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Analytical Methods

Optimized method for the quantification of pyruvic acid in onions by microplate reader and confirmation by high resolution mass spectra

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ABSTRACT

The present study describes the rapid microplate method to determine pyruvic acid content in different varieties of onions. Onion juice was treated with 2,4-dinitrophenylhydrazine to obtain hydrazone, which was further treated with potassium hydroxide to get stable colored complex. The stability of potassium complex was enhanced up to two hours and the structures of hydrazones were confirmed by LC-MS for the first time. The developed method was optimized by testing different bases, acids with varying concentrations of dinitrophenyl hydrazine to get stable color and results were comparable to developed method. Repeatability and precision showed < 9% relative standard deviation. Moreover, sweet onion juice was stored for four weeks at different temperatures for the stability; the pyruvate remained stable at all temperatures except at 25 °C. Thus, the developed method has good potential to determine of pungency in large number of onions in a short time using minimal amount of reagents.

1. Introduction

Onions (*Allium cepa* L.) are the second most important crop, based on production, after tomatoes. The United States stands in the third largest producer of onions in the world, after China and India (FAO., 2015). According to FAO reports, almost 170 countries are producing onions with a world annual production of 85,795, 191 tons of dry bulbs with 6, 521, 723 tons of them being marketed worldwide. Onions are an important vegetable crop grown in many countries and are consumed worldwide for their unique flavor (Abayomi, Terry, White, & Warner, 2006). Onions can vary in size, shape, texture, flavor, and color with yellow onions representing 87% of production, red onions representing 8%, and white onions representing 5% (Bhat, Desai, & Suleiman, 2010; NOA., 2011). The ratio of sugar to pungency determines the overall onion flavor and consumers show strong preferences for specific flavors (Vavrina & Smittle, 1993). Onions with high pungency can mask high levels of sugars, onions with low pungency and low sugar content can be perceived as bland, and onions with high levels of sugars and low pungency are considered sweet onion (Vagen & Slimestad, 2008).

The pungency in onion results from precursor compounds such as S-alk(en)yl-L-cysteine sulfoxides (ACSOs). When onion are crushed, the alliinase enzyme hydrolyses the ACSOs to form pyruvic acid, ammonia, and volatile sulphur compounds. This reaction is completed within 6 min (Schwimmer & Weston, 1961; Whitaker, 1976). These chemically unstable organosulphur compounds further rearrange to form thiopropanal S-oxide (lachrymator factor) and thiosulfinates (Imai et al., 2002). Thus, the stable pyruvic acid has been used as an indicator of onion pungency (Yoo & Pike, 1999). Depending upon the pyruvic acid level, the onions were categorized into sweet, mild, and strong. Onions with < 3.5 μmol mL^{-1} are considered as mild or sweet (Crowther, Collin, Smith, Tomsett, O'Connor, & Jones, 2005). US and European markets have shown growing demand for onions with lower pyruvic acid levels (Costa & Epperson, 2003; Ianni, Marinozzi, Scorzoni, Sardella, & Natalini, 2016). Hence, methods to measure pyruvic acid levels provide important information to ensure the quality of onions for both growers and consumers (Yoo & Pike, 2001).

The current method to measure enzymatically produced pyruvic acid uses 2,4-dinitrophenylhydrazine (DNPH) and measures absorbance at 420 nm (Schwimmer & Weston, 1961). However, this method is time consuming (74 min) from sample extraction to quantification (Randle & Bussard, 1993). To make this faster, Randle and Bussard introduced the press method to squeeze onion juice from bulbs, allowing one person to analyze 15 bulbs per hour. Further it was simplified the original method by blending onion tissue without adding water and

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analyzing pyruvic acid using undiluted juice (Yoo, Pike, & Hamilton, 1995). In addition, our group has developed custom built automated system using two HPLC pumps, an autosampler, and a detector to analyze 30 samples per hour, while reducing the human error and labor cost (Yoo et al., 1995). This method allows us to analyze a large number of samples, but uses specialized instrumentation that is not commonly available.

In addition to accuracy and time, the use of large amount of reagent also remains important issues for development of high-throughput assays. Another study used a flow injection analysis to measure pyruvic acid in onion cultivars from different regions of Venezuela. They observed non-significant differences at the 95% confidence level between the flow injection analysis and the batch method (Marcos et al., 2004). Sample dilution is not required for this method, but the sample (> 3 mL) and flow rate (6 mL min^{-1}) consumed large volumes of reagents, which is not ideal for screening of large numbers of samples. A disposable prototype pyruvate biosensor method was constructed using pyruvate oxidase immobilized on mediated meldola's blue electrodes to determine pungency; this assay also showed a strong correlation between the biosensor response and known pyruvate concentrations (2–12 μmol g^{-1} FW) in onion (Abayomi et al., 2006). Recently, non-destructive method to discriminate three "Tropea Red Onion" was developed and onion flavor has been closely linked to pungency and pyruvic acid. Although these non-destructive techniques are helpful for producers, but such instruments are not commonly available. HPLC methods also used to measure pyruvic acid (Gallina, Cabassi, Maggioni, Natalini, & Ferrante, 2012; Ianni et al., 2016), and the precursor compound S-alk(en)yl-L-cysteine sulfoxide (ACSO) in different varieties of onions (Bacon et al., 1999; Yoo, Lee, & Patil, 2012). The main drawback of HPLC methods is that they require tedious sample preparation and each analysis takes 30–40 min. Several other techniques for the determination of pyruvic acid in various samples have been reported such as fluorescence (Olsen, 1971; Zhao et al., 2008), and gas chromatography (Paik et al., 2008). Moreover, these traditionally methods use multi-step, time-consuming methods involving large volumes of solvents, making these methods potentially harmful to the environment. In recent years, the concept of green chemistry has gained tremendous momentum, promoting the judicious use of 'green' solvents, and emphasizing the "goal to minimize the environmental impact resulting from the use of chemicals" (Gałuszka, Migaszewski, & Namieśnik, 2013).

To address such drawbacks, we have optimized an assay using a microplate reader with minimal amounts of chemicals in rapid manner without degradation of color. In most of the published DNPH reaction the incubation time plays a crucial role in quantifying exact amount of pyruvic acid in onions after adding base. In general, measuring the color using spectrophotometer for individual samples will give false value due to the degradation of sodium hydrazone. For the first time, stable potassium hydrazone complex was developed for the measurement of pyruvic acid in onions. This change gives potassium hydrazone adduct, which has more stability and highly reproducible compare to sodium adduct. The developed method was optimized for various parameters such as concentration of acid, base, reagents, incubation time to get reproducible color hydrazone. The colored adduct was identified by high resolution actuate mass spectra for the first time. In addition, method was tested for commercial samples including sweet, yellow, white, and red onions as well as stability of pyruvic acid during 24 days storage period.

2. Materials and methods

2.1. Plant materials and chemicals

Yellow onions (product of Mexico), white onions (product of Mexico), red onions (product of Mexico and USA), and Texas Sweet 1015 onions (product of USA) were purchased from a local supermarket. Sodium pyruvate and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma Aldrich (St. Louis, MO, USA), Potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3) and potassium carbonate (K_2CO_3) were obtained from Fisher Scientific (Pittsburg, PA, USA).

2.2. Sample preparation

Onion bulbs were weighed; the neck, basal plate, and dry skin were removed. The onions were chopped into large cubes and blended in a mixer. The puree was incubated at room temperature for 30 min and vacuum filtered through Whatman No 1. The filtered liquid sample was transferred to 50-mL tubes and stored at −20 °C until further analysis. Depending on the pungency level, the samples were diluted 1:1 to 1:8 times with Nanopure water (NANOpure, Barnstead/Thermolyne, Dubuque, IA, USA) and used for microplate reader assays with the Synergy HT Multi-Mode Microplate Reader (BioTek, Instruments, Winooski, VT, USA).

2.3. Reagents preparation

For bioassays, 25, 50, 75, and 125 mg DNPH was dissolved in 0.5 mol L^{-1} HCl, 0.5 mol L^{-1} H_2SO_4, and 0.5 mol L^{-1} H_3PO_4 to obtain DNPH concentrations of 0.126, 0.252, 0.378, and 0.63 mM, respectively. The other reagents were prepared in nanopure water and stored at 25 °C until further use.

2.4. Sodium pyruvate

A stock solution of 10 mM was prepared by dissolving 110.04 mg of sodium pyruvate in 100 mL water. The stock solution was further diluted to obtain different concentrations (0.25, 0.5, 1, 2, 4, 6 and 8 mM). All standards were stored at −80 °C until further use.

2.5. Optimization of color development

2.5.1. Optimization of NaOH and KOH concentration

To enhance the stability of the color complex various bases such as NaOH, KOH, Na_2CO_3 and K_2CO_3 were evaluated and the resulting color was measured spectrophotometrically. Different concentrations of 0.5, 1.25, 2.5, 5 and 7.5 mol L^{-1} NaOH and KOH; and 5 mol L^{-1} Na_2CO_3 and K_2CO_3 were used for the pyruvic acid measurement.

2.5.2. Effect of incubation time for color development

A 10 μL of each different concentration of pyruvic acid (0.25, 0.5, 1, 2, 4, and 6 mM) was pipetted into 96 well plates in triplicates and treated with 90 μL of DNPH, and incubated for 0, 15, 30, and 60 min at room temperature. At each time point, 50 μL of KOH was added to each well of the respective plate and absorbance at 485 nm was recorded every 3 min in the microplate reader for 30 min at 37 °C.

2.5.3. Influence of DNPH concentration with different acids

To get a stable hydrazone complex for the quantification of pyruvic acid, 90 μL of 0.126 mM, 0.252 mM, 0.378 mM and 0.63 mM DNPH reagent in 0.5 mol L^{-1} HCl, H_2SO_4 and H_3PO_4 was added to 10 μL of pyruvic acid. The plates were incubated for 30 min at 25 °C and 50 μL of KOH was added. The kinetic readings were recorded at 485 nm for every 3 min using microplate reader for 30 min at 37 °C.

2.5.4. Stability of hydrazone

To compare the stability of sodium and potassium hydrazone, 90 μL of 0.63 mM DNPH in 0.5 mol L^{-1} H_2SO_4 was added to 10 μL of 2 mM pyruvic acid. The plates were incubated for 30 min at 25 °C and 50 μL of NaOH and KOH was added. The kinetic readings were recorded at 485 nm for every 10 min using microplate reader for 3 h at 37 °C.