Development of a Simple Pungency Indicator Test for Onions

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(Received 16 August 1991; revised version received 2 June 1992; accepted 19 August 1992)

Abstract: Long-day yellow onions differing in pungency were used to compare methods for appraisal of pungency. Pyruvate, an established indicator of pungency for Allium species, was determined by the lactate dehydrogenase and 2,4-dinitrophenyl hydrazine methods. Thiosulfinate content, another indicator of pungency, was determined spectrophotometrically by monitoring the absorbance at 515 nm after reaction with N-ethylmaleimide. Significant correlation ($R^2 = 0.871$) existed between thiosulfinate content and pyruvate concentration. In addition, a modified N-ethylmaleimide reaction was used to develop a simple pungency indicator test which adequately detected differences between bulbs. The color produced by this method was analyzed using a HunterLab reflectance colorimeter. The Hunter $a$ value and saturation index ($C$) correlated with both thiosulfinate content and pyruvate concentration ($R^2 = 0.735-0.860$). The procedure described provides the potential for screening of pungency of large numbers of Allium samples.

Key words: pyruvate, thiosulfinate, N-ethylmaleimide, pungency, onion, garlic, C-S lyase, alliinase.

INTRODUCTION

Appraisal of flavor or pungency of alliums, such as onion (Allium cepa) and garlic (Allium sativum), can be based on either subjective sensory analysis or detection of compounds generated by cysteine sulfoxide lyase (C-S lyase; EC 4.4.1.4) activity after tissue disruption. The typical flavor of Allium species is due to the conversion of endogenous alk(en)yl-L-cysteine sulfoxide flavor precursors to pyruvate, ammonia, and thiosulfinates by C-S lyase (Nock and Mazelis 1987). For example, alk(en)yl thiosulfinate products such as 1-propyl propane-thiosulfinate and methyl methanethiosulfinate are primarily responsible for the characteristic fresh flavor of onion tissue (Freeman and Whenham 1976).

Growers and breeders of alliums currently lack a facile method of screening for pungency, especially when large numbers of samples are evaluated and where only rudimentary instrumentation is available. The determination of pyruvate as an indicator of pungency is perhaps the most established method of appraisal. Pyruvate determination is based on the lactate dehydrogenase (LDH) and NADH coupled reaction or on the 2,4-dinitrophenyl hydrazine (2,4-DNPH) derivatization procedure (Schwimmer and Weston 1961). Both procedures require analytical instrumentation and are not well suited for analyzing large numbers of samples that are typically evaluated in breeding programs. Furthermore, the 2,4-DNPH method requires an additional step to correct for background carbonyls since it is nonspecific and carbonyl compounds other than pyruvate will react to give falsely high values of pyruvate (Lancaster and Boland 1990). Recognizing the need for an alternative approach to determine pungency in Allium species, Freeman and Whenham (1975) developed a procedure for the detection of thiopropanal S-oxide (the lachrymatory compound). However, since this method requires a hexane extraction step and spectrophotometric analysis, it too is not conveniently performed. Gas chromatography (GC) might be considered the best method to assess the flavor profile of Allium tissues, but the contribution of secondary reaction products (those
primarily detected by GC) to the overall pungency of the sample is uncertain (Yu et al 1989a). Moreover, GC analysis requires sophisticated and expensive instrumentation. Similar disadvantages exist for high-performance liquid chromatography, which has been used for the detection of allicin (diallyl thiosulfinate) in garlic (Jansen et al 1987).

An alternative method for the evaluation of pungency in Allium sp involves the determination of the thiosulfinates (Carson and Wong 1959; Nakata et al 1970). The procedure involves derivatizing the thiosulfinates with N-ethylmaleimide and measuring the absorbance of the conjugate at 515 nm. This procedure is quite specific for thiosulfinates, however Freeman and Whenham (1975) noted that thiopropanal S-oxide also gives a slight positive reaction (7.6% of that of 1-propyl propane-thiosulfinate). Although Carson and Wong (1959) suggested that the N-ethylmaleimide reaction for Allium thiosulfinates could be adapted as a visual test on paper, no effort to develop such a test has been reported. Therefore, the intention here was to develop a prototypic simple pungency indicator test for Allium species based on the application of the N-ethylmaleimide reaction for thiosulfinates. The efficacy of this test was determined by correlating color production with thiosulfinate content (measured spectrophotometrically) and pyruvate concentration in minced onion tissue.

**EXPERIMENTAL**

**Chemicals**

All chemicals were obtained from Sigma Chemical Co (St Louis, MO, USA). All water used was deionized, then glass-distilled.

**Onions and garlic**

Six different USDA long-day yellow onion inbreds and hybrids were used for pungency assessment. Entries evaluated included two mild inbreds (B8155 and B9161), two pungent inbreds (B4535 and B9897), and two widely grown hybrids (Spartan Banner 80 and Sweet Sandwich). Bulbs were cultivated on muck soil in a commercial production field in Palmyra, WI. In addition, yellow onion bulbs (US#1) from the same lot, and garlic cloves, were obtained from a local market and also used for analysis.

**Preparation of filtered onion puree**

For pungency assessment, three core samples were taken with a cork borer at the bulb equator from each onion and analyzed for pyruvate by the 2,4-DNPH method (see below). The outer skins were then removed and the remainder of each bulb was liquefied (Braun Juicer, Frankfurt, Germany) to yield a mixture of juice and pulp. The puree was stored in a covered container at 22-25°C for 30 min to allow for maximal pyruvate production by endogenous C-S lyase (preliminary studies verified that the reaction was essentially complete after this period) and then filtered through Whatman #50 paper to remove the pulp. The filtered puree was diluted (1:10 v) with isopropyl alcohol previously cooled to 5°C and assayed for thiosulfinate content by the spectrophotometric and reflectance colorimetric methods (see below). A portion of the undiluted filtrated puree was quickly frozen in solid CO₂ and stored at −20°C for no longer than 1 h prior to thawing and the determination of pyruvate by the LDH method (see below).

**Ether extraction of thiosulfinates**

Using the method of Nakata et al (1970), a thiosulfinate ether extract from onion and garlic was obtained as follows. One gram of tissue was blended with deionized water (10 ml) using a Brinkmann Polytron (Model PT 10/35; Brinkmann Instruments Co., Westbury, NY, USA) and the mixture filtered through Whatman #50 paper. One half of the filtrate was used for the ether extraction of thiosulfinates and the other for pyruvate determination by the LDH method (see below). Thiosulfinates were extracted with two volumes of diethyl ether by gentle agitation for 10 min. The ether phase was removed and another portion of diethyl ether (two volumes) was added for further extraction. The ether extracts were combined and evaporated to dryness. Isopropyl alcohol (5 ml) was added to the residue and the thiosulfinate preparation stored at 4°C until analyzed.

**Spectrophotometric determination of thiosulfinates**

For the spectrophotometric determination of thiosulfinates, the following reagents were added in succession to 1 ml of filtered onion puree, onion ether extract or garlic ether extract (Carson and Wong 1959; Nakata et al 1970): 1 ml of 0.05 M N-ethylmaleimide in isopropyl alcohol, 1 ml of 0.25 M KOH in isopropyl alcohol, and 1.5 ml of 10 g liter⁻¹ ascorbic acid in distilled water. After vortexing the solution for approximately 10 s, the absorbance at 515 nm was recorded using a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA). Initially, the authors attempted to quantify thiosulfinates in onion samples based on pyruvate formation and by assuming a reaction stoichiometry of 2:1 pyruvate:thiosulfinate as suggested for garlic (Nakata et al 1970). However, this assumption was found to be tenuous and not applicable to both garlic and onion (see Results and Discussion). Therefore,
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the relative thiosulfinate content as absorbance units at 515 nm is reported.

Reflectance colorimetric determination of thiosulfonates

Absorbent cotton squares (Walgreen Co., Deerfield, IL, USA), containing two raised cotton strips each, were soaked in excess 0.15 M N-ethylmaleimide (in isopropyl alcohol) for 15 min and dried in a fume hood for 1 h prior to use. Filtered onion purée (2 ml diluted with 18 ml isopropyl alcohol) was evenly layered across each square (1 ml per cotton strip) and allowed to dry for 30 min. Each square was then dipped for approximately 5 s in excess 0.25 M KOH in isopropyl alcohol to develop color. After 3 min, each cotton square was placed on the colorimeter port (covered with a black box to limit stray light) and four readings were taken one at each 90° rotation of the cotton square. Hunter L, a, and b values were determined using a HunterLab Model D-25A-9 colorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA). The average of the four readings was used in reporting each Hunter value and in determining the corresponding saturation index (C; where \(C = (a^2 + b^2)^{1/2}\); Elbe et al. 1986). The colorimeter was standardized with black, white, and red reference tiles.

Pyruvate determinations

Pyruvate was quantified by both the LDH and 2,4-DNPH methods as described by Schwimmer and Weston (1961).

Endogenous (background) pyruvate levels in onion tissue were determined as follows. Yellow onion tissue (50 g) was steeped for 24 h in 100 ml of methanol/chloroform/water (12:5:3; MCW) at -20°C. The MCW extract was removed and the tissue was steeped in an additional 50 ml of 800 ml liter⁻¹ ethanol for 2 h. The MCW extract was phase-separated by the addition of 1 volume of chloroform/water (2:3 v). The methanol/water phase was retained and combined with the 800 ml liter⁻¹ ethanol extract. The volume of this mixture was reduced to about 20 ml under vacuum at 60°C, adjusted to pH 7.5, and analyzed for pyruvate by the LDH method.

Statistics

Analysis of variance was computed using StatView® SE+ software for the Macintosh (Abacus Concepts Inc., Berkeley, CA, USA). For some studies, Duncan’s multiple range test was used for obtaining pairwise comparisons (Duncan 1955).

RESULTS AND DISCUSSION

To determine whether the pungency appraisal methods investigated herein could detect modest differences between onion bulbs, long-day yellow onions were used for analysis. Since pyruvate is an index of pungency and is directly related to the extent of C-S lyase action (Schwimmer and Weston 1961; Carson 1987), the bulbs were analyzed for pyruvate by both the LDH and 2,4-DNPH methods. Significant differences \((P < 0.05)\) in pyruvate content between different onion inbreds and hybrids were detected by both the LDH and 2,4-DNPH methods (Table 1). The average pyruvate concentration ranged from 6 to 10 \(\mu\)mol g⁻¹ fresh weight. The greatest difference in pyruvate (pungency) was between entries B8155 and B9897, onions bred to be mild and more pungent, respectively. The LDH method resulted in slightly lower average values for the pyruvate concentrations than did the 2,4-DNPH method. However, most of the variance was due to differences between bulbs of the same entry rather than the methods used, except possibly for B9161.

Significant differences \((P < 0.05)\) between entries also existed when the average thiosulfinate content was determined spectrophotometrically (Table 1). The relative amount of thiosulfonates detected ranged from 0.25 to 1.0 (absorbance units) and again, in some instances, considerable variance was detected among bulbs of the same entry. Using thiosulfinate content as a measure of pungency, similar trends with regard to bulb pungency as was indicated for pyruvate content were evident. Entries B8155 produced the least amount of thiosulfinate while B9897 produced the greatest. Comparing the thio-

<table>
<thead>
<tr>
<th>Entry</th>
<th>LDH Pyruvate (µmol g⁻¹ tissue)</th>
<th>2,4-DNPH Pyruvate (µmol g⁻¹ tissue)</th>
<th>Thiosulfinate (Abs 515 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDH method</td>
<td>2,4-DNPH method</td>
<td></td>
</tr>
<tr>
<td>B8155</td>
<td>5.9 (± 0.7)</td>
<td>6.9 (± 1.5)</td>
<td>0.262 (± 0.125)*</td>
</tr>
<tr>
<td>B9161</td>
<td>5.8 (± 0.5)</td>
<td>8.0 (± 0.3)</td>
<td>0.410 (± 0.099)*</td>
</tr>
<tr>
<td>Sweet</td>
<td>7.6 (± 1.1)</td>
<td>7.9 (± 2.6)</td>
<td>0.747 (± 0.122)**</td>
</tr>
<tr>
<td>Sandwich</td>
<td>7.7 (± 1.8)</td>
<td>9.3 (± 1.3)</td>
<td>0.632 (± 0.340)**</td>
</tr>
<tr>
<td>Spartan</td>
<td>7.6 (± 1.2)</td>
<td>9.3 (± 2.1)</td>
<td>0.692 (± 0.396)**</td>
</tr>
<tr>
<td>Banner 80</td>
<td>8.8 (± 0.1)</td>
<td>9.7 (± 0.4)</td>
<td>1.018 (± 0.039)**</td>
</tr>
<tr>
<td>B4535</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>B9897</td>
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</tbody>
</table>

* For each entry, three separate bulbs were assayed in triplicate except for B9161 and B9897 where two bulbs were analyzed. Values represent the means (± standard deviation) of the bulbs analyzed. Means sharing the same superscript in each column were not significantly different. For relative thiosulfinate content, absorbance units per ml of sample extract are reported.
sulfinate content with the pyruvate concentration, a significant correlation (Fig 1; \( R^2 = 0.871; P < 0.001 \)) was obtained, supporting the use of thiosulfinate determination as an acceptable indicator of pungency. Our results show a better relationship between thiosulfinate content and pyruvate concentration than do those of Freeman and McBreen (1973).

The relative thiosulfinate content for the onion extracts determined by the N-ethylmaleimide reaction is reported as the absorbance at 515 nm. Although thiosulfinate content appears to be an adequate indicator of pungency, no means are presently available to quantify thiosulfinates with certainty for all alliums. Nakata et al. (1970) estimated the thiosulfinate concentration of minced garlic on the basis of pyruvate concentration assuming a theoretical reaction stoichiometry of 1 mol of thiosulfinate per 2 mol of pyruvate. It was found that the pyruvate content of minced garlic (approximately 72 μmol pyruvate g\(^{-1}\)) to be 12-fold greater than in minced onion (approximately 5-8 μmol pyruvate g\(^{-1}\)) for the samples analyzed, consistent with the previous findings of Saghiri et al. (1964). It was also found that the relative thiosulfinate content of minced garlic and onion was about 1000:1 (approximately 100 and 0.1 absorbance units g\(^{-1}\) for garlic and onion, respectively). Normalized against pyruvate, garlic yielded approximately 83 times more thiosulfinate than onion. Thus, from our results, quantifying onion thiosulfonates based on the theoretical 2:1 stoichiometry of pyruvate:thiosulfinate as suggested for garlic (Nakata et al. 1970) does not appear to hold for both garlic and onion. These results are consistent with the observations of Freeman and Whenham (1975) who noted that thiopropanal S-oxide in onion (which is formed from the 1-proplyl-L-cysteine sulfoxide derivative) is stoichiometrically equivalent to pyruvate.

Given that thiosulfinates are unstable and can undergo rearrangement reactions to form mono-, di-, and tri-sulfides (Yu et al. 1989b), perhaps some of the thiosulfinates produced in the onion extracts were lost due to secondary reactions. However, no decrease in thiosulfinate content (absorbance at 515 nm) was observed for 30 min after homogenization and prior to analysis (data not shown). The low thiosulfinate:pyruvate ratio found for onion could also be due, in part, to high endogenous (background) levels of pyruvate in the intact tissue. However, analysis for pyruvate in intact onion tissues indicated that only 5-8% of the total pyruvate detected in homogenized tissue was due to background levels.

Differences in secondary reaction patterns may exist between garlic, onion, and probably other alliums. The primary reaction products of alk(en)yl-L-cysteine sulfoxides in minced alliums are the corresponding sulfenic acids (Lancaster and Boland, 1990). The 1-propenylsulfenic acid isomerizes to thiopropanal S-oxide, whereas the allyl-, propyl-, and methylsulfenic acid derivatives are converted to their respective thiosulfinates. Bodens et al. (1971) found considerably more aldehydes produced initially than sulfides in the headspace of freshly minced onion, suggesting that, in this species, further conversion of thiopropanal S-oxide to aldehyde compounds is favored over reactions leading to thiosulfinate formation. The most dominant alk(en)yl-L-cysteine sulfoxides in onion and garlic are the 1-propenyl and allyl derivatives, respectively (Lancaster and Boland, 1990; Thomas, D J and Parkin, K L, unpublished results). Therefore, a plausible explanation for the difference in relative thiosulfinate content between minced garlic and onion is that the allyl and alkyl sulfenic acid derivatives dimerize to form thiosulfinates in garlic, whereas the 1-propenylsulfenic acid in onion favors thiopropanal S-oxide and aldehyde formation. Furthermore, the relative pyruvate:thiosulfinate level of minced onion tissue is probably dependent on the relative amounts of the various alk(en)yl-L-cysteine sulfoxide substrates. Finally, greater thiosulfinate levels in garlic extracts could be attributed to less conversion of thiosulfinates to thiosulfonates compared with onion (Freeman and Whenham 1976). Differences in pH between garlic and onion homogenates could account for some of the differences in these reaction patterns. In this study it was found that the pH of garlic purées was 6.0 whereas onion purées were in the range 5.2-5.7.

The authors attempted to adapt the N-ethylmaleimide-based determination of thiosulfinates to a reflectance colorimetric method with the purpose of developing a prototype for a simple pungency indicator test. In testing various materials, it was found that the cotton worked well as a matrix for the reaction and for the retention of color. Materials such as filter paper and latex sponges...
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Fig 2. Relationship between pyruvate content (determined by the LDH method) and Hunter a values. For pyruvate content, each symbol is the mean ± SD for an individual bulb assayed in triplicate. The Hunter a values are the means ± SD for four readings from each of two replicates prepared from each bulb.

Fig 3. Relationship between pyruvate content (determined by the LDH method) and saturation index. For pyruvate content, each symbol is the mean ± SD for an individual bulb assayed in triplicate. The values for the saturation index are the means ± SD for four readings from each of two replicates prepared from each bulb.

were tried but gave fugitive results due to leaching of the reaction mixture or failure to produce resolute color.

The effect of time on the generation and stability of the Hunter a value (redness) for the reflectance colorimetric procedure indicated that maximal color production and retention occurred between 3 and 6 min, after this time the color began to fade slowly; Hunter a values were within 90% of the maximum over the time period of 2–16 min (data not shown). Although no effort was made to prove it, maintenance of color could probably be achieved by incorporating a stabilizing agent into the procedure. For example, Nakata et al (1970) showed that the addition of a reducing agent such as ascorbic acid or cysteine stabilized the color produced by the N-ethylmaleimide reaction, seemingly by protecting the colored conjugate from oxidation.

The correlation between Hunter a values, determined by the reflectance colorimetric procedure, and thiosulfinate content (determined spectrophotometrically) was significant ($R^2 = 0.828; P < 0.001$). When the saturation index was compared with the thiosulfinate content, a higher correlation ($R^2 = 0.86; P < 0.001$) was obtained. The saturation index, an indicator of the quantity of color, takes into account both the Hunter a and b (yellow) values and may better represent the red-orange color produced by the reaction of thiosulfinates with N-ethylmaleimide.

The Hunter a values and saturation index for the reflectance colorimetric procedure also significantly correlated with pyruvate content. The correlation between the Hunter a values and pyruvate concentration (Fig 2) was 0.735 ($P < 0.001$). A higher correlation ($R^2 = 0.794; P < 0.001$) was obtained when the saturation index was related to pyruvate concentration (Fig 3).

The reflectance colorimetric procedure was able to detect modest differences between onions and correlated with other pungency assessment techniques. It was the authors’ experience that color differences between onion samples could be ascertained visually using this test. Ideally, the color produced by this test could simply be compared with a color-coded chart for an estimation of allium pungency. With further development, this procedure has the potential to be used as an alternative to more complicated analytical methods and would benefit growers and breeders of alliums by providing a facile method for pungency estimation of a large number of samples.

ACKNOWLEDGEMENTS

This work was supported by the College of Agricultural and Life Sciences, and the Graduate School of the University of Wisconsin-Madison.

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