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Modified method for the determination of pyruvic acid with dinitrophenylhydrazine in the assessment of onion pungency

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Abstract: Modifications to the commonly used Schwimmer and Weston procedure for determining pyruvic acid in onions are proposed. These modifications involve changes in the concentrations and volumes of the reagents and the use of 515 rather than 420 nm as the wavelength for measurements. These changes improve both the linearity and the sensitivity of the assay and eliminate interference from coloured material found in yellow onions. With these changes, more reliable determinations of pyruvic acid and thus of onion pungency should be possible.

Keywords: onion; pungency; pyruvic acid; dinitrophenylhydrazine

INTRODUCTION

Pungency in onions is derived from a number of volatile sulphur compounds. These compounds are produced when the onion cell is mechanically disrupted, bringing the enzyme alliinase into contact with the flavour precursors, *S*-alk(en)yl-L-cysteine sulphoxides.[1,2] In addition to producing volatile sulphur compounds, the enzymatic breakdown of the *S*-alk(en)yl-L-cysteine sulphoxides also produces stoichiometric amounts of ammonia and pyruvic acid. The amount of pyruvic acid generated enzymatically upon onion homogenisation is thus a good measure of the action of alliinase on the flavour precursors and has been shown to be correlated with perceived onion pungency.[1,3] This enzymatically produced pyruvic acid is the commonly accepted measure of onion pungency. In order to determine the amount of enzymatically derived pyruvic acid, a correction for the endogenous, non-enzymatically derived pyruvic acid level must be made. This is done by inactivating the alliinase enzyme either by heating prior to homogenisation or by homogenising in trichloroacetic acid. The level of pyruvic acid in this treated sample is then determined and subtracted from the total pyruvic acid.

The pyruvic acid level in an onion homogenate can be measured by several different methods. The simplest and most commonly used involves the reaction of pyruvic acid with 2,4-dinitrophenylhydrazine (DNPH) to form a coloured product. Although simple and widely used, the determination of pyruvic acid by reaction with DNPH has problems. The procedure as originally proposed by Schwimmer and Weston[1] or with minor modifications[4] generates a non-linear standard curve. For accurate determinations of pyruvic acid an extended standard curve must be constructed and the pyruvic acid levels in samples determined by directly comparing absorbances with those on the curve. In theory one could fit a quadratic or other curve to the data points and use this to calculate values in unknown samples,[4] but unless this calculated curve gives a near-perfect fit to the data such a procedure will introduce errors. One way to minimise this problem is to keep the amount of pyruvic acid in the samples and standards at low levels, as is done in the original Schwimmer and Weston procedure. Under these conditions the deviation from linearity is small and a linear approximation of the standard curve introduces only a small error. The low levels of pyruvic acid, however, mean that all the samples have low absorbances. Using the original Schwimmer and Weston procedure, onion samples typically have absorbance values of less than 0.2 at 420 nm compared with the reagent blank. Relying on such low absorbance values is undesirable, since the small errors introduced by slight mismatches in cuvettes or instability in the spectrophotometer become significant.

A recent study showed significant variation when a single set of onion samples was analysed by different laboratories using the Schwimmer and Weston method.[5] We have examined some of the details of using DNPH for determining pyruvic acid in an onion homogenate to see if changes in the standard procedure might overcome some of the deficiencies of the assay and potentially make it more reliable.

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EXPERIMENTAL

Materials

Sodium pyruvate (Sigma ultra 99%) and DNPH were obtained from Sigma (St Louis, MO, USA). This DNPH contains approximately 30% water; no correction for this water content was made in preparing solutions. Onions were purchased from a local market.

Onion sample preparation

Onions were sliced in half longitudinally and the outer skin and ends were removed. One half of the onion was homogenised for 1 min in a Waring blender at a ratio of 1 ml of added water per gram of onion. The homogenate was allowed to stand for 10 min at room temperature, then filtered through two layers of cheesecloth. An aliquot of this filtrate was transferred to a 1.5 ml centrifuge tube and clarified by centrifugation at $10\,000 \times g$ for 5 min. We have subsequently determined that this centrifugation step can be omitted if the sample is to be analysed by the colorimetric procedures described below.

The other half of the onion was placed in a plastic bag and microwaved (microwave power equal to 1200 W) for 1 s per gram of onion weight. After standing for about 20 min to allow the sample to cool, the onion half was transferred to a blender and water was added to bring the total weight to twice the original fresh weight. This onion half was then homogenised, filtered and centrifuged as with the raw half.

Colorimetric determination of pyruvic acid

Two different procedures were used. The first closely followed the procedure outlined by Schwimmer and Weston except that the volumes of all reagents were reduced by half. In this procedure a 100-fold dilution of onion juice was made by adding 10 µl of the clarified onion filtrate with a Drummond positive displacement pipetter to 0.5 ml of water in a 13 mm × 100 mm test tube. To this was added an additional 0.5 ml of water and 0.5 ml of 0.125 g L^{-1} DNPH in 2 M HCl. The samples were placed in a 37 °C water bath, then removed after 10 min and 2.5 ml of 0.6 M NaOH was added. The absorbance at 420 nm was then determined. Standards were prepared by adding 25–200 µl of 1 mM sodium pyruvate and reducing the amount of water in the assay accordingly.

For the second procedure, several modifications to the Schwimmer and Weston method were incorporated. In this modified assay, 25 ml of the clarified onion filtrate was added to 1.0 ml of water in a 13 mm × 100 mm test tube with a Drummond positive displacement pipetter. To this was added 1.0 ml of 0.25 g l^{-1} DNPH in 1 M HCl and the samples were placed in a 37 °C water bath. After 10 min the samples were removed from the water bath and 1.0 ml of 1.5 M NaOH was added. The absorbance at 515 nm was then determined. A blank and standards were prepared by adding 25 µl of sodium pyruvate solutions, ranging in concentration from 0 to 8 mM, instead of the onion sample.

The minimum time at 37 °C needed for full colour development using the modified procedure was determined to be 8 min. Heating for up to an additional 20 min had no effect on the final absorbance. After the addition of the NaOH, two problems were observed if the samples were allowed to sit for extended periods of time before determining the absorbance values. First, the product formed by reaction of DNPH with pyruvic acid is not stable and decays at about 2.5% per hour. Second, when samples of onion extracts were analysed using the modified procedure, a faint but noticeable turbidity formed when samples were allowed to sit for 1 h or more. For these reasons it is desirable to read the samples promptly after the addition of the NaOH.

RESULTS AND DISCUSSION

In the standard procedure described by Schwimmer and Weston, the amount of pyruvic acid–DNPH adduct formed is quantified by measuring the absorbance at 420 nm. However, the absorbance spectra of the DNPH reagent and the pyruvic acid–DNPH adduct (Fig 1) indicate that 420 nm is not the preferred wavelength for this measurement. The unreacted DNPH reagent (curve a) has a significant absorbance at this wavelength, and the peak of the absorbance of the DNPH–pyruvic acid adduct (curve b) occurs at 445 nm. For increased sensitivity and a lower blank it is preferable to determine the absorbance at a wavelength of 445 nm or higher. At 515 nm the background absorbance due to the reagent is very low, while the shoulder on curve b still gives good sensitivity towards the pyruvic acid–DNPH product.

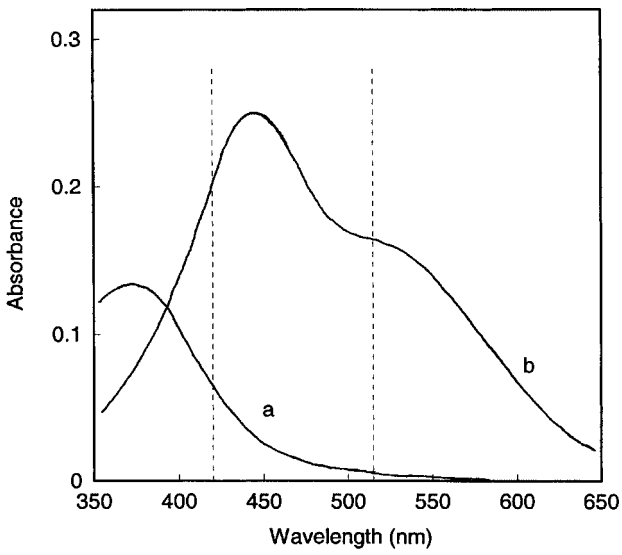

Figure 1. Absorbance spectra of DNPH reagent and pyruvic acid–DNPH adduct. Curve a is the absorbance spectrum of the reagent blank read versus water. Curve b is the absorbance spectrum of the pyruvic acid standard read versus the reagent blank. Broken vertical lines indicate 420 and 515 nm.

A second consideration in the choice of a wavelength for measurement is the possible presence of light-absorbing material other than the pyruvic acid–DNPH adduct. Visually, the diluted onion juice used for this assay appears to be colourless, implying that no interfering coloured material is being introduced with the juice. However, we have noticed that when this juice is made alkaline, as occurs in the final step of the pyruvic acid assay, it becomes yellow. This yellow material would interfere with any measurement at 420 nm. The amount of this coloured material was determined by assaying onion juice by either the standard Schwimmer and Weston method or as modified by Yoo *et al*[6] but with the omission of the DNPH from the reagents. Under these conditions the absorbance in the samples must be derived from material other than pyruvic acid in the onion. The absorbance spectra of these controls lacking DNPH (Fig 2A) indicate the presence of significant absorbing material at wavelengths below 500 nm, which is consistent with the observed yellow colour.

The extent to which coloured material in the onion sample interferes with the determination of pyruvic acid can be quantified by comparing the absorbance spectra obtained by the reaction of DNPH with a pyruvic acid standard and with a pyruvic acid-containing onion sample. If the absorbance in the onion sample is due entirely to the formation of the DNPH–pyruvic acid adduct, the two spectra should be the same and the ratio of the two should be a constant. A plot of this ratio (Fig 2B) shows that at wavelengths below 500 nm the ratio is not constant, which is consistent with the presence of other material in the onion sample absorbing light at wavelengths below 500 nm. At 420 nm the ratio of the two absorbance spectra is about 1.1, indicating that, for this particular onion sample, measuring the absorbance at 420 nm would overestimate the amount of pyruvic acid by 10%. The lack of interfering material at wavelengths above 500 nm also argues for using 515 nm as the wavelength for measurement.

A known deficiency with the standard Schwimmer and Weston procedure is the non-linearity of the pyruvic acid standard curve. We have also found this to be true (Fig 3). However, by increasing the concentration of DNPH in the assay and measuring the absorbance at 515 nm, this non-linearity can be eliminated (Fig 3). An increase in sensitivity is also obtained by reducing the final volume of the assay to 3.0 ml. By measuring the samples at 515 nm, the absorbance of the reagent blank remains low even though the final concentration of DNPH in the samples is more than five times higher than in the original procedure. The improved linearity of the modified assay is most likely the result of a higher DNPH/pyruvic acid ratio. In the original Schwimmer and Weston procedure and in various modifications the molar ratio of DNPH to pyruvic acid approaches 1:1 at the highest concentrations on the standard curve. Linearity in the reaction of DNPH and pyruvic acid has been reported to require at least a threefold excess of DNPH.[7] The non-linearity caused by a low DNPH/pyruvic acid ratio may be further exacerbated by the use of 420 nm for measurements. Since both the unreacted DNPH and the DNPH–pyruvic acid adduct absorb at this wavelength, the increase in

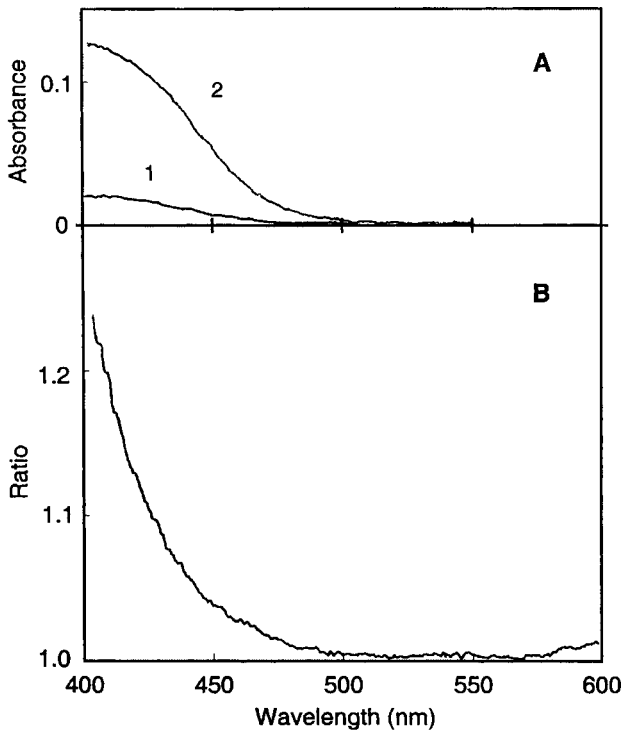

Figure 2. (A) Absorbance spectra of controls lacking DNPH. The sample for curve 1 was prepared by diluting an onion sample as described by Schwimmer and Weston;[1] the sample for curve 2 was prepared as described by Yoo *et al*.[6] In both cases the DNPH was omitted from the reagents. (B) The ratio of the absorbance of an onion sample to the absorbance of a pyruvic acid standard after reaction of each with DNPH.

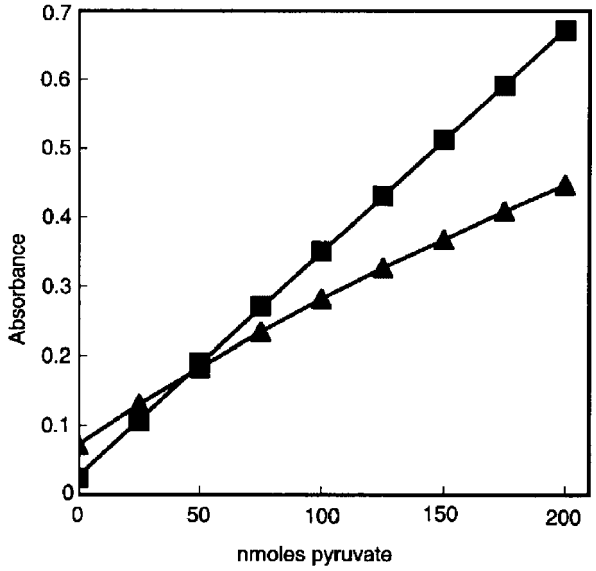

Figure 3. Standard curves for absorbance versus pyruvic acid: Squares, modified procedure with absorbances determined at 515 nm; triangles, standard procedure with absorbances determined at 420 nm.

Table 1. Comparison of total and background pyruvic acid concentrations for different onion types as determined using the Schwimmer and Weston and the modified procedures

	Pyruvic acid concentration ($\mu mol\, g^{-1}$)		
	Modified procedure	Schwimmer and Weston procedure	Difference
Total pyruvic acid			
White	6.54 ± 0.65	6.98 ± 0.69	0.44
Red	4.99 ± 0.89	5.91 ± 0.92	0.92
Yellow	10.81 ± 1.11	12.34 ± 1.17	1.52
Sweet Vidalia	3.61 ± 0.24	4.47 ± 0.24	0.86
Sweet Melody	3.43 ± 0.14	4.30 ± 0.15	0.87
Background pyruvic acid			
White	0.21 ± 0.01	0.26 ± 0.02	0.05
Red	0.38 ± 0.15	0.94 ± 0.22	0.56
Yellow	0.32 ± 0.13	1.27 ± 0.27	0.95
Sweet Vidalia	0.19 ± 0.01	0.76 ± 0.03	0.57
Sweet Melody	0.16 ± 0.02	0.78 ± 0.06	0.62

Values are mean $\pm$ S.D.

absorbance due to the formation of the adduct may be partially offset by a decrease in absorbance due to the depletion of the unreacted DNPH. Both problems are eliminated by increasing the DNPH concentration and measuring the absorbance at 515 nm.

The modified pyruvic acid method was tested on several onions and compared with the established method. In all cases the modified protocol yielded lower values than the standard Schwimmer and Weston procedure (Table 1). The higher apparent levels of pyruvic acid obtained using the standard method are consistent with the presence of interfering material at 420 nm. The samples which showed least disagreement in pyruvic acid determinations by the two methods were white onions. Consistent with this, we have found that white onions contain much less 420 nm absorbing material than yellow onions (data not shown). To test the reproducibility of the modified assay, five independent determinations were made on a single onion extract. The coefficient of variation in these pyruvate determinations was 1.7%. In addition, the recovery of known amounts of pyruvate added to the onion extracts was found to be $98.5 \pm 2.0\%$ in six experiments.

With yellow onion juice the presence of endogenous absorbing material leads to an overestimation of the pyruvic acid content of about $1.5\, \mu mol\, ml^{-1}$ when measurements are made at 420 nm (Table 1). While this difference is small, it has been reported that a difference of $1\, \mu mol\, ml^{-1}$ is perceptible when tasting onion bulbs.[8] Furthermore, some sweet onions have enzymatic pyruvic acid levels as low as $3\, \mu mol\, ml^{-1}$,[8] so an error of $1\, \mu mol\, ml^{-1}$ could be quite significant. This error is most significant in the determination of the background pyruvic acid levels. Reported values for this background pyruvic acid vary widely. One possibility, which would explain some of the reported variability, is that the amount of interfering material varies between different onion samples. The background levels determined using our modified protocol were all less than $0.5\, \mu mol\, g^{-1}$, which is in line with recent estimates made by HPLC.[9]

CONCLUSIONS

By changing the concentrations of the reagents and measuring absorbances at 515 rather than 420 nm, several deficiencies in the standard Schwimmer and Weston procedure for determining pyruvic acid in onions have been overcome. The greater sensitivity, lower background and improved linearity of the modified assay should make it more reliable for the routine determination of onion pungency. The modified procedure also eliminates the interference from endogenous absorbing material found in yellow onions. This method could easily be adapted for use in a microplate assay format[4] by simply reducing the volumes of the reagents and samples by a factor of 10.

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