the addition of 250 µL each of a series of three kit-based reagents to 3 mg of tissue will produce a sample equivalent to one produced using their standard extraction method in only five minutes. To analyze this sample, a method utilizing higher temperature ramping and increased pressure than standard analysis methods allows fatty acids ranging in chain length from 9 to 20 carbons to be identified in less than 10 minutes. Using these proposed extraction and analysis methods, the same 30 samples analyzed previously would take 7.5 hours to complete, a time reduction of 47 to 74% depending on the analysis method used.

Based on these claims, FAME analysis of nematode-infected root tissue could be greatly simplified and more easily adapted for diagnostic applications. The objective of this chapter was to determine if FAME identification of *Meloidogyne* species in root tissue can be adapted to the Instant FAME extraction and Rapid analysis to reduce sample analysis time by comparing 1) standard methods to Instant FAME and Rapid analysis using recommended quantities of infected tissue and 2) standard extraction to Instant FAME extraction using equal tissue mass.

4.2 Materials and Methods

4.2.1 Methods Evaluated

The methods evaluated in these experiments included combinations of extraction methods and analysis methods as follows: standard FAME extraction (SE), Instant FAME extraction (RE), standard FAME analysis with the EUKARY method (SA), and Rapid FAME analysis with the RTSBA6 method (RA). SE and RE FAME extraction methods vary in many ways, but primarily in the volumes of reagents used and the requirement of water bath heating in the standard extraction method (Table 4-1). Sample size requirements also differ between extraction methods with SE demanding at least 40 mg of tissue and RE needing only 3 mg of tissue. Both SA and RA methods have an initial temperature of 170°C and a final temperature of 310°C, but temperature ramping and column pressure are higher using RA (Table 4-2). Analysis run time for RA (about 6 minutes) is also considerably shorter than SA (40 minutes).

4.2.2 Nematode Populations

Four nematode populations reared in a greenhouse were used to evaluate the Instant FAME and Rapid analysis methods, *Meloidogyne incognita* races 2 and 3 and two isolates of *M. javanica* race 1. *Meloidogyne incognita* race 3 and one isolate of *M.*

javanica race 1 were maintained on *Solanum lycopersicum* 'Rutgers' whereas *M. incognita* race 2 and the second *M. javanica* race 1 isolate were maintained on *Solenostemon scutellarioides* 'Wizard Velvet.' Populations from *S. lycopersicum* were used for comparing Instant FAME methods to standard methods while the *S. scutellarioides* populations were used to compare extraction methods using equal tissue sample size.

4.2.3 Instant FAME and Rapid Analysis Evaluation

An experiment was conducted to compare the relative performance of the various combinations of extraction methods and analyses. Root tissue samples of *S. lycopersicum* 'Rutgers' infected with either *M. incognita* race 2 or *M. javanica* race 1 was subjected to extraction and analysis methods in a 2×2 factorial design with the factors "SE" and "RE" and treatments "SA" and "RA" replicated 20 times. Standard extraction and analysis was conducted using 40 mg of infected tissue as mentioned previously (Chapter 2); rapid extraction and analysis followed the methods described by MIDI (2011). Twenty samples were prepared using extractions from *M. incognita* and *M. javanica* tissue using either extraction method (80 total extractions) and analyzed with both the standard EUKARY method and the Rapid RTSBA6 method (160 total analyses).

4.2.4 Extraction Comparison Using Equal Tissue Mass

An experiment was conducted to determine if using the root tissue amount normally used for SE (40 mg) could be used for RE. Samples containing 40 mg of *S. scutellarioides* root tissue infected with either *M. incognita* race 2 or *M. javanica* race 1 were used to evaluate extracting 40 mg of tissue with RE. Each sample combination

(nematode, extraction) was replicated 20 times for a total of 80 samples. All samples were analyzed using RA.

4.2.5 Statistical Analysis

Extraction and analysis methods were scrutinized for repeatability of results and robustness of profiles while still being able to separate *M. incognita* from *M. javanica*. Profiles were imported into SAS (SAS Institute, Cary, NC) for further analysis. Mean profiles for each character or categorical "class" (SE, RA, etc.) were calculated with PROC MEANS which provided the average response for each fatty acid in all samples for the given class. Additional statistical tests were performed using with PROC STEPDISC in combination with PROC CANDISC following the method of Sekora *et al*. (2010). Stepwise discriminant analysis (SDA) by PROC STEPDISC was used to determine which fatty acids were significant for discrimination among classes using a series of stepwise analysis of variance (ANOVA) tests that evaluate the *F*-value of each fatty acid before and after inclusion (Johnson, 1998). After analysis of each fatty acid, fatty acids significant for delineation ($P < 0.15$) were used for canonical discriminant analysis (CDA) with PROC CANDISC.

Canonical discriminant analysis produces class means based on sample variance within each compared class and then represents relationships among classes in dimensional space. The dimensional space is represented by canonical variates (CAN1, CAN2, up to class *n*-1) that demonstrate class separation in graphical representation and can be assigned to *x*, *y*, or *z* axes depending on the desired class comparisons. Canonical variates are also used to describe the total multivariance within a test, and the number of variates in these tests was reduced to the fewest that could define at least 75% cumulative proportion of the total multivariance ($n\text{CAN} \leq 3$).

Separation among classes is defined by the degree of “between canonical structure” correlation (-1 to 1) of a given fatty acid along the chosen canonical variate. Absolute values approaching |1.000| indicate a high degree of correlation and help to separate classes on the specified dimension. The greater the value of correlation, the greater the spatial distance (Mahalanobis distance or D^2) among means graphically along a given canonical variate (Johnson, 1998). For the experiments described in this paper, high canonical correlation was described by correlations greater than |0.750| and significant means separation was achieved with D^2 having a $P < 0.05$.

Additional information provided by CDA is the canonical correlation and eigenvalue of each canonical variate. Canonical correlation values range from 0 to |1.000| and are an indicator of the importance of each canonical variate to the separation of classes. Canonical correlation values approaching |1.000| are considered more informative for describing the majority of multivariance within a given analysis. The eigenvalue is another statistic similar to canonical correlation that is used to rank canonical variates based on the multivariance explained by the selected variate. As with canonical correlations, higher values indicate a greater degree of explained multivariance in an analysis for the given canonical variate (Johnson, 1998).

Six classes were used in SDA and CDA to produce comparisons between extractions (SE and RE), analyses (SA and RA), nematode species infecting tissue (I2 and J1), and among all eight possible combinations (I2SESA, J1RESA, etc.). A total of 30 comparisons were made for the first experiment and 6 were made for the extraction comparison in the second experiment.

4.3 Results

4.3.1 Instant FAME and Rapid Analysis Evaluation

Of the 160 total prepared samples, 118 produced usable FAME profiles (profiles with > 2 fatty acids; Table 4-3). Samples prepared using the Instant FAME method generated 48 of 80 profiles that could be utilized for statistical analysis; 70 of the 80 standard extraction samples produced usable profiles. Additionally, all 80 of the samples prepared for Rapid analysis produced adequate FAME profiles for statistical comparison while only 38 of the samples analyzed using standard analysis yielded usable FAME profiles. Among the 160 samples analyzed, 79 fatty acids were observed with expression varying from 0 to 81.48% in 118 usable profiles (Table 4-4).

4.3.1.1 Standard extraction versus Instant FAME

Standard extraction (SE) yielded more total fatty acids (79) than RE (55), but $\geq$65% of these were expressed at concentrations less than 1.00% of the total response (Table 4-4). For SE and RE, *cis*-vaccenic acid was found at the highest concentration (23.2 and 41.2%, respectively) followed by palmitic acid (15.1%) and stearic acid (12.2%) in SE extractions and linoleic acid (12.7%) and palmitic acid (12.0%) in RE extractions.

Canonical discriminant analysis of extraction methods was able to separate profiles generated with the SE procedure from those produced with RE ($P < 0.0001$, $D^2=19$). From the 36 fatty acids selected by SDA for CDA, five were negatively correlated with the SE method (*iso*-lauric, palmitoleic/16:1 ω6c, stearic 2OH, *cis*-vaccenic, and arachidic acids; Table 4-5). All six of these fatty acids were found at higher relative concentrations in samples extracted using the RE method than in samples subjected to SE extraction.

4.3.1.2 Standard analysis compared to Rapid analysis

An average of 75 fatty acids was observed using RA versus only 9 with SA. Of these observed fatty acids, 91% and 33% were present at less than 1.00% mean concentration in samples analyzed by RA and SA, respectively. In samples analyzed with RA, *cis*-vaccenic, linoleic, and palmitic acids had the highest mean concentration, while vaccenic acid, palmitic acid, and 18:1ω8t were the most predominant in samples subjected to SA.

Canonical discriminant analysis indicated a significant degree of separation between RA and SA ($P < 0.0001$, $D^2 = 782$). Stepwise discriminant analysis chose 35 fatty acids for further analysis with CDA, five of which were negatively correlated with RA (palmitoleic acid, stearic acid, 18:1ω8t, elaidic acid, and linoleic acid; Table 4-6). The only one of these fatty acids observed in samples utilizing RA (stearic acid) had a mean concentration 40% lower than samples analyzed with SA.

4.3.1.3 Extraction\analysis coupled comparisons

A greater number of fatty acids were observed in *M. incognita* and *M. javanica* infected tissue samples utilizing the SERA combination (73 and 75, respectively) than with any other extraction/analysis combination (Table 4-4). Root samples of *M. incognita* and *M. javanica* submitted to the RESA combination had the fewest number of fatty acids observed (3 and 4, respectively), followed by SESA combinations (9 and 7, respectively) and RERA (32 and 46, respectively). More fatty acids containing hydroxyl (-OH) groups were observed in samples submitted to SE than in samples that underwent the RE procedure. Additionally, 18C unsaturated fatty acids (*cis*-vaccenic acid, elaidic acid, linoleic acid, etc.) differed in their expression between RA and SA,

regardless of extraction method. For example, elaidic acid was only observed in SA samples, while *cis*-vaccenic and linoleic acids were only observed in RA samples.

Classes utilizing SA could not be differentiated from one another in 66% of CDA comparisons ($P \geq 0.2854$, $2.95 \leq D^2 \leq 39.95$; Table 4-7). All classes using RA were significantly different from all others using CDA ($P < 0.0001$, $D^2 \geq 25.08$). Analysis methods and extraction methods within the RA category were visibly separated by canonical variates; CAN1 separated SA from RA and CAN2 separated RA into RE and SE groups (Figure 4-1). Stepwise discriminant analysis selected 41 fatty acids for use in CDA, 11 of which were significant for separating RA from SA along CAN1 (Table 4-8). From these 11 fatty acids, 5 (palmitoleic/16:1 ω6c, *cis*-vaccenic acid, 16:1ω5c, arachidic acid, and stearic 2OH) were highly correlated (canonical correlation $\geq |0.914|$) and were only found in samples analyzed using RA. Extraction methods within samples utilizing RA were demarcated by five fatty acids (17:1ω9c, 17:1ω8c, lauric 2OH, 15:1ω5c, and pentadecylic 3OH) found only in SERA combinations. The fatty acid myristic 2OH was also significant in CAN2 and was only observed in SERA combinations since the mean expression in J1RERA samples was equal to its standard error (0.03174 ± 0.03174).

Using only RA, *M. incognita* could be distinguished from *M. javanica* using either extraction method ($P < 0.0001$, $D^2 \geq 99$; Table 3-9, Figure 4-2). Stepwise discriminant analysis selected 54 fatty acids for CDA, 39 of which were significant for separating extraction methods and *Meloidogyne* species (Table 4-10). Extraction methods were separated along CAN1 by 30 highly correlated fatty acids, the majority of which only appeared in SE samples. Six of the eight fatty acids separating I2 and J1 along CAN2

within RE or SE were found at higher mean concentrations in I2RE and J1SE than in their respective counterparts, J1RE and I2SE. The single fatty acid with significant correlation along CAN3 (stearic acid, |0.813|) helped to further separate I2 and J1 within each extraction method.

4.3.2 Extraction Comparison Using Equal Tissue Mass

A total of 112 fatty acids were observed among the 80 samples analyzed, but the actual number of fatty acids found in a single nematode/extraction combination ranged from 99 to 110 (Table 4-11). The most prevalent fatty acid detected was *cis*-vaccenic acid in SE of *M. incognita* and *M. javanica*-infected tissue (35.65 and 27.91%, respectively), whereas linoleic/*anteiso*-stearic was most abundant in *M. incognita* and *M. javanica*-infected tissue that was submitted to RE (30.94 and 30.49%, respectively). Both RE and SE extraction methods produced comparable proportions of saturated (mean 2.39 and 2.28%, respectively) and unsaturated fatty acids (mean 4.30 and 4.18%, respectively). However, SE samples of *M. incognita* and *M. javanica*-infected tissue possessed proportions of hydroxylated fatty acids (mean 0.21 and 0.22%, respectively) at more than twice that of RE samples (mean 0.10 and 0.09%, respectively).

Canonical discriminant analysis coupled with SDA produced canonical means with very large D^2 values ($D^2 > 18,374,852$, Table 4-12), canonical correlations (1.00, data not shown), and eigenvalues (approaching infinity, data not shown) among classes. Although this analysis graphically demonstrated the differences among classes (Figure 4-3), it was not adequate for determining which fatty acids contributed to these differences. As a result, a truncated SDA was used that had fewer total steps (32) than the full SDA possible (80 total steps). While this reduced SDA does not demonstrate

the full degree of separation among classes, it does allow for a more complete comparison.

Employing the abridged SDA, classes were still clearly separated by extraction method and species ($P < 0.0001$, $D^2 \geq 80100$; Table 4-13). This SDA selected 74 fatty acids for CDA, 60 of which were highly correlated along the three canonical variates (Table 4-14). The greatest amount of multivariance among classes was between I2SE and J1RE, accounting for 99.5% of the total multivariance. Forty-three fatty acids were highly correlated along CAN1, nineteen of which were correlated at greater than |0.900|. The majority of these fatty acids were observed at higher concentrations in one extraction method versus the other method, but none were found exclusively in a given extraction method.

The remaining 16 fatty acids significantly correlated in CAN2 and CAN3 (8 fatty acids each), although explaining only 0.5% of the total multivariance, separated *M. incognita*-infected tissue from *M. javanica*-infected tissue within each extraction method. The best spatial separation of *M. incognita*-infected tissue from *M. javanica*-infected tissue was along CAN3, separating them within SE, but was less significant ($P = 0.0006$) than the separation of infected tissue along CAN2 and within RE ($P < 0.0001$).

4.4 Discussion

Root tissue infected with *M. incognita* race 2 or *M. javanica* race 1 could be differentiated from one another using either the Instant FAME or standard extraction methods, but standard extraction (SE) appeared to be more efficient at extracting fatty acids from tissue. However, closer inspection of the FAME profiles produced by standard extraction methods showed that more artefacts may be produced during this extraction method than the Instant FAME method. For example, fatty acids containing

hydroxyl (-OH) groups were observed in samples submitted to standard extraction at concentrations more than twice those of samples that underwent the Instant FAME procedure. This could be a result of the half hour of boiling in 3.0 M NaOH during the first step of the extraction procedure and could present artificial results.

Additionally, the five fatty acids that were negatively correlated with the standard extraction method (*iso*-lauric acid, palmitoleic/16:1 ω6c, stearic 2OH, *cis*-vaccenic acid, and arachidic acid; Table 4-5) were only found in samples that were analyzed using the Rapid analysis procedure. These differences may be due to the chemical nature of the extraction methods, but the variation of fatty acid concentrations may not be critical if separation of *Meloidogyne* species is still achieved.

Using the Rapid analysis method provided more consistent FAME profiles than the standard analysis method for separation of *Meloidogyne*-infected root tissue. All 120 samples analyzed by Rapid analysis produced FAME profiles that could be used for identification and statistical analysis, compared to 38 of 80 using standard extraction methods. Similar to differences in extraction methods, elaidic acid was only found in samples undergoing standard analysis and not in samples utilizing the Rapid method. Many fatty acids were not observed in standard analysis samples and indicated a greatly reduced sensitivity compared to Rapid analysis. This reduced sensitivity was most apparent when attempting to separate infected root tissue using either extraction method. The inability of the SA method to produce FAME profiles that can separate *Meloidogyne* species makes it inferior to the Rapid method for nematode applications.

Separation between *M. incognita* and *M. javanica*-infected tissue is possible using 3.0 mg of root tissue, but larger sample sizes coupled with Instant FAME extraction and

Rapid analysis provides greater resolution between *M. incognita* and *M. javanica*-infected tissue. Statistically there is no difference between standard extraction methods and Instant FAME extractions using 40 mg of root tissue. Discrimination of root tissue infected with *Meloidogyne* species is possible using either extraction method, but Instant FAME provides extractions that are more statistically robust and consistent than standard extraction methods.

Because of the greatly increased resolution between nematode-infected root tissues, the coupled Instant FAME and Rapid analysis procedures using 40 mg of plant tissue may provide a more rapid method for diagnostic identification of *Meloidogyne* species than traditional methods. Future work will focus on determining the sensitivity of Instant FAME extractions for nematode densities down to single individuals. We will also assess these FAME methods for quantification of *Meloidogyne* species within root tissue.

Table 4-1. Comparison of FAME extraction steps for standard and Instant FAME methods.

Extraction Step	Standard Extraction	Instant FAME Extraction
Saponification		
Reagent	3.00 M NaOH	0.82 M KOH in CH_3OH
Volume	1.0 mL	250 µL
Mixing	Vortex 10 seconds	Vortex 10 seconds
Heating	100°C waterbath for 5 minutes, repeat vortex, 100°C waterbath for 25 minutes; cool to room temperature	None
Methylation		
Reagent	2.93 M HCl in 41.25% aqueous CH_3OH	Completed during saponification
Volume	2.0 mL	
Mixing	Vortex 5 seconds	
Heating	80°C $\pm$ 1°C for 10 minutes; cool rapidly in flowing water	
Extraction		
Reagent	50/50 by volume hexane/methyl *tert*-butyl ether	*n*-Hexane
Volume	1.25 mL	250 µL
Mixing	Tumble for 10 minutes	Vortex 3 seconds
Base Wash		
Reagent	0.3 M NaOH	Neutralization step with 1.92 M HCl
Volume	3.0 mL	250 µL
Mixing	Tumble for 5 minutes followed by 10-15 minutes resting	None
Transfer		
	1.0 mL of top phase to 1.5 mL vial, evaporate, reconstitute in 75 µL extraction reagent, transfer to spring vial	150 µL of top phase to spring vial

Table 4-2. Gas chromatogram parameters required for standard FAME analysis using the EUKARY method and Rapid FAME analysis using the RTSBA6 method.

Gas Chromatography Parameter	Standard EUKARY Analysis	Rapid RTSBA6 Analysis
Column†		
Initial Temperature	170°C	170°C
Initial Pressure	9.0 psi	21.0 psi
Ramp Temperature	5°C per minute for 28:00	28°C per minute for 4:07, then 60°C per minute
Final Temperature	310°C, hold for 12:00	310°C, hold for 1:15 min
Run Time	40:00 minutes	5:48 minutes
Fatty Acids Detected	9:0 to 30:0 carbons	9:0 to 20:0 carbons
Calibration Standard	MIDI-Calibration Mix 2	MIDI-Calibration Mix 1

†=Both methods utilize an Ultra 2 Cross-linked 2% Phenyl-Methyl Siloxane 0.2 mm × 25 m column with 0.33 µm film

Table 4-3. FAME profiles produced by eight combinations of two nematode species (*Meloidogyne incognita* race 2 [I2], *M. javanica* race 1 [J1]), two extraction methods (Instant FAME extraction [RE], standard extraction [SE]), and two analysis methods (Rapid analysis [RA], and standard analysis [SA]). Usable profiles contain at least two fatty acids.

Extraction/Analysis Combination	Average Fatty Acids per Profile	Usable Profiles out of 20 Reps
I2RERA	32	20
I2RESA	3	3
I2SERA	73	20
I2SESA	9	18
J1RERA	46	20
J1RESA	4	5
J1SERA	75	20
J1SESA	7	12

Table 4-4. Mean FAME concentrations (percentage of total response) of eight combinations of two nematode species infecting root tissue (*Meloidogyne incognita* race 2 [I2], *M. javanica* race 1 [J1]), two extraction methods (Instant FAME extraction [RE], standard extraction [SE]), and two analysis methods (Rapid analysis [RA], and standard analysis [SA]).

	Nematode/Extraction/Analysis Combination							
Fatty Acid	I2RERA	I2RESA	I2SERA	I2SESA	J1RERA	J1RESA	J1SERA	J1SESA
Capric acid	0.08	--	0.07	--	0.03	--	0.28	--
Capric 2OH	--[†]	--	0.09	--	--	--	0.13	--
Capric 3OH	0.33	--	0.03	--	--	--	0.13	--
Undecylic acid	0.16	--	0.02	--	--	--	0.07	--
Undecylic 2OH	0.12	--	0.06	--	--	--	0.17	--
Lauric acid	0.21	--	0.58	--	0.41	--	0.29	--
Lauric 2OH	--	--	1.05	--	--	--	1.78	--
Lauric 3OH	--	--	0.08	--	0.02	--	0.08	--
anteiso-lauric	--	--	0.10	--	0.03	--	0.11	--
iso-lauric	0.12	--	0.03	--	0.12	--	0.08	--
Tridecylic acid	--	--	0.16	--	0.13	--	0.05	--
Tridecylic 2OH	0.18	--	0.09	--	0.03	--	0.18	--
anteiso-tridecylic	0.09	--	0.05	--	--	--	0.06	--
iso-tridecylic	0.07	--	0.07	--	0.18	--	0.22	--
13:1 at 12-13	--	--	0.09	--	0.03	--	0.06	--
Myristic acid	2.87	--	1.68	1.39	1.66	--	1.54	1.62
Myristic 2OH	--	--	0.16	--	0.03	--	0.44	--
anteiso-myristic	--	--	0.96	--	--	--	0.70	--
iso-myristic	--	--	0.10	--	0.05	--	0.07	--
Pentadecylic 2OH	--	--	0.10	--	--	--	0.26	--
Pentadecylic 3OH	--	--	0.14	--	--	--	0.18	--
anteiso-pentadecylic	0.03	--	0.17	--	0.02	--	0.27	--
iso-pentadecylic	1.47	--	1.34	--	1.09	--	1.59	--
15:1 *anteiso* A	--	--	0.66	--	--	--	0.31	--
15:1 *iso* F	--	--	--	--	--	--	0.22	--

Table 4-4. Continued.

Fatty Acid	I2RERA	I2RESA	I2SERA	I2SESA	J1RERA	J1RESA	J1SERA	J1SESA
15:1 *iso* G	--	--	1.04	--	--	--	0.52	--
15:1 ω5*c*	--	--	0.13	--	--	--	0.19	--
15:1 ω6*c*	0.03	--	--	--	0.03	--	0.19	--
Palmitic acid	11.30	11.20	11.58	21.68	11.59	17.29	8.95	21.52
Palmitic 2OH	--	--	0.03	--	0.05	--	0.13	--
Palmitic N Alcohol	0.07	--	0.03	--	0.02	--	0.19	--
anteiso-palmitic	--	--	0.19	--	0.02	--	0.17	--
iso-palmitic	0.08	--	0.11	--	0.05	--	0.25	--
iso-palmitic 3OH	--	--	0.04	--	0.04	--	0.06	--
16:1 2OH	--	--	0.56	--	--	--	0.45	--
16:1 *iso* G	0.03	--	0.04	--	--	--	0.07	--
16:1 *iso* H	--	--	0.08	--	0.02	--	0.08	--
16:1 ω5*c*	0.64	--	0.59	--	0.42	--	0.80	--
16:1 ω6c/*cis*-vaccenic	--	--	0.20	--	--	--	0.03	--
cis-vaccenic	--	--	--	0.33	--	--	--	1.62
cis-vaccenic Alcohol	0.03	--	0.04	--	0.02	--	0.11	--
cis-vaccenic/16:1 ω6*c*	2.34	--	1.72	--	1.98	--	2.41	--
Margaric acid	--	--	1.02	--	--	--	0.73	--
Margaric 10-*methyl*	--	--	0.02	--	--	--	0.06	--
Margaric 2OH	--	--	0.03	--	0.05	--	0.23	--
anteiso-margaric	--	--	0.83	--	0.08	--	0.60	--
cyclo-margaric	--	--	0.42	--	--	--	0.29	--
iso-margaric	0.95	--	1.73	--	0.76	--	1.80	--
iso-margaric 3OH	--	--	2.58	--	--	--	1.09	--
17:1 *iso* ω5*c*	--	--	0.52	--	0.05	--	0.45	--
17:1 *anteiso* A	--	--	0.06	--	0.02	--	0.07	--
17:1 *anteiso* ω9*c*	--	--	0.40	--	--	--	0.24	--

Table 4-4. Continued.

Fatty Acid	I2RERA	I2RESA	I2SERA	I2SESA	J1RERA	J1RESA	J1SERA	J1SESA
17:1 *iso* I/*anteiso*	--	--	0.05	--	0.02	--	0.08	--
17:1 ω7*c*	--	--	0.09	--	0.07	--	0.20	--
17:1 ω8*c*	--	--	0.13	--	--	--	0.24	--
17:1 ω9*c*	--	--	0.06	--	--	--	0.16	--
Stearic acid	8.18	7.32	9.46	16.98	7.20	1.62	8.50	15.74
Stearic 10-*methyl*	--	--	0.14	--	0.02	--	0.02	--
Stearic 2OH	1.24	--	0.89	--	0.71	--	1.04	--
iso-stearic	--	--	0.09	--	0.13	--	0.17	--
18:1 *iso* H	--	--	0.11	--	--	--	0.07	--
18:1 ω5*c*	1.08	--	1.00	0.07	1.09	--	1.30	--
cis-vaccenic	50.83	--	38.37	--	48.13	--	42.68	--
18:1 ω8t	--	--	--	21.12	--	--	--	21.47
18:1 ω9*c*	0.12	--	1.18	1.08	--	--	0.26	--
Elaidic acid	--	81.48	--	27.29	--	60.63	--	29.64
Linoleic/Stearic	11.30	--	9.72	--	19.20	--	7.19	--
Linoleic acid	--	--	--	5.13	--	20.47	--	5.64
γ-linolenic acid	1.58	--	0.69	--	--	--	0.77	--
Nonadecylic acid	--	--	0.33	--	--	--	0.17	--
cyclo-nonadecylic C10	--	--	0.67	--	0.03	--	1.28	--
iso-nonadecylic	--	--	0.21	--	--	--	0.09	--
19:1 ω11*c*/19:1	--	--	0.18	--	--	--	0.02	--
19:1 ω6*c*/ω7*c*/19 cyclo	--	--	0.21	--	0.02	--	0.07	--
Arachidic acid	0.67	--	1.37	--	1.13	--	1.73	--
iso-arachidic	0.13	--	0.04	--	0.11	--	0.04	--
20:1 ω7c	2.23	--	2.11	--	2.43	--	2.77	--
Eicosenoic acid	--	--	0.19	--	0.07	--	0.13	--
Arachidonic acid	0.02	--	0.03	--	0.18	--	0.37	--
n	20	3	20	18	20	5	20	12

†=Not detected

Table 4-5. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first canonical variate separating standard FAME extraction from Instant FAME extraction of *Meloidogyne*-infected root tissue.

Fatty Acid	CAN1
Capric acid	1
Capric 2OH	1
Undecylic 2OH	1
Lauric 2OH	1
anteiso-lauric	1
iso-lauric	-1
iso-myristic	1
Palmitic acid	1
anteiso-palmitic	1
16:1 ω6c/palmitoleic	1
Palmitoleic/16:1 ω6c	-1
Margaric acid	1
Margaric 10-*methyl*	1
iso-margaric 3OH	1
17:1 *iso* ω5*c*	1
17:1 *anteiso* A	1
17:1 *anteiso* ω9*c*	1
17:1 *iso* I/*anteiso*	1
17:1 ω7*c*	1
17:1 ω8*c*	1
17:1 ω9*c*	1
Stearic acid	1
Stearic 10-*methyl*	1
Stearic 2OH	-1
iso-stearic	1
18:1 *iso* H	1
18:1 ω7*c*	-1
Elaidic acid	1
Linoleic/stearic	1
Nonadecylic acid	1
cyclo-nonadecylic C10	1
19:1 ω11*c*/19:1	1
Arachidic acid	-1
20:1 ω7*c*	1
Eicosenoic acid	1
Canonical Correlation	0.908
Eigenvalue	4.67
Cumulative Proportion	1

Table 4-6. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first canonical variate separating standard FAME analysis (EUKARY method) from Rapid analysis of *Meloidogyne*-infected root tissue.

Fatty Acid	CAN1
Capric 3OH	1
Lauric acid	1
Lauric 2OH	1
Lauric 3OH	1
anteiso-lauric	1
13:1 at 12-13	1
anteiso-pentadecylic	1
iso-pentadecylic	1
15:1 *iso* G	1
Palmitic N Alcohol	1
16:1 *iso* H	1
Palmitoleic acid	-1
Palmitoleic Alcohol	1
Palmitoleic/16:1 w6c	1
Margaric acid	1
Margaric 10-*methyl*	1
iso-margaric	1
iso-margaric 3OH	1
17:1 *iso* I/*anteiso*	1
17:1 w9*c*	1
Stearic acid	-1
Stearic 2OH	1
18:1 *iso* H	1
18:1 w5*c*	1
cis-vaccenic	1
18:1 w8*t*	-1
Elaidic acid	-1
Linoleic/stearic	1
Linoleic acid	-1
γ-linolenic acid	1
cyclo-nonadecylic C10	1
iso-nonadecylic	1
Arachidic acid	1
iso-arachidic	1
20:1 w7c	1
Canonical Correlation	0.997
Eigenvalue	173.82
Cumulative Proportion	1

Table 4-7. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis comparing two nematode species infecting root tissue (*Meloidogyne incognita* race 2 [I2], *M. javanica* race 1 [J1]), two extraction methods (Instant FAME extraction [RE], standard extraction [SE]), and two analysis methods (Rapid analysis [RA], and standard analysis [SA]).

		From Analysis							
To Analysis		I2RERA	I2RESA	I2SERA	I2SESA	J1RERA	J1RESA	J1SERA	J1SESA
I2RERA	D^2	0	641.26	104.43	619.70	25.08	678.47	195.41	627.19
	P	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
I2RESA	D^2	641.26	0	848.27	12.19	791.81	39.95	1001.00	10.30
	P	<0.0001	1	<0.0001	0.9929	<0.0001	0.2854	<0.0001	0.9994
I2SERA	D^2	104.43	848.27	0	826.71	77.51	885.48	83.26	834.20
	P	<0.0001	<0.0001	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
I2SESA	D^2	619.70	12.19	826.71	0	770.25	31.58	979.17	2.95
	P	<0.0001	0.9929	<0.0001	1	<0.0001	0.0082	<0.0001	0.9999
J1RERA	D^2	25.08	791.81	77.51	770.25	0	829.02	156.09	777.74
	P	<0.0001	<0.0001	<0.0001	<0.0001	1	<0.0001	<0.0001	<0.0001
J1RESA	D^2	678.47	39.95	885.48	31.58	829.02	0	1038.00	32.21
	P	<0.0001	0.2854	<0.0001	0.0082	<0.0001	1	<0.0001	0.0181
J1SERA	D^2	195.41	1001.00	83.26	979.17	156.09	1038.00	0	986.66
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	1	<0.0001
J1SESA	D^2	627.19	10.30	834.20	2.95	777.74	32.21	986.66	0
	P	<0.0001	0.9994	<0.0001	0.9999	<0.0001	0.0181	<0.0001	1

Table 4-8. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, CAN3) for separation of extraction methods (standard/Instant FAME) and analysis methods (standard/Rapid) using root tissue infected with either *Meloidogyne incognita* or *M. javanica*. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3	Fatty Acid	CAN1	CAN2	CAN3
Capric acid	0.644	-0.613	-0.420	*iso*-margaric 3OH	0.510	-0.498	0.694
Lauric acid	**0.854**	0.020	0.480	17:1 *iso* ω5*c*	0.630	-0.676	0.381
Lauric 2OH	0.582	**-0.810**	0.018	17:1 *anteiso* A	0.722	-0.646	0.190
Lauric 3OH	0.698	-0.646	0.291	17:1 *anteiso* ω9*c*	0.557	-0.604	0.562
anteiso-lauric	0.706	-0.653	0.248	17:1 *iso* I/*anteiso*	0.698	-0.697	-0.028
iso-lauric	**0.755**	0.480	-0.443	17:1 ω8*c*	0.579	**-0.813**	-0.009
Tridecylic acid	0.658	-0.008	0.557	17:1 ω9*c*	0.537	**-0.816**	-0.206
iso-tridecylic	**0.810**	-0.186	-0.435	Stearic 10-*methyl*	0.451	-0.245	**0.858**
Myristic 2OH	0.574	**-0.794**	-0.200	Stearic 2OH	**0.914**	0.115	-0.111
Pentadecylic 3OH	0.595	**-0.783**	0.164	*iso*-stearic	**0.757**	-0.406	-0.131
anteiso-pentadecylic	0.657	-0.747	0.026	*cis*-vaccenic	**0.951**	0.272	-0.122
15:1 *iso* F	0.414	-0.743	-0.526	Elaidic acid	**-0.864**	-0.122	0.003
15:1 ω5*c*	0.593	**-0.793**	0.119	Linoleic/Stearic	**0.789**	0.528	-0.028
16:1 2OH	0.583	-0.682	0.433	Linoleic acid	-0.718	-0.105	0.003
16:1 *iso* H	0.672	-0.693	0.258	γ-linolenic acid	0.545	0.133	-0.109
16:1 ω5*c*	**0.944**	-0.138	-0.123	Nonadecylic acid	0.533	-0.547	0.638
16:1 ω6*c*/palmitoleic	0.411	-0.310	**0.852**	*iso*-nonadecylic	0.507	-0.491	0.702
Palmitoleic acid	-0.631	-0.084	0.002	19:1 ω11*c*/19:1	0.382	-0.260	**0.882**
Palmitoleic Alcohol	0.702	-0.631	-0.306	Arachidic acid	**0.937**	-0.323	0.009
Palmitoleic/16:1 ω6*c*	**0.961**	0.100	-0.209	Arachidonic acid	0.599	-0.484	-0.547
Margaric acid	0.572	-0.647	0.495				
				Canonical Correlation	0.995	0.949	0.845
				Eigenvalue	183.09	18.60	5.45
				Cumulative Proportion	0.871	0.960	0.985

Table 4-9. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis comparing two nematode species infecting root tissue (*Meloidogyne incognita* race 2 [I2], *M. javanica* race 1 [J1]) and two extraction methods (Instant FAME extraction [RE], standard extraction [SE]) using Rapid analysis .

		From Analysis			
To Analysis		I2RE	I2SE	J1RE	J1SE
I2RE	D^2	0	277.58	99.24	374.20
	P	1	<0.0001	<0.0001	<0.0001
I2SE	D^2	277.58	0	121.80	150.12
	P	<0.0001	1	<0.0001	<0.0001
J1RE	D^2	99.24	121.80	0	282.84
	P	<0.0001	<0.0001	1	<0.0001
J1SE	D^2	374.20	150.12	282.84	0
	P	<0.0001	<0.0001	<0.0001	1

Table 4-10. Correlation values between canonical structure of fatty acids significant in the three canonical variates (CAN1, CAN2, CAN3) for separating extraction method (standard/Instant FAME) and root tissue infected with either *Meloidogyne incognita* or *M. javanica*. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3	Fatty Acid	CAN1	CAN2	CAN3
Capric 2OH	**0.977**	-0.114	0.178	Palmitoleic/16:1 ω6*c*	-0.030	**-0.989**	-0.141
Capric 3OH	-0.416	**-0.786**	0.457	Margaric acid	**0.854**	0.289	0.433
Undecylic acid	-0.376	**-0.771**	0.515	Margaric 10-*methyl*	**0.908**	-0.415	-0.056
Lauric acid	0.337	**0.936**	0.106	Margaric 2OH	**0.793**	-0.467	-0.390
Lauric 2OH	**0.975**	-0.181	0.129	*cyclo*-margaric	**0.845**	0.303	0.440
Lauric 3OH	**0.964**	0.233	0.125	*iso*-margaric	**0.914**	-0.021	0.406
Tridecylic	0.310	**0.932**	-0.187	17:1 *iso* ω5*c*	**0.925**	0.226	0.305
Tridecylic 2OH	0.193	**-0.831**	0.522	17:1 *anteiso* A	**0.986**	0.169	-0.004
13:1 at 12-13	**0.808**	0.544	0.224	17:1 *anteiso* ω9*c*	**0.806**	0.359	0.470
Myristic acid	**-0.758**	-0.438	0.483	17:1 ω8*c*	**0.972**	-0.206	0.111
Myristic 2OH	**0.938**	-0.340	-0.075	Stearic acid	0.554	0.180	**0.813**
anteiso-myristic	**0.861**	0.278	0.427	Stearic 2OH	-0.162	**-0.771**	0.617
Pentadecylic 3OH	**0.973**	-0.044	0.227	*iso*-stearic	**0.760**	0.113	-0.640
anteiso-pentadecylic	**0.969**	-0.189	0.161	18:1 *iso* H	**0.812**	0.351	0.466
Palmitic N Alcohol	0.588	**-0.806**	-0.071	18:1 ω7*c*	**-0.831**	-0.465	-0.304
anteiso-palmitic	**0.928**	0.241	0.284	*cyclo*-nonadecylic C10	**0.970**	-0.232	0.067
iso-palmitic 3OH	**0.885**	0.249	-0.392	Arachidic acid	**0.971**	0.055	-0.231
16:1 2OH	**0.890**	0.225	0.397	*iso*-arachidic	**-0.961**	-0.214	-0.176
16:1 iso G	**0.799**	-0.527	0.290	Eicosenoic acid	**0.838**	0.521	0.161
Palmitoleic Alcohol	**0.840**	-0.540	-0.048				
				Canonical Correlation	0.992	0.977	0.922
				Eigenvalue	59.04	21.21	5.66
				Cumulative Proportion	0.687	0.934	1

Table 4-11. Mean FAME concentrations (percentage of total response) of four combinations of two nematode species (*Meloidogyne incognita* race 2 [I2], *M. javanica* race 1 [J1]) and two extraction methods (Instant FAME extraction [RE], standard extraction [SE]) utilizing Rapid analysis (RA). Both extractions were carried out using 40 mg of infected *Solenostemon scutellarioides* root tissue.

Fatty Acid	I2RE	I2SE	J1RE	J1SE	Fatty Acid	I2RE	I2SE	J1RE	J1SE
Capric acid	1.23	0.09	2.02	0.03	Myristic 2OH	0.06	0.18	0.08	0.19
Capric 2OH	0.06	0.53	0.08	0.45	Myristic 3OH/16:1*iso* I	0.08	0.02	0.13	0.09
Capric 3OH	0.04	0.03	0.06	0.05	*anteiso*-myristic	0.28	0.75	0.10	0.87
iso-capric	0.06	0.03	0.08	0.01	*iso*-myristic	0.18	0.17	0.23	0.15
Undecylic acid	0.06	0.02	0.04	0.01	*iso*-myristic 3OH	0.05	0.07	0.06	0.07
Undecylic 2OH	0.08	0.05	0.09	0.03	14:1 *iso* E	0.01	0.03	0.04	0.01
Undecylic 3OH	0.07	0.03	0.04	0.01	14:1 ω5*c*	0.18	0.05	0.17	0.10
anteiso-undecylic	0.05	0.07	0.07	0.14	Pentadecylic 2OH	0.11	0.04	0.07	0.07
iso-undecylic	0.13	0.01	0.15	0.01	Pentadecylic 3OH	0.05	0.03	0.06	--
iso-undecylic 3OH	0.07	0.03	0.02	0.06	*anteiso*-pentadecylic	0.09	0.20	0.19	0.14
Lauric acid	0.53	0.49	0.83	0.55	*iso*-pentadecylic	0.99	1.33	0.96	1.06
Lauric 2OH	0.10	1.20	0.21	0.85	*iso*-pentadecylic 3OH	0.14	0.05	0.04	0.03
Lauric 3OH	0.26	0.06	0.30	0.05	15:1 *anteiso* A	0.59	0.32	0.65	0.42
Lauric Aldehyde	0.12	0.03	0.12	--	15:1 *iso* F	0.09	0.05	0.22	0.02
anteiso-lauric	0.44	0.04	0.35	0.05	15:1 *iso* G	0.03	0.34	0.04	0.40
iso-lauric	--†	0.04	0.09	0.03	15:1 *iso* H/13:0 3OH	0.07	0.46	0.10	0.47
iso-lauric 3OH	0.06	0.02	0.08	0.12	15:1 *iso* ω9*c*	0.05	0.11	0.15	0.12
12:1 3OH	--	0.01	0.05	0.04	15:1 ω5*c*	0.09	0.04	0.11	0.01
Tridecylic acid	0.20	0.10	0.23	0.11	15:1 ω6*c*	0.10	0.03	0.16	0.04
Tridecylic 2OH	0.08	0.36	0.09	0.49	15:1 ω8*c*	0.06	0.02	0.17	--
13:0 3OH /15:1 *iso* H	0.03	--	0.10	--	Palmitic acid	13.23	11.62	13.34	14.47
anteiso-tridecylic	0.01	0.12	0.14	0.10	Palmitic 10-*methyl*	0.08	0.02	0.06	--
iso-tridecylic	0.24	0.10	0.20	0.08	Palmitic 2OH	0.05	0.39	0.12	0.55
iso-tridecylic 3OH	0.23	--	0.08	--	Palmitic 3OH	0.14	0.03	0.05	0.01
13:1 at 12-13	0.09	0.02	0.07	--	Palmitic N Alcohol	0.07	0.20	0.10	0.21
Myristic acid	1.24	1.89	1.66	1.55	*anteiso*-palmitic	0.06	0.09	0.05	0.29

Table 4-11. Continued.

Fatty Acid	I2RE	I2SE	J1RE	J1SE	Fatty Acid	I2RE	I2SE	J1RE	J1SE
iso-palmitic	0.06	0.02	0.12	0.02	17:1 ω9*c*	0.11	0.20	0.05	0.15
iso-palmitic 3OH	0.10	0.04	0.05	--	Stearic acid	5.76	8.43	5.90	7.61
16:1 2OH	--	0.41	0.08	0.41	Stearic 10-*methyl*	--	0.05	0.19	0.07
16:1 *iso* G	0.05	0.07	0.14	0.08	Stearic 2OH	0.39	0.65	0.19	0.57
16:1 *iso* H	0.08	0.05	0.02	0.02	*iso*-stearic	0.40	0.19	0.27	0.12
16:1 ω11*c*	0.10	0.04	0.03	--	18:1 2OH	0.05	0.04	--	0.04
16:1 ω5*c*	0.75	0.76	0.63	0.47	18:1 *iso* H	0.15	0.05	0.06	0.02
16:1 ω6*c*/16:1 ω7*c*	--	0.11	0.02	0.15	18:1 ω5*c*	1.08	1.16	0.76	0.97
Palmitoleic Alcohol	0.06	0.02	0.24	0.03	*cis*-vaccenic	27.39	35.65	25.47	27.91
16:1 ω7*c*/16:1 ω6*c*	1.76	1.96	1.46	1.45	*cis*-vaccenic 11-*methyl*	0.13	0.15	0.03	0.07
16:1 ω9c	0.01	0.01	0.05	0.06	Oleic acid	0.30	1.39	--	0.63
Margaric acid	0.47	0.57	0.48	0.67	Linoleic/*anteiso*-stearic	30.94	16.72	30.49	25.25
Margaric 10-*methyl*	0.03	0.02	0.09	--	γ-linolenic acid	0.04	0.23	0.26	0.21
Margaric 2OH	0.07	0.30	0.13	0.31	Nonadecylic acid	0.17	0.21	0.12	0.12
Margaric 3OH	0.05	--	0.06	--	*anteiso*-nonadecylic	0.07	0.02	0.03	--
anteiso-margaric	0.15	0.13	0.20	0.14	*cyclo*-nonadecylic C10	0.04	0.62	0.16	0.65
cyclo-margaric	0.08	0.10	0.08	0.28	*cyclo*-nonadecylic C8	0.19	0.03	0.11	0.03
iso-margaric	0.49	0.78	0.63	0.67	*iso*-nonadecylic	0.12	0.25	0.06	0.19
iso-margaric 3OH	0.27	0.51	0.16	0.33	19:1 *iso* I	0.13	0.07	0.17	0.10
17:1 *iso* ω5*c*	0.09	0.30	0.12	0.36	19:1 ω11*c*/19:1 ω9*c*	0.14	0.20	0.09	0.20
17:1 *anteiso* A	0.09	0.03	0.06	0.01	19:1 ω6*c*/ω7*c*/19*cyclo*	0.07	0.31	0.03	0.18
17:1 *anteiso* B/*iso* I	0.07	0.02	0.08	0.04	19:1 ω9*c*/19:1 ω11*c*	0.04	0.02	0.05	--
17:1 *anteiso* ω9*c*	0.11	0.29	0.09	0.33	19:1ω7*c*/19:1 ω6*c*	0.08	0.11	0.09	0.03
17:1 *iso* I/*anteiso* B	0.02	0.00	0.07	0.02	Arachidic acid	2.05	1.72	2.63	2.00
17:1 *iso* ω10*c*	0.08	0.01	0.01	0.01	*iso*-arachidic	0.04	0.31	0.12	0.25
17:1 ω5*c*	0.08	0.01	0.02	0.01	20:1 ω7c	1.25	1.66	1.36	1.20
17:1 ω6*c*	0.05	0.15	0.01	0.07	Eicosenoic acid	0.32	0.25	0.28	0.25
17:1 ω7*c*	0.10	0.07	0.18	0.16	Arachidonic acid	0.27	0.33	0.47	0.33
17:1 ω8*c*	0.09	0.05	0.16	0.05	Pelargonic acid	0.18	0.05	0.24	0.02

Table 4-11. Continued.

Fatty Acid	I2RE	I2SE	J1RE	J1SE
Pelargonic 3OH	0.05	0.03	0.01	0.01
unknown 10.9525	0.10	0.05	0.03	0.02
n	20	20	20	20

†=Not detected

Table 4-12. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis (with full stepwise discriminant analysis prior) comparing two FAME extraction methods (Instant FAME extraction [RE], standard extraction [SE]) on 40 mg of *Solenostemon scutellarioides* root tissue infected with either *Meloidogyne incognita* race 2 (I2) or *M. javanica* race 1 (J1) utilizing Rapid analysis.

To Analysis		I2RE	I2SE	J1RE	J1SE
I2RE	D^2	0	113,385,980	83,340,926	40,487,884
	P	1	<0.0001	<0.0001	<0.0001
I2SE	D^2	113,385,980	0	213,256,435	18,374,852
	P	<0.0001	1	<0.0001	<0.0001
J1RE	D^2	83,340,926	213,256,435	0	132,766,463
	P	<0.0001	<0.0001	1	<0.0001
J1SE	D^2	40,487,884	18,374,852	132,766,463	0
	P	<0.0001	<0.0001	<0.0001	1

Table 4-13. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis (with truncated stepwise discriminant analysis prior) comparing two FAME extraction methods (Instant FAME extraction [RE], standard extraction [SE]) on 40 mg *Solenostemon scutellarioides* root tissue infected with either *Meloidogyne incognita* race 2 (I2) or *M. javanica* race 1 (J1) utilizing Rapid analysis.

To Analysis		I2RE	I2SE	J1RE	J1SE
I2RE	D^2	0	2,175,521	80,100	751,766
	P	1	<0.0001	0.0002	<0.0001
I2SE	D^2	2,175,521	0	2,987,898	374,119
	P	<0.0001	1	<0.0001	<0.0001
J1RE	D^2	80,100	2,987,898	0	1,257,481
	P	0.0002	<0.0001	1	<0.0001
J1SE	D^2	751,766	374,119	1,257,481	0
	P	<0.0001	<0.0001	<0.0001	1

Table 4-14. Correlation values between canonical structure and fatty acids selected by truncated stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, CAN3) for separating extraction methods (standard/Instant FAME) and *Solenostemon scutellarioides* root tissue infected with either *Meloidogyne incognita* or *M. javanica* in 40-mg tissue samples. Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3	Fatty Acid	CAN1	CAN2	CAN3
Capric acid	**0.923**	0.252	0.291	15:1 *iso* G	**-0.885**	0.088	-0.458
Capric 2OH	**-0.967**	0.148	-0.208	15:1 *iso* H/13:0 3OH	**-0.924**	0.146	-0.354
Capric 3OH	0.625	0.356	-0.695	15:1 *iso* ω9c	-0.049	**0.969**	-0.241
iso-capric	**0.831**	0.140	0.538	15:1 ω5*c*	**0.812**	0.142	0.566
Undecylic 2OH	**0.788**	0.025	0.615	Palmitic acid	0.426	-0.128	**-0.896**
anteiso-undecylic	-0.381	0.178	**-0.907**	Palmitic 10-*methyl*	**0.793**	-0.267	0.547
iso-undecylic	**0.951**	-0.006	0.309	Palmitic 2OH	**-0.789**	0.175	-0.588
iso-undecylic 3OH	0.204	**-0.933**	-0.296	Palmitic 3OH	0.589	-0.667	0.456
Lauric acid	0.721	0.691	-0.047	Palmitic N Alcohol	**-0.895**	0.249	-0.370
Lauric 2OH	**-0.975**	0.208	-0.072	*anteiso*-palmitic	-0.404	-0.090	**-0.910**
Lauric 3OH	**0.939**	0.038	0.341	*iso*-palmitic	**0.894**	0.371	0.252
Lauric Aldehyde	**0.847**	-0.064	0.528	16:1 *iso* H	-0.026	**-0.872**	0.489
anteiso-lauric	**0.915**	-0.258	0.310	16:1 ω11*c*	0.301	-0.675	0.673
iso-tridecylic	**0.852**	-0.290	0.437	16:1 ω5*c*	-0.019	-0.241	**0.970**
iso-tridecylic 3OH	0.711	-0.616	0.339	16:1 ω6*c*/16:1 ω7*c*	**-0.823**	0.155	-0.546
Myristic 2OH	**-0.895**	0.201	-0.398	Palmitoleic Alcohol	**0.773**	0.626	0.097
Myristic 3OH	**0.821**	0.329	-0.466	16:1 ω7*c*/16:1 ω6*c*	-0.535	-0.340	**0.774**
iso-myristic	0.740	0.479	0.471	16:1 ω9*c*	0.241	0.465	**-0.852**
iso-myristic 3OH	**-0.820**	0.493	-0.292	Margaric acid	-0.689	0.044	-0.724
14:1 *iso* E	0.023	**0.856**	0.516	Margaric 2OH	**-0.886**	0.277	-0.371
14:1 ω5*c*	**0.986**	-0.166	0.008	Margaric 3OH	**0.950**	0.059	0.307
Pentadecylic 2OH	0.693	-0.721	0.006	*cyclo*-margaric	-0.381	-0.095	**-0.919**
15:1 *anteiso* A	**0.998**	0.043	0.047	*iso*-margaric	**-0.810**	0.586	0.005

Continued on next page

Table 4-14. Continued.

Fatty Acid	CAN1	CAN2	CAN3	Fatty Acid	CAN1	CAN2	CAN3
iso-margaric 3OH	**-0.957**	-0.158	0.242	γ-linolenic acid	-0.299	**0.935**	-0.192
17:1 *iso* ω5*c*	**-0.850**	0.138	-0.508	Nonadecylic acid	-0.492	-0.341	**0.801**
17:1 *anteiso* A	0.743	-0.383	0.550	*cyclo*-nonadecylic C10	**-0.899**	0.235	-0.370
17:1 *anteiso* ω9*c*	**-0.899**	-0.008	-0.437	*cyclo*-nonadecylic C8	**0.780**	-0.538	0.321
17:1 ω6*c*	**-0.933**	-0.151	0.327	*iso*-nonadecylic	**-0.980**	-0.198	-0.005
17:1 ω8*c*	**0.821**	0.505	0.265	19:1 *iso* I	**0.966**	0.252	-0.055
Stearic acid	**-0.983**	0.164	-0.081	19:1 ω11*c*/19:1 ω9*c*	**-0.903**	-0.326	-0.279
Stearic 10-*methyl*	0.391	**0.912**	-0.125	19:1 ω6*c*/ω7*c*/19*cyclo*	**-0.994**	0.014	0.107
Stearic 2OH	**-0.952**	-0.296	-0.085	19:1ω7*c*/19:1 ω6*c*	0.037	0.233	**0.972**
iso-stearic	0.683	-0.444	0.580	Arachidic acid	**0.857**	0.502	-0.118
18:1 2OH	-0.523	**-0.850**	0.052	20:1 ω7c	-0.579	0.396	0.713
18:1 ω5*c*	-0.703	-0.625	0.339	Eicosenoic acid	0.733	-0.583	0.349
Linoleic/*anteiso*-stearic	**0.960**	-0.182	-0.211	Arachidonic acid	0.368	**0.929**	-0.035
				Canonical Correlation	1	1	0.999
				Eigenvalue	499291	2037	441
				Cumulative Proportion	0.995	0.999	1

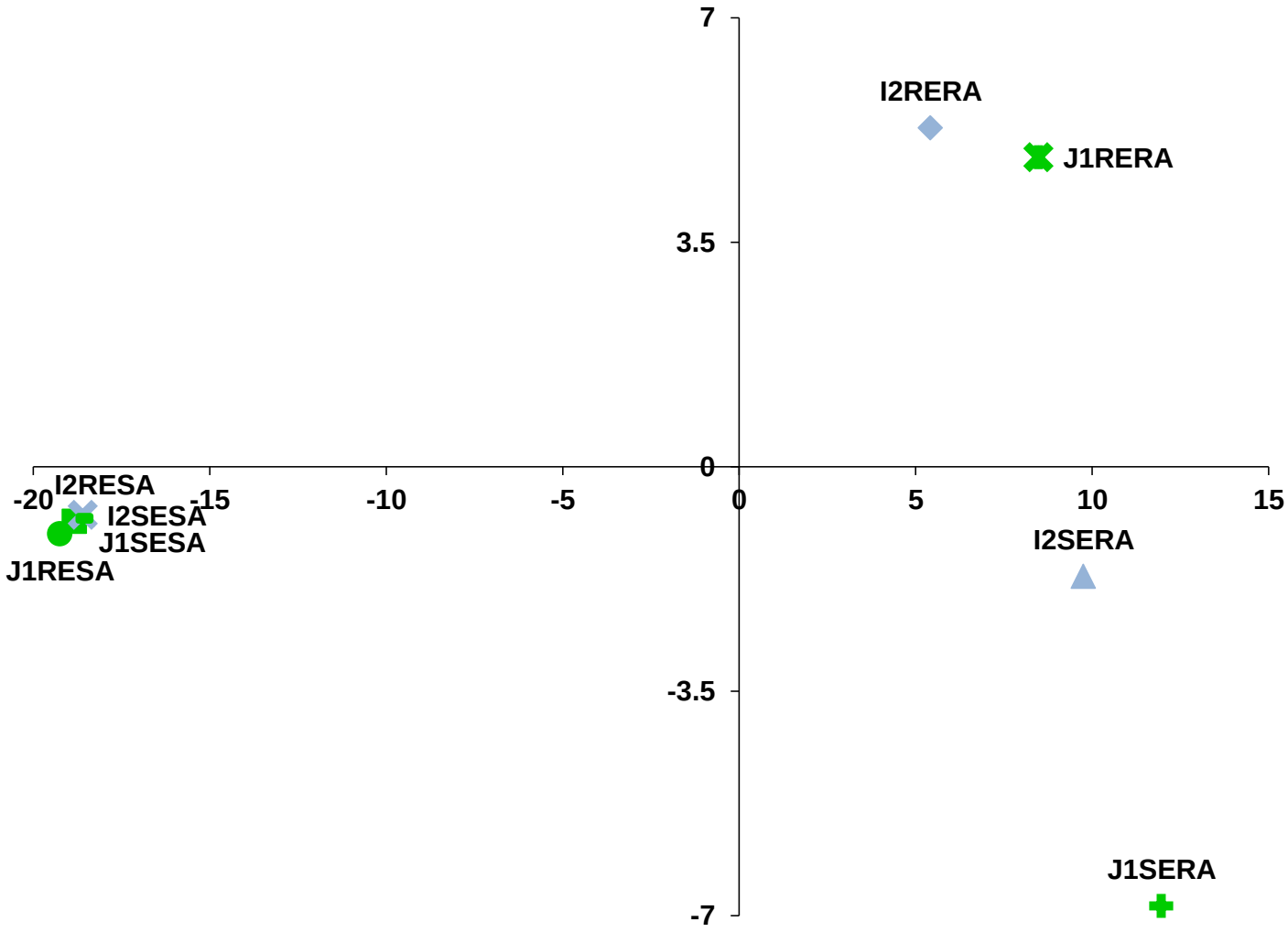


Figure 4-1. Canonical distribution comparing *M. javanica*-infected tomato root tissue (J1) FAME profiles to *M. incognita*-infected tissue (I2) using either standard FAME extraction (SE) or Instant FAME extraction (RE) in combination with either Rapid FAME analysis (RA) or standard analysis (SA). Sample sizes were 40 mg for SE and 3 mg for RE.

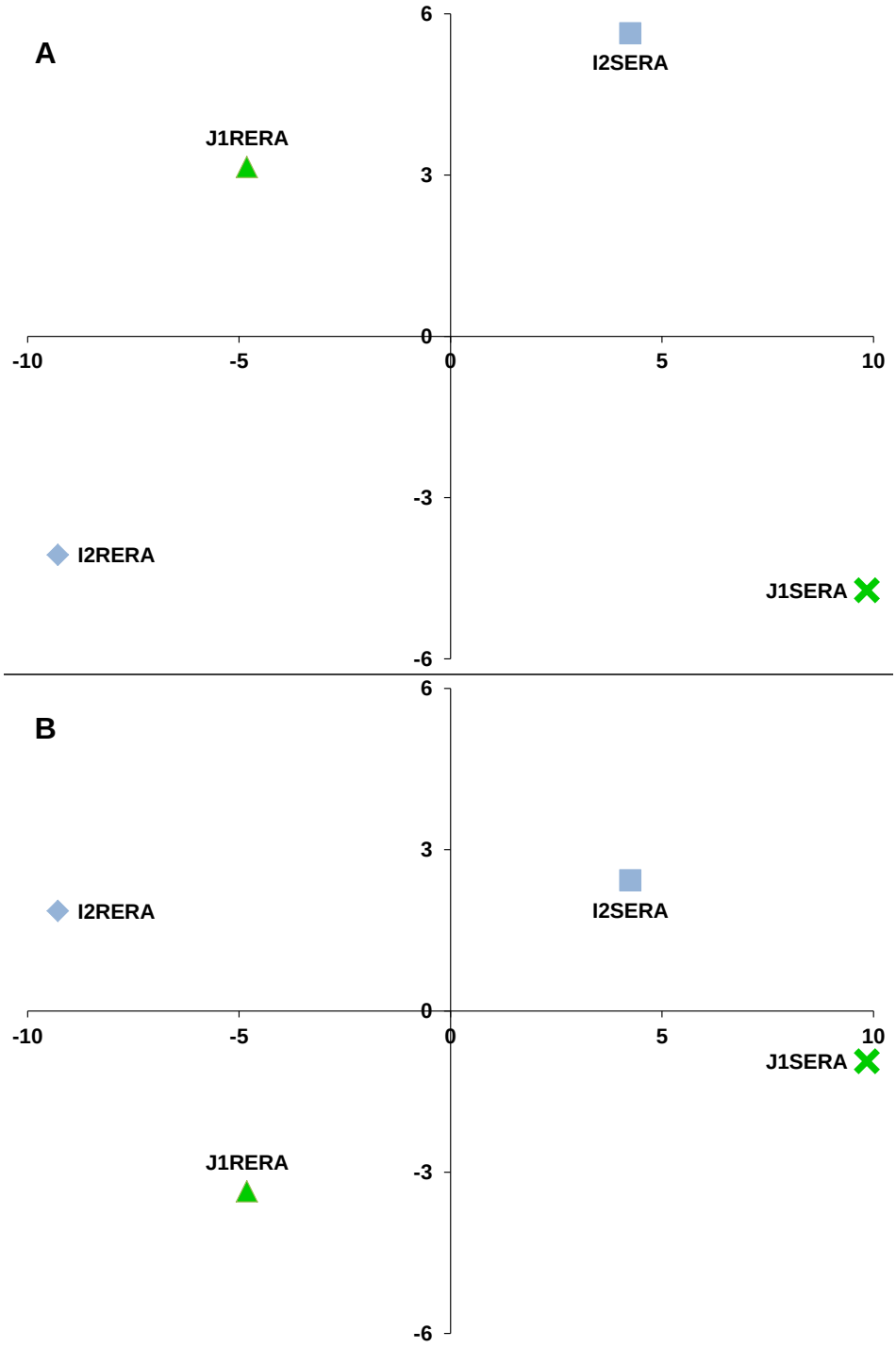


Figure 4-2. Canonical distribution comparing *Meloidogyne javanica*-infected tomato root tissue (J1) FAME profiles to *M. incognita*-infected tissue (I2) using either standard FAME extraction (SE) or Instant FAME extraction (RE). All extractions were analyzed using Rapid FAME analysis (RA). Sample sizes were 40 mg for SE and 3 mg for RE. A) CAN1 (*x*-axis) versus CAN2 (*y*-axis) and B) CAN1 (*x*-axis) versus CAN3 (*y*-axis).

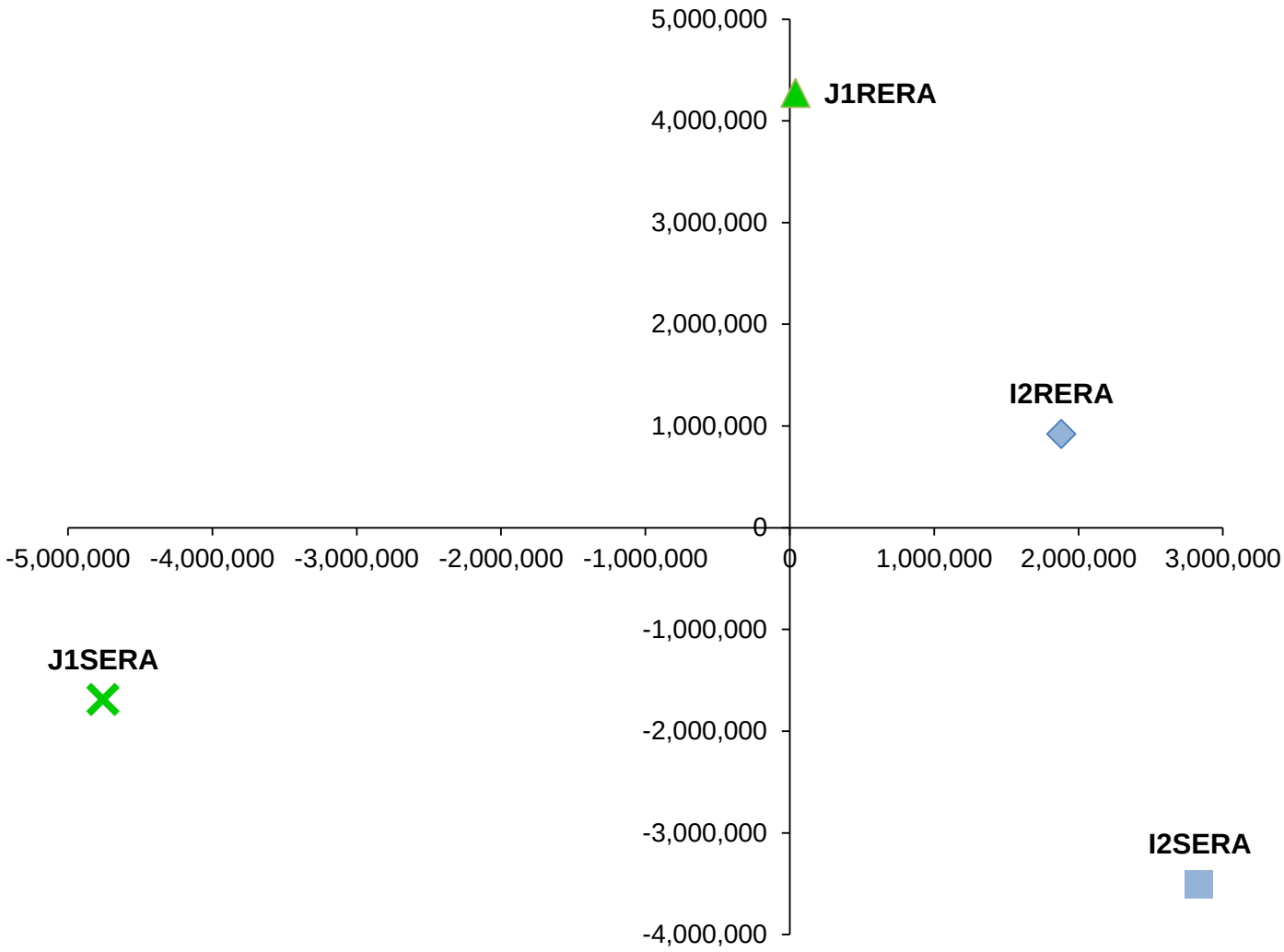


Figure 4-3. Canonical discriminant analysis after full stepwise discriminant analysis comparing 40 mg of *Meloidogyne javanica*-infected *Solenostemon scutellarioides* root tissue (J1) FAME profiles to 40 mg of *M. incognita*-infected tissue (I2) using either standard FAME extraction (SE) or Instant FAME extraction (RE). All extractions were analyzed using Rapid FAME analysis (RA).

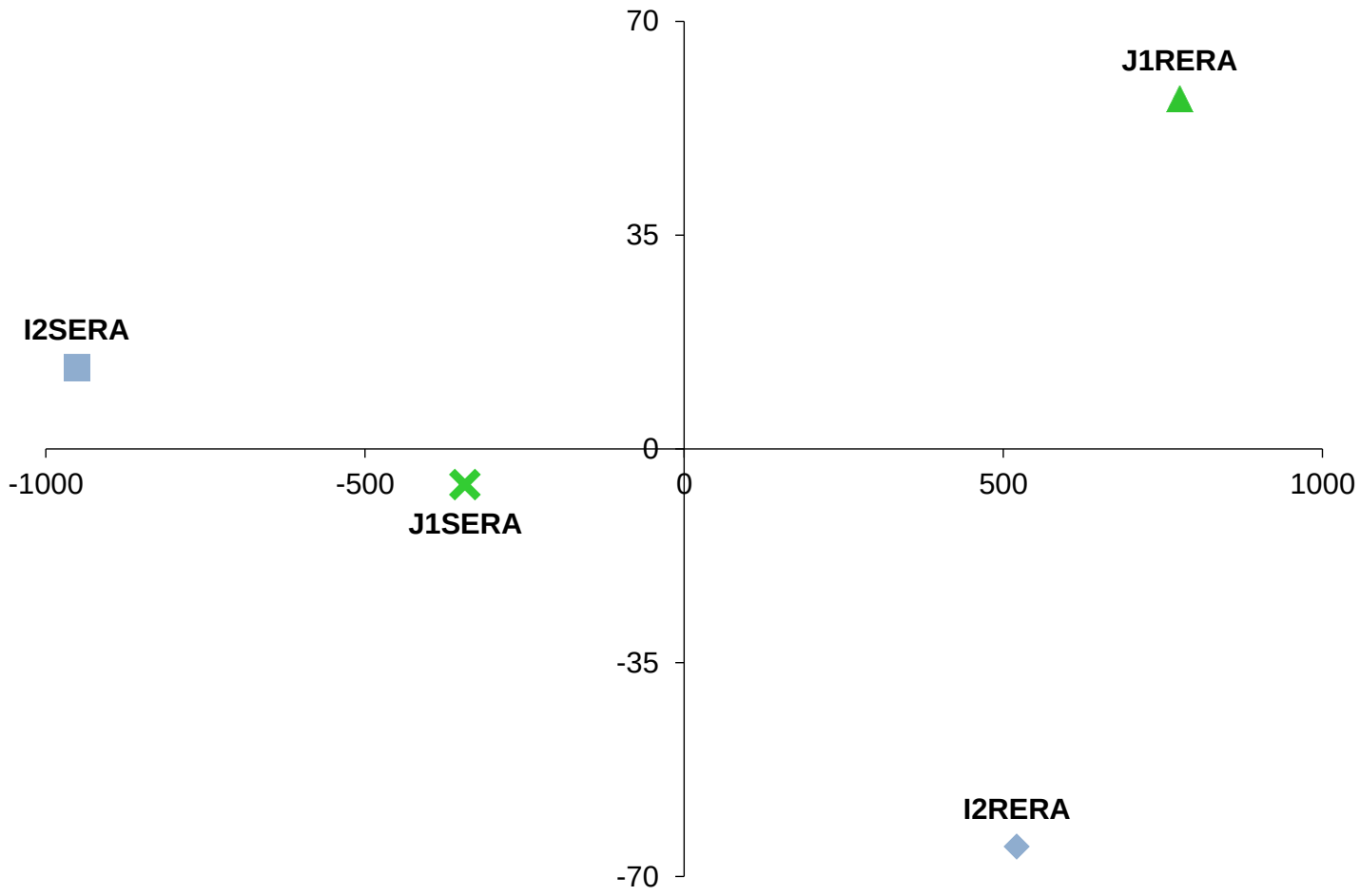


Figure 4-4. Canonical discriminant analysis after truncated stepwise discriminant analysis comparing 40 mg of *Meloidogyne javanica*-infected *Solenostemon scutellarioides* root tissue (J1) FAME profiles to 40 mg *M. incognita*-infected tissue (I2) using either standard FAME extraction (SE) or Instant FAME extraction (RE). All extractions were analyzed using Rapid FAME analysis (RA).

CHAPTER 5
POPULATION DENSITY AND DETECTION OF *MELOIDOGYNE* SPECIES USING FATTY ACID METHYL ESTER ANALYSIS

5.1 Introduction

Previous work by Sekora *et al.* (2009) comparing fatty acid profiles of *Rotylenchulus reniformis* and *Meloidogyne incognita* at multiple population densities has indicated that it may be possible to use the information gained from fatty acid methyl ester (FAME) analysis of a specific nematode species to estimate the number of individuals of the given species in a sample. Each analysis provides a "total response," the amount of identified lipid in the sample as a function of electrical response (mV), as a subset of information that could be used to set up prediction intervals for the expected response of a sample given a certain number of individuals.

Since fatty acid compositions can vary depending on the life stage analyzed (Krusberg *et al*., 1973; Sekora *et al.*, 2008; Sekora *et al*., 2009), it is crucial to study the effects of increasing numbers of individuals on FAME profiles and response before accurate quantification can be achieved. It is expected that lipid compositions will change as the tissues within a nematode develop into an adult, but the degree of those changes between juvenile and adult may or may not influence quantification using FAME profiles. Sekora *et al*. (2009) found significant differences among life stages of *Heterodera glycines*; egg producing females produced FAME profiles distinct from those of cysts and eggs. Quantification of a sample containing mixed life stages of *H. glycines*, or any other nematode species, would likely require profiles based on increasing numbers of each life stage.

Another critical aspect of quantification is the use of extraction and analysis methods that are sensitive enough to detect minute quantities of fatty acids. More

efficient and precise methods of FAME extraction and analysis (Instant FAME and Rapid analysis) have recently been developed by MIDI (Newark, DE), and these methods have been shown to be excellent at increasing the ability of FAME analysis to separate *Meloidogyne* species within root tissue (Sekora and Crow, 2011). It is expected that the increased resolution of the Instant FAME and Rapid analysis methods will reduce the number of individuals required for FAME analysis from those published by Sekora *et al*. (2009). Therefore, the objective of this study is to determine the minimum number of nematodes of various life stages required to produce a FAME profile adequate for quantification using the improved extraction and analysis methods.

5.2 Materials and Methods

5.2.1 Sample Preparation

Mature females (F), males (M), and second-stage juveniles (J) of *Meloidogyne graminis* were hand-picked from a stock population maintained under greenhouse conditions on *Paspalum notatum* (bahiagrass) at the University of Florida. Picked nematodes were immediately placed in 0.25 mL of the first extraction reagent (5% KOH in methanol) and the remainder of the extraction procedure was carried out once enough nematodes had been picked to complete an entire replicate (within 10 minutes; Figure 5-1). Twelve replications of five treatments (samples containing one, two, or five females, one male, or one juvenile) were prepared for FAME extraction using the Instant FAME method (MIDI, Newark, DE). A total of 60 samples were prepared for FAME analysis.

5.2.2 Statistical Analysis

Comparisons were made using the fatty acid analyses of samples containing each life stage of *M. graminis* as well as the multiple densities of *M. graminis* females. FAME

profiles were imported into SAS (SAS Institute, Cary, NC) for further analysis. Mean profiles for each categorical "class" (F5, J1, etc.) were calculated with PROC MEANS which provided the average response for each fatty acid in all samples for the given class. Additional statistical tests were performed using PROC STEPDISC in combination with PROC CANDISC following the method of Sekora *et al*. (2010). Stepwise discriminant analysis (SDA) by PROC STEPDISC was used to determine which fatty acids were significant for discrimination among classes using a series of stepwise analysis of variance (ANOVA) tests that evaluate the *F*-value of each fatty acid before and after inclusion (Johnson, 1998). After analysis of each fatty acid, fatty acids significant for delineation ($P < 0.15$) were used for canonical discriminant analysis (CDA) with PROC CANDISC.

Canonical discriminant analysis produces class means based on sample variance within each compared class and then represents relationships among classes in dimensional space. The dimensional space is represented by canonical variates (CAN1, CAN2, up to class *n*-1) that demonstrate class separation in graphical representation and can be assigned to *x*, *y*, or *z* axes depending on the desired class comparisons. Canonical variates are also used to describe the total multivariance within a test, and the number of variates in these tests was reduced to the fewest that could define at least 75% cumulative proportion of the total multivariance ($n\text{CAN} \leq 3$). Separation among classes is defined by the degree of "between canonical structure" correlation (-1 to 1) of a given fatty acid along the chosen canonical variate. Absolute values approaching |1.000| indicate a high degree of correlation and help to separate classes on the specified dimension. The greater the value of correlation, the greater the

spatial distance (Mahalanobis distance or D^2) among means graphically along a given canonical variate (Johnson, 1998). For the experiments described in this paper, high canonical correlation was described by correlations greater than |0.750| and significant mean separation was achieved with D^2 having $P < 0.05$.

Additional information provided by CDA is the canonical correlation and eigenvalue of each canonical variate. Canonical correlation values range from 0 to |1.000| and are indicators of the importance of each canonical variate to the separation of classes. Canonical correlation values approaching |1.000| are considered more informative for describing the majority of multivariance within a given analysis. The eigenvalue is another statistic similar to canonical correlation that is used to rank canonical variates based on the multivariance explained by the selected variate. As with canonical correlations, higher values indicate a greater degree of explained multivariance in an analysis for the given canonical variate (Johnson, 1998).

Regression analysis of total response against increasing numbers of *M. graminis* females was performed using the PROC REG procedure. The best-fit linear regression was obtained, and the predicted values and 95% confidence intervals were graphed overlaying the actual response values.

5.3 Results

In total, 51 of the 60 prepared samples produced FAME profiles that were useful for further statistical analysis. Samples containing females of *M. graminis* had fewer fatty acids than profiles derived from males or juveniles, and the number of fatty acids present did not increase as more females were added to a sample (Table 5-1). Oleic acid was observed as the most prevalent fatty acid in samples containing males or females, but myristic acid was most abundant in samples containing juveniles. Juvenile

and male samples contained 17 of 25 and 18 of 24 fatty acids, respectively, at concentrations between 2.0% and 6.0%. Less predominant fatty acids (linolenic/stearic, arachidonic acid, etc.) were detected as the number of females in a sample increased, which reduced the overall proportion of palmitic and stearic acids. Both were found in higher concentrations in single female samples than samples containing single males or juveniles.

Canonical discriminant analysis following SDA of the FAME profiles distinctly separated juveniles and males from the three female densities (Table 5-2; Figure 4-3). While the three female profile densities could not be statistically separated ($D^2 \leq 11.69$, $P \geq 0.3625$), single males and juveniles were distinctive from each other ($D^2 = 1009.00$, $P < 0.0001$) as well as the clustered female profiles ($D^2 \geq 412.56$, $P < 0.0001$). Single males and juveniles were separated by five fatty acids along CAN1 (64.1% of multivariance; Table 5-3), all of which were found at higher mean concentrations in single-juvenile samples than samples containing single males (Table 5-1). CAN2 (34.7% of total multivariance) separated single-male samples from the three female densities with 11 fatty acids (Table 5-3). Of these 11 fatty acids, only stearic acid was observed at a higher concentration in samples containing females of *M. graminis* (Table 5-1). Although CAN3 was not significant for separating the five classes ($P = 0.7090$), pinoleic acid was still significant for separation of the three female densities (Table 5-3; Figure 5-3).

Total responses of individual males (5,342.3 $\pm$ 817.1) were similar to those of individual females (5,650.7 $\pm$ 1833.2). Total response increased seemingly linearly from single-female samples as the number of individuals increased to two (11,115.6 $\pm$

3,141.2) and five (54,737.0 ± 13,042.0) per sample. Total responses of samples containing single juveniles (3,777.0 ± 931.9) were less than either single males or females, but still more than half that of a single mature female. Regression analysis of female total response indicated that response was linearly correlated with the number of females per sample ($R^2 = 0.5928$, $P < 0.0001$), but the observed responses appeared to increase with a conical spread as female numbers increased (Figure 5-2).

5.4 Discussion

Variation in response was observed in all life stages with positive signals and ranged from 406.13 to 18,912.23 for a single female (data not shown). Some variation was expected since each individual may differ in its FAME composition based on its size, age, maturity, time since feeding, and possibly by the presence of internal parasites. These variations were inflated when observing five mature females in a sample (total response ranged from 9192.96 to 103,530.70) and indicate that further studies need to be conducted to determine the factors for this variation before advanced utilization, such as quantification, is pursued.

Another type of variation in response had an impact on the fatty acids detected. By increasing the number of nematodes in a sample, fatty acids that were found at low levels in a single individual (or not at all, like *anteiso*-pentadecylic acid) had a greater chance of detection. These fatty acids appear to decrease the proportion of other fatty acids, like stearic and palmitic acids, but in reality do not affect their responses. Therefore, the reduction in proportion for stearic and palmitic acids is only an adjustment to account for the responses of the newly detected fatty acids.

The Agilent 6890N Gas Chromatography System (Agilent Technologies, Santa Clara, CA) that this study employed has the ability to increase or decrease sensitivity of

an injected sample through use of a split/splitless valve. The split/splitless valve allows for a portion of the injected sample to be shunted away from the chromatography column to prevent overload and damage to the column from samples with high lipid content. The proportion of sample analyzed is diluted with carrier gas (N_2) that moves the compounds through the column. The dilution is defined by the ratio *N*:*S* where *N* is the proportion of N_2 and *S* is the proportion of sample by volume. Samples are typically run at a split/splitless ratio of 25:1 with the Rapid analysis method. By reducing this ratio it is possible to detect more minute quantities of lipid within a sample. However, reducing this ratio requires more strict scrutiny of the FAME profiles produced since the detection of artefacts may also be increased.

Using the coupled Instant FAME and Rapid extraction methods it was possible to obtain distinct FAME profiles from single nematodes as opposed to the 250 reported for standard extraction and analysis methods (Sekora *et al.*, 2008). This reduction in the required number of individuals makes using FAME methods for population studies possible since these studies are typically conducted using single individuals. Currently, single females would be the likely choice for analysis, but work to further increase the sensitivity of the system may indicate that it may be possible to use individual males of juveniles.

Table 5-1. Mean FAME concentrations (percentage of total response) of *Meloidogyne graminis* females (F) at densities of 1, 2, or 5 individuals per sample, single juveniles (J1), and individual males (M1).

Fatty Acid	F1	F2	F5	J1	M1
Undecylic acid	0.79	--	--	4.85	3.83
Lauric 2OH	3.35	6.97	1.09	1.38	--
Lauric 3OH	--[†]	--	--	7.63	2.25
Tridecylic 3OH/15:1 iso	--	--	--	2.39	5.57
Myristic acid	0.77	2.42	3.20	14.25	0.88
Myristic 2OH	--	0.18	--	0.59	3.21
anteiso-pentadecylic	--	1.32	0.87	5.17	--
15:1 ω5c	0.23	0.29	--	2.13	2.84
Palmitic acid	18.45	14.30	7.50	5.40	9.70
Palmitic 2OH	--	--	--	5.65	1.65
Palmitic 3OH	--	--	--	3.55	3.10
16:1 ω5c	0.18	1.55	1.37	1.77	2.61
Palmitoleic/16:1 ω6c	0.87	0.41	2.25	0.50	2.60
Stearic acid	16.35	14.98	8.19	3.51	4.19
Stearic 3OH	--	--	--	4.08	2.26
iso-stearic	--	--	--	4.33	3.97
18:1 ω5c	0.28	1.43	1.70	--	2.32
cis-vaccenic	43.12	45.06	61.42	1.70	21.36
Linoleic/Stearic	--	0.22	1.23	--	0.86
γ-linoleic acid	9.07	1.99	2.57	6.35	2.07
cyclo-nonadecylic C10	--	--	--	1.32	2.75
iso-nonadecylic	--	--	--	3.12	3.01
19:1 iso I	--	--	--	2.62	2.98
19:1 ω7c/19:1 ω6c	--	--	--	7.11	--
Arachidic acid	1.97	1.79	2.81	2.51	6.59

Table 5-1. Continued.

Fatty Acid	F1	F2	F5	J1	M1
20:1 ω7c	4.57	7.09	5.47	2.92	4.54
Arachidonic acid	--	--	0.33	5.15	4.89
Total Response (mV)	5650.72	11115.59	54,736.99	3776.96	5342.32
n	11	12	12	9	7

†=Not detected

Table 5-2. Mahalanobis distances (D^2) and *P*-values from canonical discriminant analysis comparing FAME profiles of *Meloidogyne graminis* females (F) at densities of 1, 2, or 5 individuals per sample, individual juveniles (J1), and individual males (M1).

		From Count				
To Count		F1	F2	F5	J1	M1
F1	D^2	0	11.69	15.90	608.55	412.56
	P	1	0.3625	0.4326	<0.0001	<0.0001
F2	D^2	11.69	0	12.59	572.35	495.72
	P	0.3625	1	0.6096	<0.0001	<0.0001
F5	D^2	15.90	12.59	0	518.16	425.56
	P	0.4326	0.6096	1	<0.0001	<0.0001
J1	D^2	608.55	572.35	518.16	0	1009.00
	P	<0.0001	<0.0001	<0.0001	1	<0.0001
M1	D^2	412.56	495.72	425.56	1009.00	0
	P	<0.0001	<0.0001	<0.0001	<0.0001	1

Table 5-3. Correlation values between canonical structure and fatty acids selected by stepwise discriminant analysis in the first three canonical variates (CAN1, CAN2, and CAN3) for separating FAME profiles of *Meloidogyne graminis* females (1, 2, and 5 individuals per sample), males (one individual per sample), and juveniles (one individual per sample). Values listed in bold (greater than |0.750|) indicate significant correlation within the given canonical variate.

Fatty Acid	CAN1	CAN2	CAN3
Undecylic acid	0.556	**0.814**	0.166
Lauric 2OH	-0.148	-0.739	-0.020
Lauric 3OH	**0.851**	0.522	0.045
Tridecylic 3OH/15:1 iso	-0.021	**0.995**	-0.020
Myristic acid	**0.976**	0.198	-0.094
Myristic 2OH	-0.304	**0.938**	-0.063
anteiso-pentadecylic	**0.971**	0.119	-0.144
15:1 w5c	0.260	**0.951**	0.034
Palmitic acid	-0.583	-0.526	0.590
Palmitic 2OH	**0.852**	0.519	0.045
Palmitic 3OH	0.515	**0.853**	0.017
Palmitoleic/16:1 w6c	-0.522	0.594	-0.313
Stearic acid	-0.449	**-0.801**	0.346
Stearic 3OH	0.708	0.703	0.033
iso-stearic	0.489	**0.868**	0.015
cis-vaccenic	-0.654	-0.669	-0.247
Linoleic/Stearic	-0.451	0.309	-0.706
γ-linoleic	0.303	-0.183	**0.862**
cyclo-nonadecylic C10	0.032	**0.995**	-0.017
iso-nonadecylic	0.462	**0.883**	0.013
19:1 iso I	0.369	**0.925**	0.006
19:1w7c/19:1 w6c	**0.959**	0.276	0.057
20:1 w7c	-0.579	-0.546	-0.425
Arachidonic acid	0.472	**0.880**	-0.016
Canonical Correlation	0.995	0.992	0.743
Eigenvalue	109.75	59.60	1.233
Cumulative Proportion	0.641	0.988	0.996

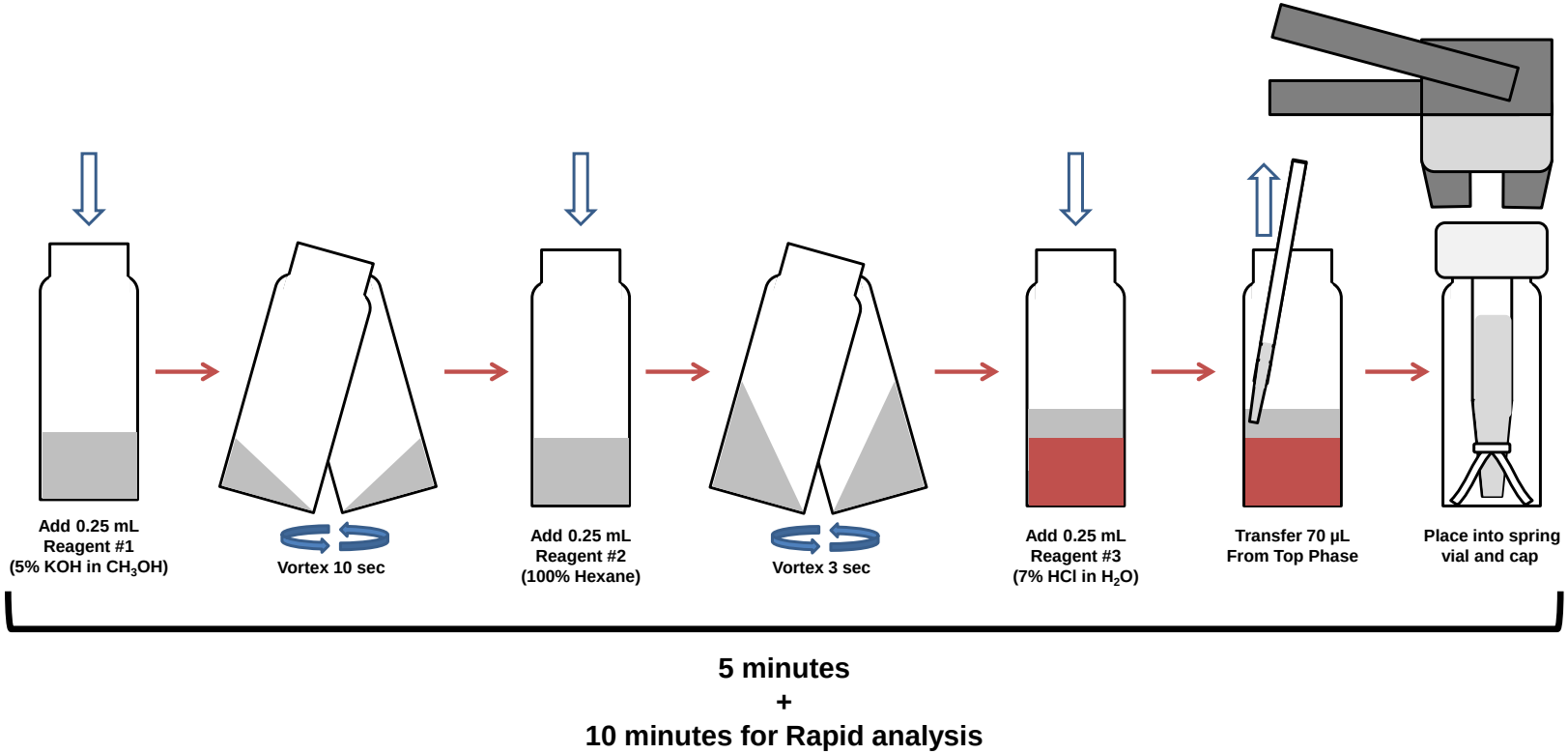


Figure 5-1. Instant FAME extraction and Rapid analysis method developed by MIDI (Newark, DE).

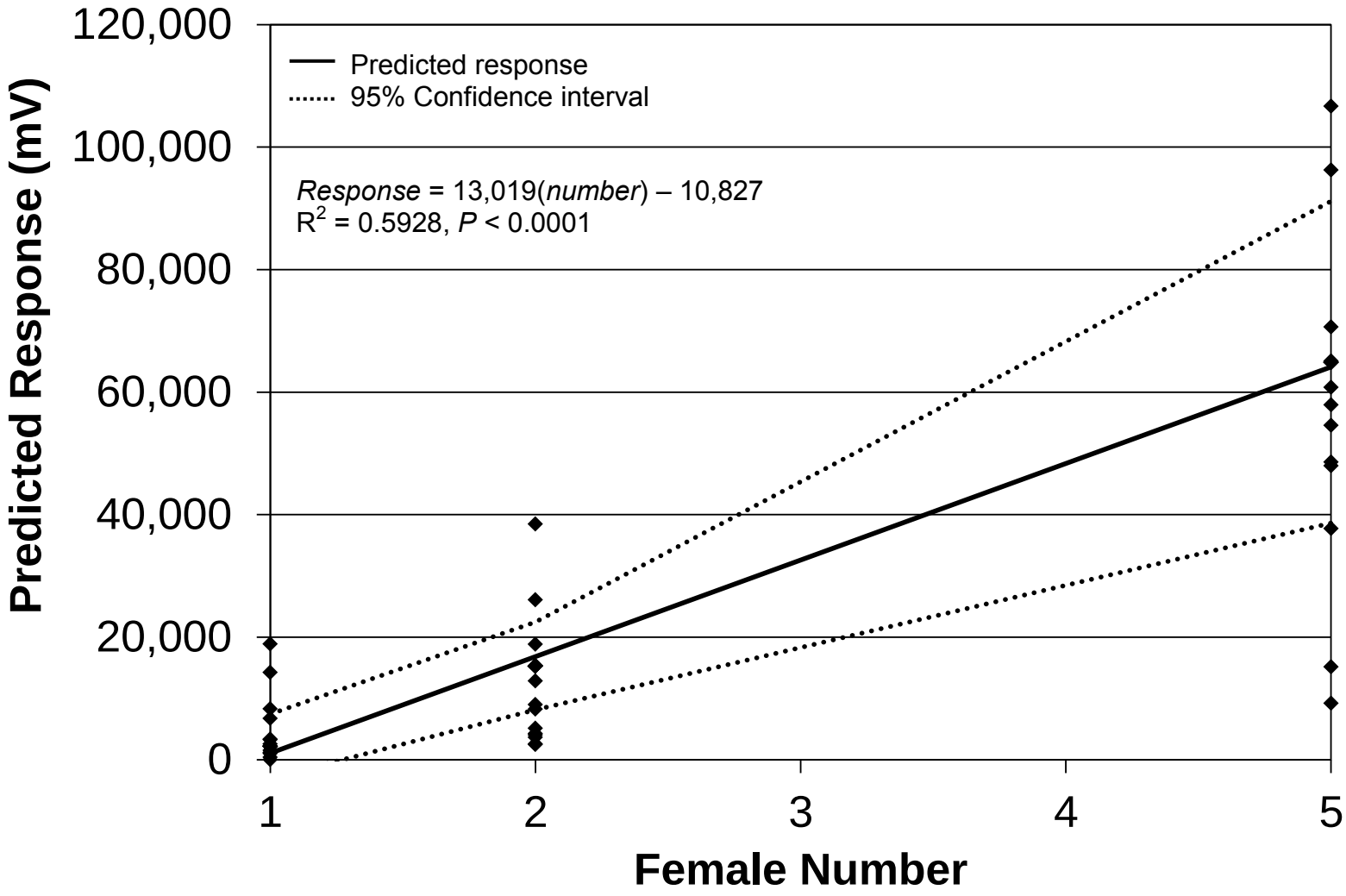


Figure 5-2. Regression of predicted FAME response (mV) against increasing numbers of *Meloidogyne graminis* females per sample.

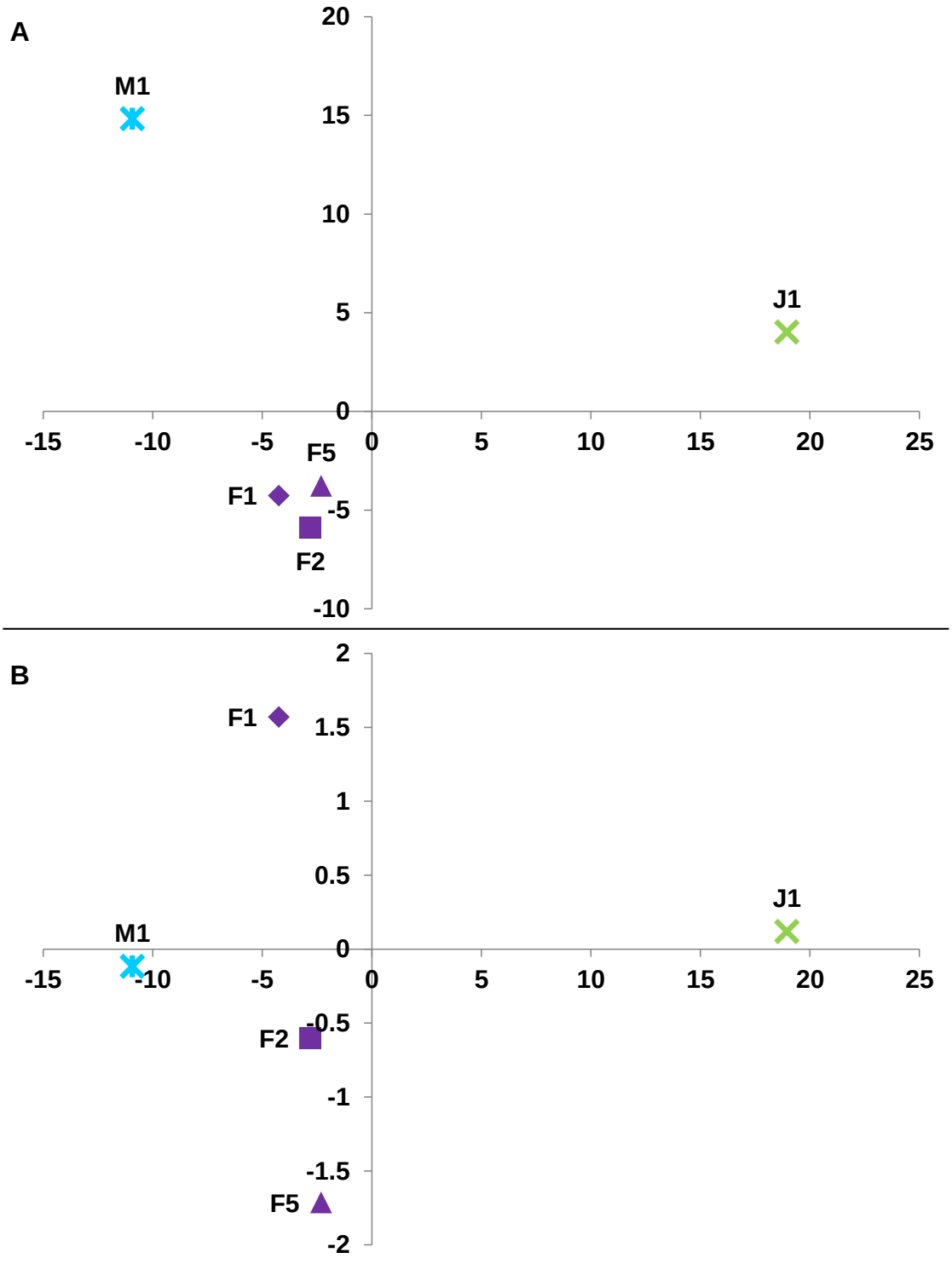


Figure 5-3. Canonical discriminant analysis after stepwise discriminant analysis comparing FAME profiles of *Meloidogyne graminis* females (F) at densities of 1, 2, or 5 individuals per sample, individual males (M1), and individual juveniles (J1). A) CAN1 (*x*-axis) versus CAN2 (*y*-axis) and B) CAN1 (*x*-axis) versus CAN3 (*y*-axis).

APPENDIX A
FATTY ACID PEAK NAMING TABLE FOR THE EUKARY METHOD

Fatty Acid[v]	Nom. ECL[w]	Nom. RT[x]	Cal/Ind[y]	Qnt Cal[z]
Unknown 8.281	8.281	2.059	.	No
Pelargonic acid	9.000	2.401	C	Yes
Caprylic 3OH	9.385	2.584	.	No
Capric primary alcohol	9.468	2.623	.	No
Unknown 9.521	9.521	2.648	.	No
iso-capric	9.605	2.688	.	No
Capric acid	10.000	2.876	C	Yes
Pelargonic 3OH	10.408	3.145	.	No
Unknown 10.531	10.531	3.226	.	No
iso-undecylic	10.605	3.275	.	No
anteiso-undecylic	10.693	3.333	.	No
Lauric aldehyde	10.914	3.478	.	No
Unknown 10.928	10.928	3.488	.	No
Undecylic acid	11.000	3.535	C	Yes
Unknown 11.097	11.097	3.620	.	No
Capric 2OH	11.157	3.672	I	No
Suberic (C8 dicarbox)	11.195	3.706	.	No
Capric 3OH	11.423	3.905	I	No
Lauric primary alcohol	11.490	3.964	.	No
Unknown 11.541	11.541	4.008	.	No
iso-lauric	11.608	4.067	.	No
anteiso-lauric/12:2 ω6*c*	11.689	4.138	.	No
12:2 ω6*c*/*anteiso*-lauric	11.699	4.147	.	No
12:1 ω9*c*	11.772	4.211	.	No
12:1 ω8*c*	11.789	4.225	.	No
12:1 ω7*c*	11.806	4.240	.	No
12:1 ω6*c*	11.850	4.279	.	No
12:1 ω5*c*	11.897	4.320	.	No
12:1 ω3*c*	11.932	4.351	.	No
Unknown 11.981	11.981	4.393	.	No
Lauric acid	12.000	4.410	C	Yes
iso-undecylic 3OH	12.090	4.509	.	No
Unknown 12.112	12.112	4.534	.	No
Undecylic 2OH	12.158	4.584	.	No
Pelargonic dicarbox	12.213	4.645	.	No
Lauric $2CH_3$	12.314	4.757	.	No
Undecylic 3OH	12.441	4.897	.	No
Undecylic DMA	12.468	4.927	.	No
Unknown 12.486	12.487	4.948	.	No
Unknown 12.553	12.553	5.021	.	No
iso-tridecylic	12.612	5.086	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
anteiso-tridecylic	12.701	5.184	.	No
13:1 ω9*c*	12.772	5.262	.	No
13:1 ω8*c*	12.790	5.282	.	No
13:1 ω7*c*	12.809	5.303	.	No
13:1 ω6*c*	12.852	5.351	.	No
13:1 ω5*c*	12.900	5.404	.	No
13:1 ω3*c* or ω12	12.932	5.439	.	No
Tridecylic acid	13.000	5.514	C	Yes
iso-lauric 3OH	13.098	5.643	.	No
Lauric 2OH	13.178	5.748	.	No
Sebacic C10 dicarbox	13.230	5.817	.	No
12:1 3OH	13.289	5.895	.	No
14:1 iso E	13.388	6.025	.	No
Lauric 3OH	13.455	6.113	.	No
Lauric DMA	13.471	6.134	.	No
Myristic N alcohol	13.518	6.196	.	No
Unknown 13.566	13.566	6.259	.	No
iso-myristic	13.618	6.328	.	No
Unknown 13.671	13.671	6.398	.	No
14:2 ω6*c*/14:0 anteiso	13.705	6.442	.	No
14:1 ω11*c*	13.754	6.507	.	No
14:1 ω9*c*	13.773	6.532	.	No
14:1 ω8*c*	13.791	6.556	.	No
14:1 ω7*c*	13.812	6.583	.	No
14:1 ω6*c*	13.854	6.639	.	No
14:1 ω5*c*	13.901	6.701	.	No
14:1 ω3*c*	13.933	6.743	.	No
11:1 2OH	13.946	6.760	.	No
Unknown 13.962	13.962	6.781	.	No
Myristic acid	14.000	6.831	C	Yes
iso-tridecylic 3OH	14.110	6.995	.	No
Tridecylic 2OH	14.191	7.117	.	No
Unknown 14.258	14.258	7.217	.	No
14:1 *cis* 7 DMA	14.291	7.266	.	No
Lauric $2CH_3$	14.316	7.303	.	No
iso 15:1 AT 5	14.387	7.410	.	No
15:1 iso F	14.414	7.450	.	No
15:1 iso G	14.441	7.490	.	No
Tridecylic 3OH	14.470	7.534	.	No
Unknown 14.503	14.503	7.583	.	No
Pentadecylic N alcohol	14.534	7.629	.	No
Myristic 2,4-dimethyl	14.573	7.688	.	No
iso-pentadecylic	14.621	7.759	.	No
anteiso-pentadecylic	14.711	7.894	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
15:1 ω11*c*	14.754	7.958	.	No
15:1 ω9*c*	14.772	7.985	.	No
15:1 ω8*c*	14.792	8.015	.	No
15:1 ω7*c*	14.815	8.049	.	No
Phytanic acid	14.835	8.079	.	No
15:1 ω6*c*	14.856	8.111	.	No
15:1 ω5*c*	14.905	8.184	.	No
15:1 ω3*c*	14.937	8.232	.	No
Lauric aldehyde	14.952	8.254	.	No
Unknown 14.967	14.967	8.277	.	No
Pentadecylic acid	15.000	8.326	C	Yes
iso-myristic 3OH	15.117	8.515	.	No
Unknown 15.176	15.176	8.611	.	No
Myristic 2OH	15.205	8.658	I	No
Unknown 15.273	15.273	8.768	.	No
16:1 *cis* Alcohol ω7	15.386	8.951	.	No
16:1 Alcohol ω7*t*	15.415	8.997	.	No
16:1 *iso* G	15.442	9.041	.	No
16:1 *iso* H	15.460	9.070	.	No
16:1 *iso* I/14:0 3OH	15.482	9.106	.	No
Myristic 3OH/16:1 iso I	15.490	9.119	I	No
Palmitic N alcohol	15.549	9.214	.	No
iso-palmitic	15.626	9.339	.	No
Unknown 15.665	15.665	9.402	.	No
16:2 ω6*c*	15.714	9.481	.	No
16:1 ω11*c*	15.755	9.548	.	No
16:1 ω9*c*	15.773	9.577	.	No
16:1 ω8*c*	15.793	9.609	.	No
16:1 ω7*c*	15.817	9.648	.	No
16:1 ω7*t*	15.835	9.677	.	No
iso-pentadecylic 2OH/16:1ω6*c*	15.851	9.703	.	No
16:1 ω6*c* / 15 *iso* 2OH	15.856	9.711	.	No
16:1 ω5*c*	15.909	9.797	.	No
16:1 ω3*c*	15.939	9.845	.	No
Palmitic acid	16.000	9.944	C	Yes
iso-pentadecylic 3OH	16.135	10.172	.	No
Pentadecylic 2OH	16.217	10.311	.	No
16:1 *cis* 7 DMA (ω 9)	16.240	10.350	.	No
Unknown 16.286	16.286	10.428	.	No
17:1 alcohol (ω 8?)	16.371	10.572	.	No
ISO 17:1 AT 10	16.387	10.599	.	No
ISO 17:1 AT 9	16.415	10.646	.	No
ISO 17:1 G	16.434	10.678	.	No
ISO 17:1 ω5*c*	16.460	10.722	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
17:1 *iso* I/*anteiso* B	16.477	10.751	.	No
17:1 *anteiso* B/i I	16.486	10.766	.	No
Pentadecylic 3OH	16.505	10.798	.	No
anteiso 17:1 AT 9	16.524	10.831	.	No
17:1 *anteiso* A	16.540	10.858	.	No
2,3 dihydroxy pentadecylic	16.553	10.880	.	No
Margaric primary alcohol	16.558	10.888	.	No
Margaric alcohol	16.562	10.895	.	No
iso-margaric	16.629	11.008	.	No
anteiso-margaric	16.722	11.166	.	No
17:1 ω11*c*	16.757	11.225	.	No
17:1 ω9*c*	16.772	11.250	.	No
17:1 ω8*c*	16.793	11.286	.	No
17:1 ω7*c*	16.819	11.330	.	No
17:1 ω6*c*	16.860	11.399	.	No
cyclo-margaric	16.888	11.446	.	No
17:1 ω5*c*	16.914	11.490	.	No
17:1 ω3*c*	16.941	11.536	.	No
Unknown 16.975	16.975	11.594	.	No
Margaric acid	17.000	11.636	C	Yes
16:1 2OH	17.047	11.717	.	No
Unknown 17.154	17.154	11.901	.	No
anteiso-margaric DMA	17.196	11.974	.	No
Palmitic 2OH	17.235	12.041	I	No
Myristic dicarboxilic	17.256	12.077	.	No
Unknown 17.300	17.300	12.153	.	No
Linoleic alcohol	17.322	12.190	.	No
Oleic alcohol	17.361	12.258	.	No
α-linoleic alcohol	17.379	12.289	.	No
18:1 ω12*c* alcohol	17.387	12.302	.	No
Elaidic alcohol	17.410	12.342	.	No
18:1 *iso* G	17.440	12.394	.	No
18:1 *iso* H	17.460	12.428	.	No
Margaric DMA	17.469	12.444	.	No
18:1 (ω ?) alcohol	17.495	12.488	.	No
Palmitic 3OH	17.520	12.531	.	No
γ-linoleic acid	17.574	12.624	.	No
Stearidonic acid	17.640	12.738	.	No
Unknown 17.678	17.678	12.804	.	No
Linoleic acid	17.719	12.874	.	No
18:1 ω11*c*	17.753	12.933	.	No
Oleic acid	17.770	12.962	.	No
γ-linoleic /18:1 ω8*c*	17.786	12.989	.	No
18:1 ω8*c*/ γ-linoleic	17.795	13.005	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
iso-elaidic	17.822	13.051	.	No
Elaidic acid	17.825	13.057	.	No
Unknown 17.838	17.838	13.079	.	No
18:1 ω8*t*	17.840	13.082	.	No
18:1 ω6*c*	17.860	13.117	.	No
18:1 ω5*c*	17.918	13.217	.	No
18:1 ω3*c*	17.943	13.260	.	No
Stearic acid	18.000	13.358	C	Yes
19:1 (ω 11?) alcohol	18.124	13.571	.	No
iso-margaric 3OH	18.164	13.640	.	No
Unknown 18.197	18.197	13.697	.	No
Unknown 18.218	18.218	13.733	.	No
18:1 ω9*c* DMA	18.224	13.744	.	No
cis-vaccenic DMA	18.285	13.848	.	No
Unknown 18.316	18.316	13.902	.	No
19:1 (ω 8?) alcohol	18.390	14.029	.	No
Unknown 18.473	18.473	14.172	.	No
Nonadecylic N alcohol	18.592	14.377	.	No
iso-nonadecylic	18.633	14.447	.	No
19:2 ω6*c*.	18.720	14.597	.	No
19:1 ω11*c*	18.754	14.656	.	No
19:1 ω9*c*	18.771	14.685	.	No
19:1 ω8*c*	18.796	14.728	.	No
19:1 ω7*c*/19:1 ω9*t*	18.823	14.774	.	No
19:1 ω9*t*/19:1 ω7*c*	18.828	14.783	.	No
19:1 ω8t	18.845	14.812	.	No
19:1 ω6*c* /*cyclo*-nonadecylic	18.862	14.842	.	No
cyclo-nonadecylic C10 /19:1	18.868	14.852	.	No
cyclo-nonadecylic C11	18.901	14.909	.	No
19:1 ω5*c*	18.923	14.946	.	No
19:1 ω3*c*	18.945	14.984	.	No
Nonadecylic acid	19.000	15.079	C	Yes
Unknown 19.055	19.055	15.174	.	No
18:1 2OH	19.088	15.231	.	No
Unknown 19.225	19.225	15.468	.	No
Stearic 2OH	19.264	15.535	.	No
Unknown B	19.276	15.556	.	No
cyclo-nonadecylic C9 DMA	19.322	15.636	.	No
Arachidonic acid	19.392	15.757	.	No
Eicosapentaenoic acid	19.453	15.862	.	No
Unknown 19.470	19.470	15.892	.	No
Unknown 19.521	19.521	15.980	.	No
Dihomo-γ-linoleic acid	19.556	16.040	.	No
Arachidic N alcohol	19.600	16.116	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
iso-arachidic	19.635	16.177	.	No
Docosadienoic acid	19.660	16.220	.	No
Oleic 12OH	19.681	16.256	.	No
cis-stearic 9,10 epoxy	19.703	16.294	.	No
Eicosadienoic acid	19.726	16.334	.	No
20:1 ω12*c*	19.744	16.365	.	No
20:1 ω11*c*	19.754	16.383	.	No
Eicosenoic acid	19.771	16.412	.	No
20:1 ω8*c*/eicosatrienoic	19.796	16.455	.	No
eicosatrienoic /20:1 ω8*c*	19.807	16.474	.	No
20:1 ω7*c*/20:1 ω9t	19.825	16.505	.	No
20:1 ω9*t*/20:1 ω7*c*	19.833	16.519	.	No
20:1 ω6*c*	19.867	16.578	.	No
20:1 ω5*c*	19.923	16.675	.	No
20:1 ω3*c*	19.948	16.718	.	No
Stearic 12OH	19.975	16.765	.	No
Arachidic acid	20.000	16.808	C	Yes
Unknown 20.084	20.084	16.950	.	No
cyclo-nonadecylic C11 2OH	20.189	17.126	.	No
13-eicosynoic	20.209	17.160	.	No
Unknown 20.241	20.241	17.214	.	No
Unknown 20.257	20.257	17.241	.	No
Nonadecylic 2OH	20.279	17.278	.	No
Unknown 20.343	20.343	17.386	.	No
Nonadecylic 3OH	20.566	17.762	.	No
Unknown 20.588	20.588	17.799	.	No
Heneicosylic primary alcohol	20.613	17.841	.	No
iso-heneicosylic	20.637	17.881	.	No
anteiso-heneicosylic	20.738	18.052	.	No
21:1 ω11*c*	20.755	18.080	.	No
21:1 ω9*c*	20.772	18.109	.	No
21:1 ω7*c*	20.828	18.203	.	No
21:1 ω6*c*	20.866	18.267	.	No
21:1 ω5*c*	20.928	18.372	.	No
21:1 ω3*c*	20.949	18.407	.	No
Heneicosylic acid	21.000	18.493	C	Yes
Unknown 21.111	21.111	18.676	.	No
Unknown 21.252	21.252	18.908	.	No
Stearic N alcohol	21.283	18.959	.	No
Cervonic acid	21.334	19.042	.	No
22:1 (ω 11?) alcohol	21.370	19.102	.	No
Adrenic acid	21.384	19.125	.	No
22:1 ω9*c* alcohol	21.400	19.151	.	No
Clupianodonic acid	21.466	19.260	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
Arachidic 3OH	21.578	19.444	.	No
Behenic primary alcohol	21.627	19.524	.	No
Docosadienoic acid	21.744	19.717	.	No
22:1 ω11*c*	21.751	19.728	.	No
Erucic acid	21.774	19.766	.	No
Unknown 21.808	21.808	19.822	.	No
22:1 ω7*c*/22:3 ω3*c*	21.833	19.863	.	No
22:1 ω6*c*	21.871	19.926	.	No
22:1 ω5*c*	21.931	20.024	.	No
22:1 ω3*c*	21.951	20.057	.	No
Behenic acid	22.000	20.138	C	Yes
Unknown 22.138	22.138	20.358	.	No
Unknown 22.267	22.267	20.563	.	No
Heneicosylic 2OH	22.308	20.628	.	No
Unknown 22.374	22.374	20.733	.	No
Heneicosylic 3OH	22.597	21.088	.	No
Tricosylic primary alcohol	22.643	21.161	.	No
Unknown 22.682	22.682	21.223	.	No
23:1 ω9*c*	22.787	21.390	.	No
23:1 ω7*c*	22.836	21.468	.	No
Tricosylic acid	23.000	21.729	C	Yes
Unknown 23.166	23.166	21.985	.	No
Unknown 23.283	23.283	22.166	.	No
Behenic 2OH	23.325	22.231	.	No
Unknown 23.390	23.390	22.332	.	No
Nervonic alcohol	23.434	22.400	.	No
Tetracosapentaenoic acid	23.467	22.451	.	No
Behenic 3OH	23.608	22.668	.	No
Unknown 23.670	23.670	22.764	.	No
24:2 ω6*c*	23.752	22.891	.	No
Nervonic acid	23.787	22.945	.	No
24:1 ω6*c*	23.872	23.076	.	No
24:1 ω3*c*	23.954	23.203	.	No
Lignoceric acid	24.000	23.274	C	Yes
Unknown 24.098	24.098	23.421	.	No
Unknown 24.196	24.196	23.568	.	No
Tricosylic 2OH	24.337	23.779	.	No
Unknown 24.407	24.407	23.884	.	No
Tricosylic 3OH	24.625	24.211	.	No
Pentacosylic N alcohol	24.669	24.277	.	No
Pentacosylic acid	25.000	24.773	C	Yes
Unknown 25.052	25.052	24.856	.	No
Coprostane	25.138	24.993	.	No
Unknown 25.339	25.338	25.313	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
Lignoceric 2OH	25.355	25.340	.	No
5-Cholestane	25.511	25.589	.	No
Unknown 25.545	25.545	25.644	.	No
Lignoceric 3OH	25.640	25.796	.	No
Cholesteryl-palmitate	25.938	26.272	.	No
Unknown 26.295	26.295	26.842	.	No
Unknown 26.335	26.335	26.906	.	No
Pentacosylic 2OH	26.366	26.955	.	No
Pentacosylic 3OH	26.654	27.416	.	No
Cerotic 3OH	27.668	29.036	.	No
Montanic acid	28.000	29.566	C	Yes
Cholesterol	28.210	30.045	.	No
Cholestanol	28.295	30.239	.	No
Campesterol	29.250	32.420	.	No
Stigmasterol	29.577	33.166	.	No
Melissic acid	30.000	34.132	C	Yes
β-sitosterol	30.236	34.671	.	No
Fucosterol	30.262	34.730	.	No

[v] Structural notations: (ω) functional group located at carbon numbered from terminal carbon, (*c* or *cis*) all double bonds in *cis* or *Z* configuration, (*t* or *trans*) all double bonds in *trans* or *E* configuration, (OH) hydroxyl group at indicated carbon, (*iso*-) fatty acid is in *iso* configuration, (*anteiso*-) fatty acid is in *anteiso* configuration, (*cyclo*-) fatty acid has a cyclic –O– bond at indicated carbon, (DMA) fatty acid structure contains dimethyl acetal tail, (-*methyl*) fatty acid has a methyl group at the indicated carbon, (aldehyde) fatty acid structured with an aldehyde tail, (alcohol) fatty acid tail has CH_2OH tail instead of CH_3, (Unknown …) fatty acid peak structure has yet to be determined as is listed by retention time.

[w] Nominal estimated chain length (ECL) of given fatty acid.

[x] Nominal retention time (RT) of given fatty acid after sample injection.

[y] Fatty acid is used for calibration mix (C) or indicator (I) of calibration shift

[z] Amount of fatty acid can be quantified (Qnt) during calibration (Cal).

APPENDIX B
FATTY ACID PEAK NAMING TABLE FOR THE RTSBA6 METHOD

Fatty Acid[v]	Nom. ECL[w]	Nom. RT[x]	Cal/Ind[y]	Qnt Cal[z]
Pelargonic acid	9.0000	1.0670	C	Yes
Caprylic 3OH	9.4120	1.1285	.	No
Unknown 9.560	9.5600	1.1506	.	No
iso-capric	9.6160	1.1589	.	No
Capric acid	10.0000	1.2162	C	Yes
Pelargonic 3OH	10.4310	1.2980	.	No
iso-undecylic	10.6180	1.3335	.	No
anteiso-undecylic	10.7050	1.3500	.	No
Lauric aldehyde	10.9300	1.3927	.	No
Unknown 10.9525	10.9525	1.3970	.	No
Undecylic acid	11.0000	1.4060	C	Yes
Capric 2OH	11.1774	1.4470	I	No
Capric 3OH	11.4480	1.5095	I	No
Unknown 11.543	11.5430	1.5314	.	No
iso-lauric	11.6210	1.5495	.	No
anteiso-lauric	11.7100	1.5700	.	No
Unknown 11.825	11.8250	1.5966	.	No
12:1 at 11-12	11.9250	1.6197	.	No
Lauric acid	12.0000	1.6370	C	Yes
iso-undecylic 3OH	12.1080	1.6657	.	No
Undecylic 2OH	12.1910	1.6878	.	No
Undecylic 3OH	12.4650	1.7607	.	No
Unknown 12.502	12.5020	1.7705	.	No
iso-tridecylic	12.6230	1.8027	.	No
anteiso-tridecylic	12.7140	1.8269	.	No
13:1 at 12-13	12.9580	1.8918	.	No
Tridecylic acid	13.0000	1.9030	C	Yes
iso-lauric 3OH	13.1200	1.9382	.	No
Lauric 2OH	13.2040	1.9628	.	No
12:1 3OH	13.3250	1.9982	.	No
14:1 *iso* E	13.3960	2.0190	.	No
Lauric 3OH	13.4830	2.0445	.	No
Unknown 13.591	13.5910	2.0762	.	No
iso-myristic	13.6280	2.0870	.	No
anteiso-myristic	13.7180	2.1134	.	No
14:1 ω5*c*	13.9160	2.1714	.	No
Unknown 13.951	13.9510	2.1816	.	No
Myristic acid	14.0000	2.1960	C	Yes
iso-tridecylic 3OH	14.1320	2.2367	.	No
Tridecylic 2OH	14.2240	2.2651	.	No
Unknown 14.263	14.2630	2.2772	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
15:1 *iso* ω9*c*	14.4130	2.3235	.	No
15:1 *iso* F	14.4420	2.3324	.	No
15:1 *iso* G	14.4600	2.3380	.	No
15:1 *iso* H/13:0 3OH	14.4760	2.3429	.	No
13:0 3OH/15:1 *iso* H	14.5030	2.3513	.	No
Unknown 14.502	14.5160	2.3553	.	No
15:1 *anteiso* A	14.5510	2.3661	.	No
iso-pentadecylic	14.6320	2.3911	.	No
anteiso-pentadecylic	14.7250	2.4198	.	No
15:1 ω8*c*	14.8140	2.4473	.	No
15:1 ω6*c*	14.8750	2.4661	.	No
15:1 ω5*c*	14.9260	2.4819	.	No
Unknown 14.969	14.9690	2.4951	.	No
Pentadecylic acid	15.0000	2.5047	C	No
iso-myristic 3OH	15.1470	2.5515	.	No
Myristic 2OH	15.2332	2.5789	I	No
Palmitoleic alcohol	15.4140	2.6365	.	No
16:1 *iso* G	15.4550	2.6495	.	No
16:1 *iso* H	15.4820	2.6581	.	No
16:1 *iso* I/14:0 3OH	15.5011	2.6642	.	No
14:0 3OH/16:1 *iso* I	15.5153	2.6687	I	No
Palmitic N alcohol	15.5740	2.6874	.	No
iso-palmitic	15.6330	2.7062	.	No
Unknown 15.669	15.6690	2.7176	.	No
anteiso-palmitic	15.7270	2.7361	.	No
16:1 ω11*c*	15.7820	2.7536	.	No
16:1 ω9*c*	15.8000	2.7593	.	No
16:1 ω7*c*/16:1 ω6*c*	15.8400	2.7721	.	No
16:1 ω6*c*/16:1 ω7*c*	15.8750	2.7832	.	No
16:1 ω5*c*	15.9280	2.8001	.	No
Palmitic acid	16.0000	2.8230	C	Yes
iso-pentadecylic 3OH	16.1620	2.8748	.	No
Pentadecylic 2OH	16.2550	2.9046	.	No
17:1 *iso* ω10*c*	16.4140	2.9555	.	No
Palmitic 10-*methyl*	16.4350	2.9622	.	No
17:1 *iso* ω9*c*	16.4470	2.9660	.	No
17:1 *iso* ω5*c*	16.4830	2.9776	.	No
17:1 *iso* I/*anteiso* B	16.4980	2.9824	.	No
17:1 *anteiso* B/*iso* I	16.5120	2.9868	.	No
Pentadecylic 3OH	16.5330	2.9936	.	No
17:1 *anteiso* ω9*c*	16.5520	2.9996	.	No
17:1 *anteiso* A	16.5710	3.0057	.	No
Unknown 16.586	16.5860	3.0105	.	No
iso-margaric	16.6370	3.0268	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
anteiso-margaric	16.7330	3.0576	.	No
17:1 ω9*c*	16.7830	3.0736	.	No
17:1 ω8*c*	16.8150	3.0838	.	No
17:1 ω7*c*	16.8360	3.0905	.	No
17:1 ω6*c*	16.8810	3.1049	.	No
17:0 *cyclo*	16.9150	3.1158	.	No
17:1 ω5*c*	16.9300	3.1206	.	No
Margaric acid	17.0000	3.1430	C	Yes
16:1 2OH	17.0980	3.1740	.	No
iso-palmitic 3OH	17.1740	3.1980	.	No
anteiso-margaric	16.7330	3.0576	.	No
17:1 ω9*c*	16.7830	3.0736	.	No
17:1 ω8*c*	16.8150	3.0838	.	No
17:1 ω7*c*	16.8360	3.0905	.	No
17:1 ω6*c*	16.8810	3.1049	.	No
cyclo-margaric	16.9150	3.1158	.	No
17:1 ω5*c*	16.9300	3.1206	.	No
Palmitic 2OH	17.2655	3.2269	I	No
Margaric 10-*methyl*	17.4150	3.2741	.	No
18:1 *iso* H	17.4900	3.2978	.	No
Palmitic 3OH	17.5480	3.3162	.	No
γ-linolenic acid	17.6000	3.3326	.	No
iso-stearic	17.6360	3.3440	.	No
anteiso-stearic/linoleic	17.7310	3.3740	.	No
Linoleic/*anteiso*-stearic	17.7560	3.3819	.	No
Oleic acid	17.7940	3.3939	.	No
cis-vaccenic acid	17.8475	3.4108	.	No
18:1 ω6*c*	17.9020	3.4280	.	No
18:1 ω5*c*	17.9370	3.4391	.	No
Stearic acid	18.0000	3.4590	C	Yes
cis-vaccenic 11-*methyl*	18.0860	3.4857	.	No
iso-margaric 3OH	18.1930	3.5190	.	No
Margaric 2OH	18.2880	3.5486	.	No
Stearic 10-methyl	18.3950	3.5818	.	No
19:1 *iso* I	18.4980	3.6139	.	No
Margaric 3OH	18.5650	3.6347	.	No
iso-nonadecylic	18.6380	3.6574	.	No
anteiso-nonadecylic	18.7380	3.6885	.	No
19:1 ω11*c*/19:1 ω9*c*	18.7750	3.7000	.	No
19:1 ω9*c*/19:1 ω11*c*	18.7920	3.7053	.	No
Unknown 18.815	18.8150	3.7125	.	No
19:1 ω7*c*/19:1 ω6*c*	18.8370	3.7193	.	No
19:1 ω6*c*/ω7*c*/19*c*y	18.8570	3.7255	.	No
cyclo-nonadecylic C10	18.8870	3.7349	.	No

Fatty Acid	Nom. ECL	Nom. RT	Cal/Ind	Qnt Cal
cyclo-nonadecylic C8	18.9320	3.7489	.	No
Nonadecylic acid	19.0000	3.7700	C	Yes
18:1 2OH	19.1460	3.8142	.	No
Stearic 2OH	19.2980	3.8603	.	No
Nonadecylic 10-*methyl*	19.3720	3.8827	.	No
Arachidonic acid	19.4660	3.9112	.	No
Stearic 3OH	19.5800	3.9457	.	No
iso-arachidic	19.6360	3.9627	.	No
Eicosadienoic acid	19.7390	3.9939	.	No
Eicosenoic acid	19.7910	4.0097	.	No
20:1 ω7*c*	19.8500	4.0276	.	No
Arachidic acid	20.0000	4.0730	C	Yes

[v] Structural notations: (ω) functional group located at carbon numbered from terminal carbon, (*c* or *cis*) all double bonds in *cis* or *Z* configuration, (*t* or *trans*) all double bonds in *trans* or *E* configuration, (OH) hydroxyl group at indicated carbon, (*iso*-) fatty acid is in *iso* configuration, (*anteiso*-) fatty acid is in *anteiso* configuration, (*cyclo*-) fatty acid has a cyclic –O– bond at indicated carbon, (DMA) fatty acid structure contains dimethyl acetal tail, (-*methyl*) fatty acid has a methyl group at the indicated carbon, (aldehyde) fatty acid structured with an aldehyde tail, (alcohol) fatty acid tail has CH_2OH tail instead of CH_3, (Unknown …) fatty acid peak structure has yet to be determined as is listed by retention time.

[w] Nominal estimated chain length (ECL) of given fatty acid.

[x] Nominal retention time (RT) of given fatty acid after sample injection.

[y] Fatty acid is used for calibration mix (C) or indicator (I) of calibration shift

[z] Amount of fatty acid can be quantified (Qnt) during calibration (Cal).

APPENDIX C
COMMON NAMES FOR SATURATED FATTY ACIDS

Common Name	Chain Length	Structural Formula
Pelargonic acid	9:0	$CH_3(CH_2)_7COOH$
Capric acid	10:0	$CH_3(CH_2)_8COOH$
Undecylic acid	11:0	$CH_3(CH_2)9COOH$
Lauric acid	12:0	$CH_3(CH_2)_{10}COOH$
Tridecylic acid	13:0	$CH_3(CH_2)_{11}COOH$
Myristic acid	14:0	$CH_3(CH_2)_{12}COOH$
Pentadecylic acid	15:0	$CH_3(CH_2)_{13}COOH$
Palmitic acid	16:0	$CH_3(CH_2)_{14}COOH$
Margaric acid	17:0	$CH_3(CH_2)_{15}COOH$
Stearic acid	18:0	$CH_3(CH_2)_{16}COOH$
Nonadecylic acid	19:0	$CH_3(CH_2)_{17}COOH$
Arachidic acid	20:0	$CH_3(CH_2)_{18}COOH$
Heneicosylic acid	21:0	$CH_3(CH_2)_{19}COOH$
Behenic acid	22:0	$CH_3(CH_2)_{20}COOH$
Tricosylic acid	23:0	$CH_3(CH_2)_{21}COOH$
Lignoceric acid	24:0	$CH_3(CH_2)_{22}COOH$
Pentacosylic acid	25:0	$CH_3(CH_2)_{23}COOH$
Cerotic acid	26:0	$CH_3(CH_2)_{24}COOH$
Heptacosylic acid	27:0	$CH_3(CH_2)_{25}COOH$
Montanic acid	28:0	$CH_3(CH_2)_{26}COOH$
Nonacosylic acid	29:0	$CH_3(CH_2)_{27}COOH$
Melissic acid	30:0	$CH_3(CH_2)_{28}COOH$
Henatriacontylic acid	31:0	$CH_3(CH_2)_{29}COOH$
Lacceroic acid	32:0	$CH_3(CH_2)_{30}COOH$
Psyllic acid	33:0	$CH_3(CH_2)_{31}COOH$
Geddic acid	34:0	$CH_3(CH_2)_{32}COOH$
Ceroplastic acid	35:0	$CH_3(CH_2)_{33}COOH$
Hexatriacontylic acid	36:0	$CH_3(CH_2)_{34}COOH$

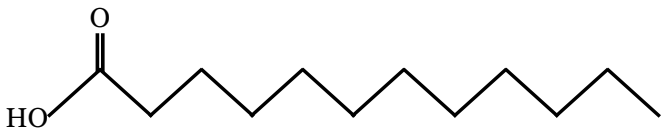
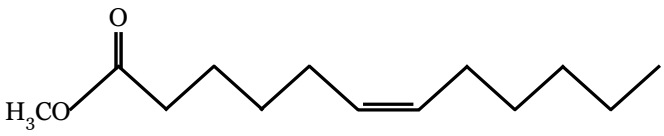
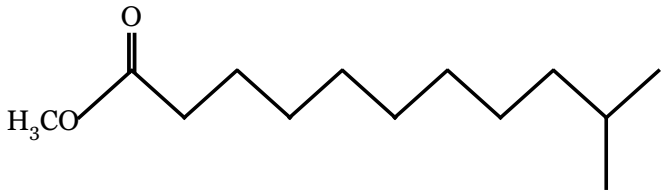
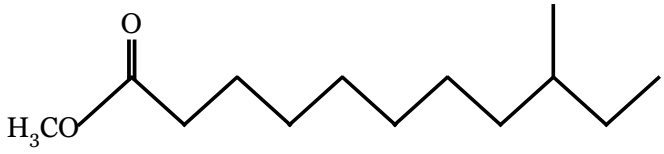
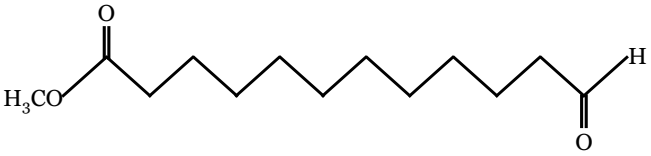
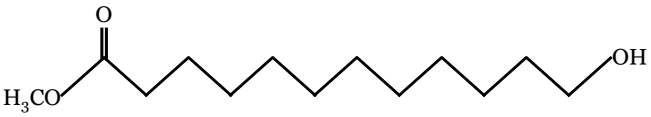
APPENDIX D
COMMON NAMES FOR UNSATURATED FATTY ACIDS

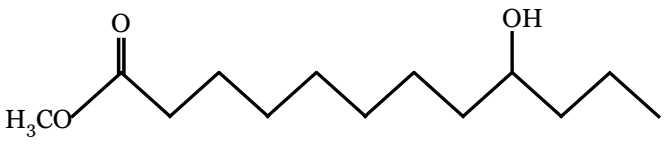
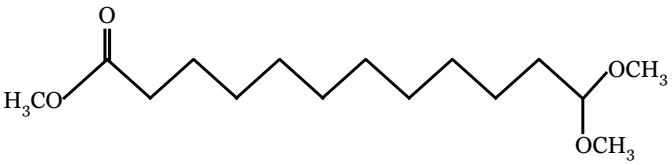
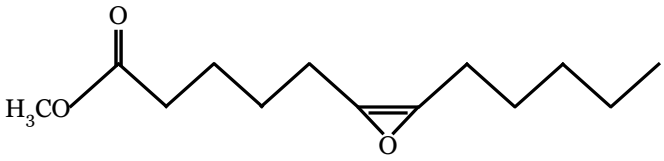
Common Name	Structure[†]
Palmitoleic acid	16:1 ω7*c*
Hexadecatrienoic acid	16:3 ω5,7,9*c*
Vaccenic acid	18:1 ω7*t*
cis-vaccenic acid	18:1 ω7*c*
Oleic acid	18:1 ω9*c*
Elaidic acid	18:1 ω9*t*
Linoleic acid	18:2 ω6,9*c*
Rumenic acid	18:2 ω7*Z*,9*E*
α-linolenic acid	18:3 ω3,6,9*c*
Rumelenic acid	18:3 ω3*E*,7*Z*,9*E*
α-Eleostearic acid	18:3 ω5*E*,7*E*,9*Z*
β-Eleostearic acid	18:3 ω5*E*,7*E*,9*E*
Catalpic acid	18:3 ω5*E*,7*Z*,9*Z*
Punicic acid	18:3 ω5*Z*,7*E*,9*Z*
γ-linolenic acid	18:3 ω6,9,12*c*
α-Calendic acid	18:3 ω6*Z*,8*E*,10*E*
β-Calendic acid	18:3 ω6*E*,8*E*,10*E*
Jacaric acid	18:3 ω6*Z*,8*E*,10*Z*
Pinolenic acid	18:3 ω6*c*,9,13*c*
Stearidonic acid	18:4 ω3,6,9,12*c*
α-Parinaric acid	18:4 ω3*E*,5*Z*,7*Z*,9*E*
β-Parinaric acid	18:4 ω3,5,7,9*t*
Eicosenoic acid	20:1 ω9*c*
Eicosadienoic acid	20:2 ω6,9*c*
Eicosatrienoic acid	20:3 ω3,6,9*c*
Dihomo-γ-linolenic acid	20:3 ω6,9,12*c*
Podocarpic acid	20:3 ω6*c*,9*c*,15*c*
Mead acid	20:3 ω9,12,15*c*
Eicosatetraenoic acid	20:4 ω3,6,9,12*c*
Arachidonic acid	20:4 ω6,9,12,15*c*
Eicosapentaenoic acid (Timnodonic acid)	20:5 ω3,6,9,12,15*c*
Bosseopentaenoic acid	20:5 ω6*Z*,8*E*,10*E*,12*Z*,15*Z*
Heneicosapentaenoic acid	21:5 ω3,6,9,12,15*c*
Erucic acid	22:1 ω9*c*
Docosadienoic acid	22:2 ω6,9*c*
Adrenic acid	22:4 ω6,9,12,15*c*
Docosapentaenoic acid (Clupanodonic acid)	22:5 ω3,6,9,12,15*c*
Docosapentaenoic acid (Osbond acid)	22:5 ω6,9,12,15,18*c*
Docosahexaenoic acid (Cervonic acid)	22:6 ω3,6,9,12,15,18*c*
Nervonic acid	24:1 ω9*c*
Tetracosatetraenoic acid	24:4 ω6,9,12,15*c*
Tetracosapentaenoic acid	24:5 ω3,6,9,12,15*c*

Tetracosapentaenoic acid	24:5 ω6,9,12,15,18*c*
Tetracosahexaenoic acid (Nisinic acid)	24:6 ω3,6,9,12,15,18*c*

†Structural notations: (*c*) all double bonds in *cis* or *Z* configuration, (*t*) all double bonds in *trans* or *E* configuration, (*Z*) preceding double bond location is in *cis* configuration, (*E*) preceding double bond is in *trans* configuration.

APPENDIX E
COMMON FATTY ACID STRUCTURES

Fatty acid	Structure
Saturated fatty acid	
Saturated fatty acid methyl ester (FAME)	
trans-unsaturated FAME	
cis-unsaturated FAME	
iso-FAME	
anteiso- FAME	
FAME aldehyde	
FAME alcohol	

FAME hydroxyl (OH)	
FAME dimethylacetal (DMA)	
cyclo-FAME	

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BIOGRAPHICAL SKETCH

Nicholas Sekora grew up trying to escape from hot, humid weather, but to date has had no luck. He completed his B.S. in environmental biology with a chemistry minor and concentration in botany at the University of North Alabama in 2007. While attending UNA, Nick was active in Beta Beta Beta as both a chapter officer and a regional officer. Nick went on to complete his M.S. in plant pathology at Auburn University. During his time at Auburn, Nick presented more than ten papers and posters for his work to evaluate identification of plant-parasitic nematodes using FAME analysis. Nick was invited to present his thesis work to the Entomology and Nematology Department at the University of Florida in January of 2009, which allowed him to secure a position in that department to begin his doctoral degree later that year with Dr. Billy Crow. In May 2009, Nick assisted Dr. David Weaver of Auburn University in teaching a crop breeding class at Northwest Agricultural and Forestry University in Yangling, Shaanxi, China. His responsibilities included helping Dr. Weaver communicate with the Chinese students, aiding Chinese and American students with homework assignments, as well as instructing Dr. Weaver how to survive using only chopsticks to eat. Currently, Nick is working with clarifying the taxonomic nightmare of *Meloidogyne* and hopes to develop a quick and accurate field identification system for growers. In his free time, Nick works with Dr. Tesfa Mekete conducting taxonomic studies. He also practices applied mechanical engineering of automobiles and dabbles in atomic and light physics.