Direct determination of 1-aminocyclopropane-1-carboxylic acid in plant tissues by using a gas chromatograph with flame-ionization detection

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Direct determination of 1-aminocyclopropane-1-carboxylic acid in plant tissues by using a
gas chromatograph with flame-ionization detection

by

Katey H. Warnberg

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Horticulture

Program of Study Committee:
Richard J. Gladon, Major Professor
William R. Graves
A. Susana Goggi

Iowa State University

Ames, Iowa

2011
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ABSTRACT

1-Aminocyclopropane-1-carboxylic acid is the immediate precursor of ethylene, a phytohormone that is produced in many plant tissues, and is effective in trace amounts (< 1 µl/l).

Quantitative determination of ACC in biological tissues is paramount to understanding the regulation of this metabolic process. The most widely accepted method for quantifying 1-aminocyclopropane-1-carboxylic acid requires its oxidation to ethylene, which then is measured by gas chromatography. Our objective was to develop a method for the rapid, direct quantification of 1-aminocyclopropane-1-carboxylic acid by using a gas chromatograph with a flame-ionization detector. Ethylene production was measured in tissues from fruits, leaves, seeds, florets, and flower petals. The remaining tissue samples were ground to a fine powder in liquid nitrogen, and 1-aminocyclopropane-1-carboxylic acid was extracted from the powder with a methanol:chloroform:water mixture (5:12:3 v/v/v). 1- Aminocyclopropane-1-carboxylic acid was quantified by using an EZ:faast™ gas chromatography mass-spectrometry free amino acid analysis kit, was identified by using gas chromatography mass-spectrometry, and was confirmed with authentic 1-aminocyclopropane-1-carboxylic acid. Benefits of this method include a short run time of less than six minutes and the absence of a potentially hazardous tracer, such as $^{14}$C. We show that this method is effective for accurate, direct measurement of 1-aminocyclopropane-1-carboxylic acid from a variety of both reproductive and vegetative plant tissues.
CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

One manuscript is presented in this thesis. Chapter 2 contains a manuscript titled "Direct determination of 1-aminocyclopropane-1-carboxylic acid in plant tissues by using a gas chromatograph with flame-ionization detection." This manuscript is intended for submission to Analytical Biochemistry and has been formatted for that journal. Overall conclusions emerging from this manuscript are presented in chapter 3. An appendix, which follows the conclusions, presents observations and data that were collected but not incorporated into a manuscript.

Introduction

Ethylene (C\textsubscript{2}H\textsubscript{4}) is a phytohormone that is produced in many plant tissues, and it is effective in minute amounts (< 1 µl/l). C\textsubscript{2}H\textsubscript{4} is involved in many metabolic processes, including fruit ripening; leaf, flower, and fruit senescence; leaf and flower abscission; regulation of stress responses; and seed germination [1]. Methionine, the earliest precursor of C\textsubscript{2}H\textsubscript{4}, is converted enzymatically to S-adenosyl methionine [3], which then is converted enzymatically to 1-aminocyclopropane-1-carboxylic acid (ACC). The formation of ACC by ACC synthase is the rate-controlling step in the C\textsubscript{2}H\textsubscript{4} biosynthesis pathway [17]. ACC is converted rapidly to C\textsubscript{2}H\textsubscript{4} in the presence of molecular oxygen. Because ACC is an important intermediate in C\textsubscript{2}H\textsubscript{4} biosynthesis [2], quantification of ACC has been a central component of studies in C\textsubscript{2}H\textsubscript{4} physiology. In addition, a recent study suggests that ACC
may have a role in plants that is independent of ethylene, which makes the quantification of ACC more important [16].

The most widely accepted assay for ACC requires oxidation of ACC to \( \text{C}_2\text{H}_4 \), which is measured by gas chromatography [11]. This indirect method has an average conversion rate of about 80%, but conversion ranges from 60% to \( \geq 100\% \) [9]. The Lizada and Yang method requires equipment to measure \( \text{C}_2\text{H}_4 \) and has several drawbacks, including reliance on indirect measurement of ACC [5]. The conversion of ACC to \( \text{C}_2\text{H}_4 \) can be variable and is vulnerable to interference from other \( \text{C}_2\text{H}_4 \)-generating or -absorbing compounds such as ethanol and perchloric acid [4,12]. A direct, rapid, and accurate method for ACC quantification would be helpful to biologists working with \( \text{C}_2\text{H}_4 \).

Several methods have been developed to overcome these difficulties, including gas chromatography with nitrogen/phosphorus detection [7] and capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) [10]. Methods using high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), such as HPLC with UV detection [5,9], HPLC with MS [6], HPLC-MS-MS [14], HPLC with fluorescence detection [13], and GC-MS [12,15], also have been developed. Although sensitive and highly specific, methods that require HPLC and GC-MS require expensive equipment not available in many laboratories. Sample run times can exceed 8 min for HPLC-UV [9], HPLC with fluorescence detection [13], GC with a nitrogen/phosphorus detector [7], and CE-LIF [10]. In addition, many methods involve \(^{14}\text{C} \) as a radioactive tracer [7,8,11], which can be hazardous.
Our overall objective was to develop a method for quantifying ACC by using gas chromatography with flame-ionization detection (GC-FID). A FID is one of the more common and inexpensive GC detectors, and we know of no reports of an assay for direct measurement of ACC by GC-FID. Our specific objectives were to develop a method for direct measurement of ACC that does not rely on a radioactive tracer, an internal standard, nor expensive laboratory equipment, and to reduce the sample run time and quantify the least detectable quantity of ACC.

References


CHAPTER 2. DIRECT DETERMINATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID IN PLANT TISSUES BY USING A GAS CHROMATOGRAPH WITH FLAME IONIZATION DETECTION

A paper to be submitted to *Analytical Biochemistry*

Katey H. Warnberg¹,², Richard J. Gladon¹, M. Ann Perera³, Jennifer R. Robinson³

**ABSTRACT.** 1-Aminocyclopropane-1-carboxylic acid is the immediate precursor of ethylene, a phytohormone that is produced in many plant tissues, which is effective in trace amounts (< 1 µl/l). Quantitative determination of ACC in biological tissues is paramount to understanding the regulation of this metabolic process. The most widely accepted method for quantifying 1-aminocyclopropane-1-carboxylic acid requires its oxidation to ethylene, which then is measured by gas chromatography. Our objective was to develop a method for the rapid, direct quantification of 1-aminocyclopropane-1-carboxylic acid by using a gas chromatograph with a flame-ionization detector. Ethylene production was measured in tissues from fruits, leaves, seeds, florets, and flower petals. The remaining tissue samples were ground to a fine powder in liquid nitrogen, and 1-aminocyclopropane-1-carboxylic acid was extracted from the powder with a methanol:chloroform:water mixture (5:12:3 v/v/v). 1-

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³W. M. Keck Metabolomics Research Laboratory, The Office of Biotechnology, Iowa State University.
Aminocyclopropane-1-carboxylic acid was quantified by using an EZ:faast™ gas chromatography mass-spectrometry free amino acid analysis kit, was identified using gas chromatography mass-spectrometry, and was confirmed with authentic 1-aminocyclopropane-1-carboxylic acid. Additional benefits include a short run time of less than six minutes and the absence of a potentially hazardous tracer, such as $^{14}$C. We show that this method is effective for accurate, direct measurement of 1-aminocyclopropane-1-carboxylic acid from a variety of both reproductive and vegetative plant tissues.

Ethylene is a phytohormone that is produced in many plant tissues, and it is effective in minute amounts (< 1 µl/l). Ethylene is involved in many metabolic processes, including fruit ripening; leaf, flower, and fruit senescence; leaf and flower abscission; regulation of stress responses; and seed germination [1]. Methionine, the earliest precursor of ethylene (C$_2$H$_4$), is converted enzymatically to S-adenosyl methionine [3], which then is converted enzymatically to 1-aminocyclopropane-1-carboxylic acid (ACC). The formation of ACC by ACC synthase is the rate-controlling step in the C$_2$H$_4$ biosynthesis pathway [19]. ACC is converted rapidly to C$_2$H$_4$ in the presence of molecular oxygen. Because ACC is an important intermediate in C$_2$H$_4$ biosynthesis [2], quantification of ACC has been a central component of studies in C$_2$H$_4$ physiology. In addition, a recent study suggests that ACC may have a role in plants that is independent of ethylene, which makes the quantification of ACC more important [18].

The most widely accepted assay for ACC requires oxidation of ACC to C$_2$H$_4$, which then is measured by gas chromatography [12]. This indirect method has an average
conversion rate of about 80%, but conversion ranges from 60% to $\geq 100\%$ [10]. The Lizada and Yang method requires equipment to measure $\text{C}_2\text{H}_4$ and has several drawbacks, including reliance on indirect measurement of ACC [5]. The conversion of ACC to $\text{C}_2\text{H}_4$, can be variable and is vulnerable to interference from other $\text{C}_2\text{H}_4$-generating or -absorbing compounds such as ethanol and perchloric acid [4,13]. A direct, rapid, and accurate method for ACC quantification would be helpful to biologists working with $\text{C}_2\text{H}_4$.

Several methods have been developed to overcome these difficulties, including gas chromatography with nitrogen/phosphorus detection [8] capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) [11]. Methods using HPLC and GC-MS, such as HPLC with UV detection [5,10], HPLC with MS [6], HPLC-MS-MS [16], HPLC with fluorescence detection [15], and GC-MS [13,17], also have been developed. Although sensitive and highly specific, methods that require HPLC and GC-MS require expensive equipment not available in many laboratories. Sample run times can exceed 8 min for HPLC-UV [10], HPLC with fluorescence detection [15], GC with a nitrogen/phosphorus detector [8], and CE-LIF [11]. In addition, many methods involve $^{14}\text{C}$ as a radioactive tracer [8,9,12], which can be hazardous.

Our overall objective was to develop a method for quantifying ACC by using gas chromatography with flame-ionization detection (GC-FID). A FID is one of the more common and inexpensive GC detectors, and we know of no reports of an assay for direct measurement of ACC by GC-FID. Our specific objectives were to develop a method for direct measurement of ACC that does not rely on a radioactive tracer, an internal standard,
nor expensive laboratory equipment, and to reduce the sample run time and quantify the least detectable quantity of ACC.

Materials and Methods

Chemicals

1-Aminocyclopropane-1-carboxylic acid was purchased from Sigma (Allentown, PA). All reagents used were HPLC grade unless otherwise noted. Methanol, chloroform, and iso-propanol were obtained from Sigma (Milwaukee, WI). An EZ:faast™ kit for amino acid analysis was purchased from Phenomenex® (Torrance, CA).

Plant material

Ethylene production and ACC content were analyzed in tissues from fruits, leaves, seeds, florets, and flower petals, and all tissues were obtained from local sources except seeds of impatiens (PanAmerican Seed™, West Chicago, IL). Drupe tissue of grape (Vitis vinifera L.), florets of broccoli (Brassica oleracea L.), and leaves of spinach (Spinacia oleracea L.) were incubated in 1-L mason jars at 25 °C for 3 h before C2H4 analysis. Individual fruits of apple (Malus ×domestica Borkh. ‘Delicious’) and fruits of cucumber (Cucumis sativus L.) were incubated separately in 4-L jars for 1 and 3 h, respectively, before C2H4 analysis. Seeds of impatiens (Impatiens wallerana Hook. F. ‘Super Elfin White’) were placed on two layers of blue blotter paper that was saturated with deionized water and kept in a petri dish sealed with parafilm. Petri dishes were placed in an incubator held at 25 °C
under constant irradiance for 170 h. At 170 h, seeds were removed from the petri dish and incubated in 40-ml vials for 1.5 h before C$_2$H$_4$ analysis. Flower petals of geranium (Pelargonium ×hortorum L.H. Bailey ‘Rocky Mountain Dark Red’) and ray florets of gerbera (Gerbera ×hybrida ‘Royal Yellow Dark Eye’) were incubated in 40-ml vials for 4.5 h before C$_2$H$_4$ analysis. Fruits of single mature-green, pink, and red cherry tomato (Lycopersicon esculentum Mill.) were incubated separately in 1-L mason jars for 2 h before C$_2$H$_4$ analysis. Fruits of tomato (Lycopersicon esculentum L.) were used as a model species for C$_2$H$_4$ and ACC analysis. Fruits of single, mature-green, breaker, turning, pink, light-red, and red tomatoes were incubated separately in 1-L mason jars for 1 h before C$_2$H$_4$ analysis.

C$_2$H$_4$ determination

All mason jars and vials were flushed with air and sealed with lids with a sampling port. After respective incubation times, gas samples were removed with a gas-tight syringe and analyzed for C$_2$H$_4$ by GC-FID with a Varian Star 3600 CX system (Palo Alto, CA) with an activated alumina column. After C$_2$H$_4$ analysis, tissue samples (0.25 x 0.25 x 3.5 cm; approximately 1 g) were cut from tissue of tomato, grape, apple, and cucumber. Samples of florets of broccoli, leaves of spinach, seeds of impatiens, petals of geranium, and ray florets of gerbera were 0.5 to 1 g. Tissue samples were placed in individual plastic 15-ml centrifuge tubes, frozen in liquid N$_2$, and stored at -80 °C for subsequent ACC analysis. Four replicates of each tissue type were analyzed for ethylene and frozen for ACC analysis.
ACC extraction

Frozen tissue was ground to a fine powder in liquid N\textsubscript{2}, and ACC was extracted with three successive 1.7-ml aliquots of a methanol:chloroform:water mixture (5:12:3 v/v/v). After adding 1.5 ml of water and 1 ml of chloroform to the 5-ml extract, the sample was agitated on a vortex mixer and allowed to phase-separate on ice for 1 h. The upper, aqueous phase was recovered and stored at -20 °C for < 72 h, and the remainder was discarded. The sample (approximately 2 ml) was dried to ~ 400 µl under a stream of gaseous N\textsubscript{2}, centrifuged at 550 g\textsubscript{n} for 10 min, filtered through a PTFE filter that was 13 mm x 0.2 µm (Supelco, Bellefonte, PA), and then stored at -20 °C for < 72 h. Tissues such as tomato fruits and broccoli florets, which contained amounts of ACC too great to be quantified accurately, were dried to 1 ml under a stream of gaseous N\textsubscript{2}. 1-Aminocyclopropane-1-carboxylic acid was quantified after a 1:1 dilution of the final extract:iso-propanol.

Qualitative and quantitative determination of ACC

Analysis of ACC was conducted with an EZ:faast\textsuperscript{TM} GC-MS free amino acid analysis kit (Phenomenex\textsuperscript{®}; part number KGO-7166; Torrance, CA). 1-Aminocyclopropane-1-carboxylic acid was identified by using GC-MS and confirmed with authentic ACC. Both GC-FID and GC-MS chromatographic analyses were conducted using a gas chromatograph (6890 series, Agilent, Santa Clara, CA), equipped with either an FID or a mass detector (Model No. 5973, Agilent, Santa Clara, CA). Amino acid extracts containing ACC were separated on a 10-m-long, 0.25-mm-interior diameter Zebron ZB-AAA capillary column with He as the carrier gas. The injector and detector were held at 250 °C. Initially, the oven
was 110 °C, and it was heated at a rate of 30 °C /min until reaching 320 °C. The mass
detector for GC-MS analyses was held at 240 °C for source, and quads at 180 °C. Scan range
was 45-45- m/z. Identification and quantification of ACC were facilitated by using HP
enhanced ChemStation™ G1701 BA version D.01.00 with a Windows NT™ operating
system (Agilent, Santa Clara, CA). A standard curve of several ACC concentrations was
used to obtain the absolute amount of ACC in each extract. The same column and instrument
settings were used to generate the standard curve and to quantify ACC in plant tissues by
using the curve. Four 200-µl solutions containing 0.01-M ACC diluted with a 1:1
methanol:water solution were used to generate a dilution series of 2, 5, 10, and 15 mmol, and
a 3-µl injection was used.

**Extraction efficiency**

A standard curve from several concentrations of norvaline, a synthetic amino acid,
was used to calculate extraction efficiency of ACC. Four 200-µl solutions using 0.2-mM
norvaline diluted with iso-propanol were used to generate a dilution series of 25, 30, 35, and
40 nmol norvaline. Three samples of seeds of imatiens were extracted according to the
method described above. After the tissue was ground in liquid N₂ and 1.7 ml of the
methanol:chloroform:water mixture was added, 125 µl of norvaline was added to each
sample. The remainder of the extraction procedure was done as described previously. The
samples were analyzed for norvaline by using GC-FID, using the same methods and
conditions for ACC quantification as described previously. Using the standard curve for
norvaline, we calculated the amount of norvaline in each sample of imatiens. Extraction
efficiency was the percentage of calculated norvaline in the sample of impatiens compared with the expected value from the standard curve.

**Data analysis**

Data for all experiments were analyzed using the regression-procedure option of Statistical Analysis Software (SAS Institute Inc., 1998). Analysis of variance was performed to assess how nanomoles of ACC influenced peak area. Regression analysis was used to test linear effects of nanomoles of ACC on peak area.

**Results**

We developed a method for quantification of ACC that uses the same instrument used for quantification of C$_2$H$_4$, a GC-FID. Our sample run time was 6 min, the ACC peak was present at about 3.75 min, and no peaks were present after 6 min (Fig. 1A). Quantification of ACC was conducted by use of a standard curve that ranged from 6 to 45 nmol of ACC (Fig. 2). The plot of peak area (relative area units) as a function of ACC content (nmol) yielded a correlation coefficient ($r^2$) of 0.97, a slope of the regression line of 0.23, and an intercept of -1.75 (Fig. 2). Injection of an ACC solution with a concentration of < 100 pmol did not yield a readable peak, and an injection of an ACC solution at > 1 x 10$^7$ nmol yielded a saturated peak that was unreadable. Thus, the usable range for this analysis is about 0.1 nmol to about 10.0 mmol.
Seed tissue of impatiens was used to calculate ACC extraction efficiency, via use of norvaline, a synthetic amino acid. The norvaline peak was present at about 1.7 min, and it did not interfere with the quantification of any other compounds. The standard curve made from norvaline had an $r^2$ of 0.96, a slope of the regression line of 8.17, and an intercept of -4.07. The extraction efficiency was 91.5%, with a standard error of 4.8.

Complete baseline separation of ACC from its common ACC conjugate, malonyl-ACC, was achieved (Fig. 1B). An extract of seeds of impatiens was derivatized by using the EZ-faast™ kit, analyzed by GC-MS, and the ACC peak occurred at about 3.7 min (Fig. 1A). According to the derivatization protocol in the EZ-faast™ kit, 42 and 86 m/z would be added to the carboxylic acid and amino ends, respectively (Fig. 1B). A mass spectrum of the peak identified as ACC (m/z 100) was increased by 42 (i) or 86 (ii) m/z due to derivatization (Fig. 1B). Malonyl-ACC (m/z 185) would be increased by 42 (227 total m/z) or 86 (271 total m/z) m/z due to derivatization, and none of these three peaks was detected (Fig. 1B).

To assess the utility of this method on economically important crops, several tissue types were analyzed for $C_2H_4$ production and ACC content (Table 1). Climacteric fruits (apple, tomato) produced $C_2H_4$ and contained ACC (Table 1). Nonclimacteric fruits (grape, cucumber, broccoli florets) produced no detectable or little $C_2H_4$, but they contained ACC (Table 1). Spinach leaves produced no detectable $C_2H_4$, but they contained ACC. Ethylene was not detected in seeds of impatiens that were held in the previously described conditions conducive to germination, although ACC was present. Floral tissues (geranium petals, gerbera ray florets) produced little $C_2H_4$, and neither contained detectable ACC. In addition, six stages of tomato ripening were analyzed for $C_2H_4$ production and ACC content (Fig. 3).
Ethylene production increased during ripening, peaked during stage 4 (pink stage), and then decreased. 1-Aminocyclopropane-1-carboxylic acid content remained relatively constant throughout ripening (Fig. 3).

**Discussion**

Our method provides a new way to quantify ACC, after derivatization, by direct measurement on a GC-FID, the same instrument used to analyze for C\textsubscript{2}H\textsubscript{4}. This method overcomes the primary limitation to the method of Lizada and Yang (1979), which is the reliance on an indirect measurement of ACC. Our method also has a short run time of < 6 min. Other methods for direct measurement of ACC [8,10,11,15] have run times of approximately 8 min [11] to more than 20 min [15]. Our short run time allows for 50 to 100% more samples to be processed per unit of time. Moreover, our method does not require expensive or uncommon equipment. We showed that the performance of GC-FID using an ACC derivative is similar to that of analysis by costly and often-unavailable GC-MS (Fig. 1).

Another benefit of our method is that it does not require the potentially hazardous \textsuperscript{14}C tracer involved in other protocols [8,9,12]. Our use of pure ACC to construct a standardized curve (Fig. 2) eliminated the need for an internal standard. Furthermore, a standardized curve constructed with seed extracts of impatiens with the addition of norvaline, a synthetic amino acid, demonstrated that the efficiency of the extraction was at least 90%. This extraction efficiency compares favorably with the method of Lizada and Yang, in which conversion of C\textsubscript{2}H\textsubscript{4} of ACC averaged 80%, but ranged from 60% to over 100% [10].
We have documented the efficacy of this direct method of ACC quantification after derivatization for use with both vegetative and reproductive structures (Table 1). Our results show that fruits of tomato and apple, which are classic examples of C₂H₄-producing tissues, had abundant amounts of C₂H₄ and its precursor, ACC. Conversely, tissues such as geranium petals and gerbera ray florets contained little C₂H₄ and no detectable ACC. We were interested to find that several vegetative and reproductive tissue types produced low to nondetectable C₂H₄, but contained at least 600 nmol g⁻¹ ACC, similar to findings by Clark et al. (1997) and Mencarelli et al. (1995). The functions of ACC in these tissues remain to be determined. Furthermore, results from tomatoes at the six stages of ripening showed, varying rates of C₂H₄ production, but greater-than-expected ACC that was relatively constant during ripening (Fig. 3).

A requirement for determination of ACC is that liquid samples used for this method should be prepared within the range of 1 nmol to < 1 x 10⁷ nmol of ACC to ensure accurate quantification. For example, analysis of broccoli florets and tomato berries proved to be more difficult than that of other tissues because of a greater content of ACC, and because of the existence of unidentified compounds that had retention times near that of the ACC peak. To circumvent these challenges, we made dilutions of the samples until they were within the acceptable range of ACC concentration.

Practical implementation of this method should be straightforward for many laboratories equipped for C₂H₄ analysis. Two ports and two columns must be installed into a gas chromatograph, with one port set for FID for analysis of C₂H₄, and the second port set for injection of liquid samples containing ACC derivatized with the EZ-faast™ GC-MS free
amino acid analysis kit. Based on our results, we suggest that run times for analysis of ACC with this instrumentation could be reduced to 6 min because ACC was detected at about 3.75 min, and no additional peaks were detected after 6 min (Fig. 1B).

References


[19] Yang, S.F. and N.E. Hoffman, Ethylene biosynthesis and its regulation in higher plants,

Table 1. Ethylene production rate and 1-aminocyclopropane-1-carboxylic acid (ACC) content of several types of plant tissue\textsuperscript{a}. Values are means of four replications. Standard errors are in parentheses.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ethylene production (ml C\textsubscript{2}H\textsubscript{4} g\textsuperscript{-1} h\textsuperscript{-1})</th>
<th>ACC content (nmol g\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptacle of Malus \textit{×}domestica Borkh. ‘Delicious’</td>
<td>7.4 (0.6)</td>
<td>516.2 (18.9)</td>
</tr>
<tr>
<td>Drupe tissue of Vitis vinifera L.</td>
<td>ND\textsuperscript{b}</td>
<td>1131.2 (243.7)</td>
</tr>
<tr>
<td>Florets of Brassica oleracea L.</td>
<td>1.1 (0.1)</td>
<td>4518.6 (231.1)</td>
</tr>
<tr>
<td>Pepo tissue of Cucumis sativus L.</td>
<td>1.8 (1.5)</td>
<td>1171.8 (133.6)</td>
</tr>
<tr>
<td>Leaves of Spinacia oleracea L.</td>
<td>ND</td>
<td>697.8 (61.7)</td>
</tr>
<tr>
<td>Germinated seeds of Impatiens wallerana Hook. F. ‘Super Elfin White’</td>
<td>ND</td>
<td>12791.9 (3481.6)</td>
</tr>
<tr>
<td>Petals of Pelargonium \textit{×hortorum} L.H. Bailey ‘Rocky Mountain Dark Red’</td>
<td>1.3 (0.3)</td>
<td>ND</td>
</tr>
<tr>
<td>Ray florets of Gerbera \textit{×hybrida} ‘Royal Yellow Dark Eye’</td>
<td>0.3 (0.2)</td>
<td>ND</td>
</tr>
<tr>
<td>Mature green fruit of Lycopersicon esculentum Mill.</td>
<td>10.8 (6.4)</td>
<td>639.7 (68.1)</td>
</tr>
<tr>
<td>Pink fruit of Lycopersicon esculentum Mill.</td>
<td>72.5 (4.6)</td>
<td>919.9 (33.8)</td>
</tr>
<tr>
<td>Red fruit of Lycopersicon esculentum Mill.</td>
<td>17.9 (6.3)</td>
<td>900.1 (72.7)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}All tissues were obtained from local sources except seeds of impatiens (PanAmerican Seed\textsuperscript{TM}, West Chicago, IL). Cultivar names were unavailable from most local sources of tissue.

\textsuperscript{b}Not detectable.
Figure Captions

Fig. 1. A. Chromatogram of germinated impatiens seeds tissue extract after derivatization with the EZ-faast™ GC-MS free amino acid analysis kit and analyzed by using a gas chromatograph with flame-ionization detection. The ACC peak appeared at about 3.75 min, with a total run time of 6. The last 2 min of an 8-min run time did not contain any peaks and are not shown fully. B. Mass spectrum of the peak identified as ACC. According to the derivatization protocol in the EZ-faast™ kit, m/z 42 and m/z 86 will be added to the carboxylic acid and amino ends, respectively. Therefore, ACC (m/z 100) will be increased by 42 (i) or 86 (ii) m/z units due to derivatization.

Fig. 2. Standard curve for quantification of 1-aminocyclopropane-1-carboxylic acid (ACC) by gas chromatography-flame ionization detection (GC-FID) with a 3-µl injection. Derivatization was conducted with an EZ-Faast™ GC-MS free amino acid analysis kit. Four 200-µl solutions of 0.01-M ACC diluted with a 1:1 methanol:water solution were used to generate a dilution series of 2, 5, 10, and 15 nmol × 10^2. \( r^2 = 0.97 \) and \( y = 0.2251x - 1.7524 \).

Fig. 3. Change in \( \text{C}_2\text{H}_4 \) production rate and ACC content of tomato fruit in the six USDA stages of ripening. Stages 1 through 6 represent mature-green, breaker, turning, pink, light-red, and red fruit, respectively. Four single-fruit replications were monitored individually for \( \text{C}_2\text{H}_4 \) production immediately before ACC analysis. Vertical bars represent the SE of means based on an average of four fruits. When absent, the SE bars fall within the dimensions of the symbol.
Fig. 1.
Fig. 2.
Fig. 3.
CHAPTER 3. GENERAL CONCLUSIONS

Our method provides a new way to quantify ACC, after derivatization, by direct measurement on a GC-FID, the same instrument used to quantify \( \text{C}_2\text{H}_4 \). This method overcomes the primary limitation to the method of Lizada and Yang (1979), which is the reliance on an indirect measurement of ACC. Our method also has a short run time of < 6 min. Other methods for direct measurement of ACC \([1,3,4,6]\) have run times of approximately 8 min \([4]\) to more than 20 min \([6]\). Our short run time allows for 50 to 100\% more samples to be processed per unit of time. Moreover, our method does not require expensive or uncommon equipment. We showed that the performance of GC-FID using an ACC derivative is similar to that of analysis by costly and often-unavailable GC-MS (Fig. 1).

Another benefit of our method is that it does not require the potentially hazardous \(^{14}\text{C}\) tracer involved in other protocols \([1,2,5]\). Our use of pure ACC to construct a standardized curve (Fig. 2) eliminated the need for an internal standard. Furthermore, a standardized curve constructed with seed extracts of impatiens with the addition of norvaline, a synthetic amino acid, demonstrated that the efficiency of the extraction was at least 90\%. This extraction efficiency compares favorably with the method of Lizada and Yang, in which conversion of \( \text{C}_2\text{H}_4 \) of ACC averaged 80\%, but ranged from 60\% to over 100\% \([3]\).

We have documented the efficacy of this direct method of ACC quantification after derivatization for use with both vegetative and reproductive structures (Table 1). Our results show that fruits of tomato and apple, which are classic examples of \( \text{C}_2\text{H}_4 \)-producing tissues, had abundant amounts of \( \text{C}_2\text{H}_4 \) and its precursor, ACC. Conversely, tissues such as geranium petals and gerbera ray florets contained little \( \text{C}_2\text{H}_4 \) and no detectable ACC. We were interested to find
that several vegetative and reproductive tissue types produced low to nondetectable \( \text{C}_2\text{H}_4 \), but contained at least 600 nmol g\(^{-1}\) ACC, similar to findings by Clark et al. (1995) and Mencarelli et al. (1997). The functions of ACC in these tissues remain to be determined. Furthermore, results from tomatoes at the six stages of ripening showed varying rates of \( \text{C}_2\text{H}_4 \) production, and greater-than-expected ACC that was relatively constant during ripening (Fig. 3).

A requirement for determination of ACC is that liquid samples used for this method should be prepared within the range of 1 nmol to \(< 1 \times 10^7\) nmol of ACC to ensure accurate quantification. For example, analysis of broccoli florets and tomato berries proved to be more difficult than that of other tissues because of a greater content of ACC, and because of the existence of unidentified compounds that had retention times near that of the ACC peak. To circumvent these challenges, we made dilutions of the samples until they were within the acceptable range of ACC concentration.

Practical implementation of this method should be straightforward for many laboratories equipped for \( \text{C}_2\text{H}_4 \) analysis. Two ports and two columns must be installed into a gas chromatograph, with one port set for FID for analysis of \( \text{C}_2\text{H}_4 \), and the second port set for injection of liquid samples containing ACC derivatized with the EZ-faast™ GC-MS free amino acid analysis kit. Based on our results, we suggest that run times for analysis of ACC with this instrumentation could be reduced to 6 min because ACC was detected at about 3.75 min, and no additional peaks were detected after 6 min (Fig. 1B).

References


Suggestions for Further Research

This project was originally intended to improve upon the sowing of germinated seeds, specifically impatiens, to lead to 100% stand establishment, speed seedling production time, and reduce profit loss from poorly germinated seeds. Rate of establishment, seedling production, and profit loss have become increasingly important to the greenhouse industry, as many bedding
plant varieties have low germination rates. To achieve 100% stand establishment, low-oxygen environments have proven beneficial, specifically by suppressing radicle growth to control radicle length for use in mechanical transplanting. However, if the O₂ concentrations are too low (< 0.5% O₂), or if seeds are exposed for too long (> 72 hours), severe irreversible abnormalities of the seedlings can occur. Future research may include using this method to determine 1-aminocyclopropane-1-carboxylic acid (ACC) content within germinating impatiens seeds. This will help to determine if the cause of abnormal seedling formation in Impatiens wallerana Hook. F. ‘Super Elfin White’ is due to the accumulation of ACC in the tissues during low-oxygen treatments.
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