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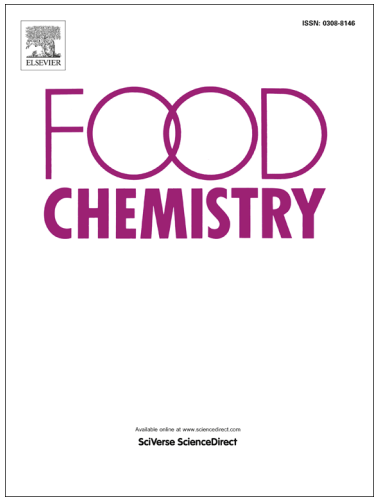

Short communication

Variability in spectrophotometric pyruvate analyses for predicting onion pungency and nutraceutical value

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Variability in spectrophotometric pyruvate analyses for predicting onion pungency and nutraceutical value

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Abstract

Onion pyruvate concentration is used as a predictor of flavor intensity and nutraceutical value. The protocol of Schwimmer and Weston (SW) (1961) is the most widespread methodology for estimating onion pyruvate. Anthon and Barret (AB) (2003) proposed modifications to this procedure. Here, we compared these spectrophotometry-based procedures for pyruvate analysis using a diverse collection of onion cultivars. The SW method always led to over-estimation of pyruvate levels in colored, but not in white onions, by up to 65%. Identification of light-absorbance interfering compounds was performed by spectrophotometry and HPLC analysis. Interference by quercetin and anthocyanins, jointly, accounted for more than 90% of the over-estimation of pyruvate. Pyruvate determinations according to AB significantly reduced absorbance interference from compounds other than pyruvate. This study provides evidence about the mechanistic basis underlying differences between the SW and AB methods for indirect assessment of onion flavor and nutraceutical value.

Keywords: Pyruvate, onion, *Allium cepa*, spectrophotometry, HPLC, pungency

1. Introduction

Onion (*Allium cepa* L.) is cultivated and consumed worldwide, mainly due to its distinctive odor and taste. In addition, various health-promoting effects have been associated with the consumption of onion and other *Allium* species, such as garlic and leek. Among them, antiplatelet, antihypertensive, antioxidant, hypoglycemic, anticancer, and hypolipidemic properties have been reported (reviewed by Corzo-Martínez, Corzo & Villamiel, 2007; and Block, 2010).

Allium flavor (pungency) constituents arise from interaction of the vacuolar enzyme alliinase with the cytoplasmic flavor precursors S-alk(en)yl-L-cysteine sulfoxides (ACSOs) after cutting or crushing of fresh tissues (Lancaster and Boland,1990). Alliinase-mediated cleavage of the ACSOs produces volatile thiosulfinates (TSs), pyruvate and ammonia (Block, 2010). TSs are responsible for the pungency (Macpherson et al., 2005) and, together with other sulfur-compounds derived from TSs degradation (for a comprehensive review on *Allium* biochemistry see Block, 2010), they contribute greatly to most of the health-enhancing properties of *Allium* (Corzo-Martínez et al., 2007). Because pyruvate and TSs are formed stoichiometrically in the ACSOs-alliinase reaction, pyruvate content correlates positively with pungency intensity (Wall & Corgan, 1992), and is used commonly as an estimator of the total TS content (Goldman, Kopelberg, Debaene & Schwartz, 1996). Therefore, pyruvate content is also used to predict both flavor intensity and nutraceutical value in onion and garlic.

To date, the most widespread method for estimating pyruvate levels in *Allium* has been the spectrophotometry-based protocol of Schwimmer and Weston (1961) (SW). For example, in onion, at least 56 scientific papers have used this procedure (Suppl. Table 1). Despite its general use, variation in pyruvate results among and within laboratories, using the SW technique and the

same lot of onion samples, was reported in a previous study, although the observed variations were attributed to factors other than the analytical method (Havey et al., 2002). The SW method is relatively rapid and inexpensive, advantages that have led to its widespread adoption. The method determines total 2,4-dinitrophenyl-hydrazine-reacting carbonyls, resulting from the addition of excess 2,4-dinitrophenyl-hydrazine (DNPH) to pyruvate-containing aqueous onion extracts. Color development in the solution, due to the formation of chromogenic DNPH-pyruvate adducts, is measured at 420 nm.

Anthon and Barrett (2003) (AB) proposed modifications to the SW method, specifically changes in reagent concentrations and the use of 515 nm (instead of 420 nm). The authors proposed that such modifications could improve linearity and sensitivity of the assay, and eliminate interferences at 420 nm from other compounds that may be present in onion bulbs.

Given the relevance of pyruvate determinations for inferring indirectly onion flavor and functional value, it is important to compare the two methods in a consistent and systematic way. Also, the identification of interfering compounds in onion bulbs (as proposed by AB), and quantification of their relative contribution to such interferences, would shed light on the mechanistic basis for this source of variation, providing a rationale for predicting the extent of methodology-based variations that can be expected when using either method for estimating onion pyruvate levels. Thus, the objectives of the present study were to: 1) compare the SW and AB spectrophotometry-based procedures for pyruvate analysis in a diverse collection of onion cultivars over three growing seasons; and 2) investigate sources of variation due to interfering compounds and, once identified, estimate their magnitude and contribution for different onion color types. Our results highlight the importance of examining critically the method of choice for estimating pyruvate levels, which is used to predict onion flavor and putative health benefits.

2. Materials and methods

2.1. Plant materials

Eleven Argentine onion cultivars (Galmarini 2000) were characterized for their bulb pyruvate concentration using the methods of Schwimmer and Weston (1961) (SW) and Anthon and Barret (2002) (AB). Pyruvate levels were determined during three growing seasons, 2012, 2014 and 2015. Nine cultivars were grown at the experimental station of INTA La Consulta (Mendoza, Argentina), using conventional agricultural practices, whereas cultivars "Morada1" and "Morada2", two phenotypically different red onions were obtained from a local market during 2012 and 2015.

2.2. Processing of samples

Sample processing and preparation of aqueous extracts from onion bulbs were performed as previously described by Galmarini et al. (2001) and Cavagnaro & Galmarini (2012). Briefly, three replicates composed of five bulbs were used. The outer dry scales were removed and the onions cut in half longitudinally. One half of each bulb was bulked and juiced in a 1:1 volume (w/v) of distilled water using a blender (Braun MR 400 Plus, Kronberg, Germany). The homogenates were then filtered, centrifuged, and the clear supernatants were stored at -20°C.

2.3. Pyruvate content analyses

Both the methods of SW and the AB were used strictly as indicated by the authors. Briefly, for the SW method, 2 mL of diluted extracts were added to 1 mL of 0.0125% DNPH in 2 M HCl and incubated at 37°C for 10 min before 5 mL of NaOH 0.6 mol/l was added. The absorbance at 420

nm was measured using a Beckman DU Series 500 UV/Visible spectrophotometer (Beckman Coulter Inc., Brea, USA). Pyruvate concentrations were calculated using standard curves for sodium pyruvate (Sigma ultra 99%, Sigma-Aldrich, Buenos Aires, Argentina), performed independently for each method, and expressed as µmol pyruvate/g fw.

The AB procedure included the following modifications to the SW protocol: DNPH concentration was increased from 0.0125% to 0.25%; final sample volume was reduced; the NaOH concentration, which is used to stop the DNPH-pyruvate reaction, modified from 2.5 mL of 0.6 mol/l NaOH (in SW) to 1 mL of 1.5 mol/l NaOH; and the absorbance wavelength used was 515 nm not 420 nm.

2.4. Analysis of total flavonoids

Total flavonoids content was estimated in 11 onion cultivars (Table 1) during 2012 and 2015, according to Yang, Meyers, Van der Heide & Liu. (2004). Flavonoid content was calculated using a quercetin standard curve and expressed as mg quercetin equivalents % g fw.

2.5. Light absorbance interference by compounds different from pyruvate

In order to quantify the absorbance of compounds other than pyruvate (i.e., the absorbance of onion compounds that may interfere with pyruvate determinations), aqueous extracts from three white (Refinta 20, Alfredo, Antártica), three yellow (Grano de Oro, Valcatorce, Navideña), and two red (Morada1, Morada2) cultivars were used. Five replicates per cultivar were prepared as described above. Absorbance was measured as described above but without the addition of DNPH to the reaction mixture, in order to avoid formation of yellow DNPH-pyruvate adducts. Since colorless pyruvate (i.e., pyruvate not bond to DNPH) does not absorb light at the

wavelengths used by either methods, only compounds absorbing light at 420 nm (SW) and 515 nm (AB) contributed to the readings. These compounds represent a source of error in pyruvate determination. Absorbance values obtained with both methods were compared. In addition, absorption spectra (400-550 nm) for white, yellow and red onion extracts, as well as for the flavonoid quercetin, with and without the addition of NaOH (SW, 0.6N and AB, 1.5M), were characterized.

2.6. Identification of light-absorbance interfering compounds

To test if the interference observed in pyruvate determinations of colored onion extracts was due to flavonoid compounds, the following was performed: Extracts of a white onion cultivar (Refinta20), characterized for its very low quercetin content and no anthocyanins, were spiked with quercetin and/or anthocyanins to a final concentration equivalent to that found for these flavonoids in a yellow (Valcatorce; 41.7 mg quercentin % g fw) and a red (Morada1; 80.7 mg quercentin % g fw, 23 mg cyanidine % g fw) onion cultivars. A commercial standard of cyanidine-3 glucoside (Sigma-Aldrich, St. Louis, USA) was used for the anthocyanin assay. Absorbance was measured at 420 nm. Total anthocyanins content was measured spectrophotometrically according to Fulecky and Francis (1968). Quercetin was considered to represent ~85% and ~30% of the total flavonoids content of yellow and red onions, respectively, as indicated in a previous study (Slimestad, Fossen & Molund Vagen 2007).

2.7. HPLC analysis of interfering compounds

Pure standards (Sigma > 95%, Sigma-Aldrich, St. Louis, USA) for seven phenolic compounds commonly found in onion [quercetin, myricetin, kaempferol, rutin, catechin, epicatechin gallate

(ECG), and epigallocatechin gallate (ECGC)] were analyzed using high performance liquid chromatography (HPLC). The resulting absorbance data were used to plot absorption spectra between 400 and 540 nm for all the compounds. Additionally, bulb contents for these seven phenolic compounds were estimated, using HPLC analysis, in five yellow (Valcatorce, Cobriza, Valuno, Angaco, Navidena) and three white (Ancasti, Antartica, Refinta 20) onion cultivars (three replicates of eight bulbs per cultivar were analyzed), and 10 yellow-, 16 red- and 11 white-bulb onions from an F_2 population, following methods described previously (Insani et al., 2016).

2.8. Reproducibility and linearity

Twenty samples of a yellow cultivar were analyzed according to the SW and AB methods. Coefficients of variation (CV) values were calculated from absorbance tests and used to infer reproducibility. To compare the extent of linearity in pyruvate-absorbance dose-response curves when using the SW and AB methods, pyruvate standard solutions with a concentration range of 0.01-0.9 mM were prepared and absorbance measured at 420 and 515 nm. The concentration range below which the dose-response curve was linear was used as a criterion for comparing the two procedures.

2.9. Statistical analysis

Analysis of variance (ANOVA) and correlations were performed using InfoStat version 2014 software for Windows 4.0 (Di Rienzo, Casanovesm Balzarini, Gonzalez, Tablada & Robledo 2014). Means comparisons were performed by the least significant difference (LSD Fisher) test and P values <0.05 were considered significant.

3. Results and discussion

Comparison of pyruvate levels estimated by SW and AB methods

The SW method yielded higher pyruvate values than the AB method for all the cultivars across all three years studied (Table 1). Regardless of year and cultivar, the SW method generated significantly higher pyruvate values than the AB method for 10 of the 31 (32.2%) samples evaluated. Percent relative difference between both methods varied from 3.6 to 65.6%, depending on the cultivar and year. On average, pyruvate levels measured with the SW method were 33.6%, 18.5%, and 15.7% higher than the respective mean AB values for 2012, 2014, and 2015, and such differences were always significant (*P* values ranged from 0.0001 to 0.045).

Comparative analysis of both procedures by bulb color revealed significant differences in pyruvate concentration for different onion colors (Fig. 1). Differences between the methods (SW-AB) were significantly higher for colored onions compared to white onions across the three years studied. Methodological differences were 2-3 fold higher in colored onions than in white onions, suggesting that colored onions contain compounds other than pyruvate that absorb light at 420 nm, the wavelength used for pyruvate determinations in the SW procedure, but not at 515 nm, the wavelength used by AB. Presumably, in white onions, the levels of such compounds (i.e., compounds that preferentially absorb light at ~ 420 nm) is less than in colored onions, since pyruvate concentration measured using either methods were more alike in the former (Fig. 1).

The extent of differences found between the methods was correlated positively with flavonoid content for years 2012 (r=0.52, *P*=0.003), 2014 (r=0.46, *P*=0.041), and 2015 (r=0.56, *P*<0.001), suggesting that onion flavonoids interfere with pyruvate determination when using the SW procedure.

The absorbance of onion compounds other than pyruvate that might interfere with pyruvate determination was estimated in white, yellow and red onions (Fig. 2). Light absorbance varied substantially and significantly between both methods for yellow and red onion cultivars. For these colored onions, absorbance readings using the SW method were four-fold (for cv. Morada1) to 22-fold (for cv. Grano de oro) higher than the respective readings obtained with the AB method. Conversely, for white onions, differences between absorbance readings obtained with both methods were small and not statistically different from each other. It should be noted that, with only one exception (Morada1), all the absorbance readings obtained at 515 nm, using the AB method, were very small and statistically similar, regardless of bulb color (Fig. 2).

Altogether, these data suggest that yellow and red onions contain high levels of compounds that, other than pyruvate, absorb light at 420 nm. Thus, this absorbance, which accounted for 12.8 to 48.6 % of the total absorbance of colored onions using standard pyruvate determinations (Fig. 2), represents a significant source of errors in pyruvate determinations when using the SW method. These errors, which led to substantial pyruvate over-estimation in yellow and red onions by the SW method, were not observed in white onions, suggesting that white onions have very low content of compounds that, other than pyruvate, absorb light at 420 nm.

The differences in absorbance observed between white and colored onions, due to compounds other than pyruvate, were investigated further. Figure 3 presents absorption spectra for white, yellow and red onion extracts, as well as for the flavonoid quercetin, with and without the addition of NaOH in concentrations, as used by the SW (0.6 M) and AB (1.5 M) methods. In extracts of yellow and red onions, a progressive and substantial increase in light absorbance was observed for wavelengths < 475 when the onion juice was alkalinized. For wavelengths greater

than 475 nm, absorbance readings became progressively smaller as wavelength increased, for both the standard and alkalinized extracts, with negligible differences observed between the two at 515 nm. The same trend was observed in the absorption spectra of quercetin, the most abundant flavonoid found in yellow and red onions. These results suggest that quercetin might interfere with pyruvate determinations when using absorbance wavelengths shorter than 475 nm. Conversely, in white onion, minimal variation in absorbance was observed across the wavelength range assayed, and were unaffected by alkalinization. Altogether, these data indicate that at 515 nm (wavelength used by AB), there is little interference by compounds other than pyruvate, regardless of bulb color and alkalinization, whereas, at 420 nm (SW), there is substantial interference in extracts from yellow and red onions (but not in white onions), presumably due to quercetin and perhaps other flavonoids.

Identification of interfering compounds

Yellow coloring in onion bulbs is due mainly to quercetin (Rhodes and Price, 1996) whereas red onions accumulate -in addition to quercetin- anthocyanins (Fossen et al., 1996). To test whether interference observed at 420 nm in colored onions was due mainly to these flavonoids, white onion extracts were spiked with quercetin and or anthocyanins (see materials and methods, Section 2.6). It was found that quercetin content explained all the interference in yellow onion Valcatorce, as evidenced from the statistically similar absorbance in Valcatorce and the spiked extracts (Fig. 4). In the red onion Morada1, quercetin and anthocyanin content together explained most of the interference, although not all of it, as indicated by the higher absorbance of Morada1 compared to spiked extracts. Interestingly, anthocyanins had minimal contribution to this interference (Fig. 4). So, estimation of the interference was performed for all colored onions.

'Anthocyanin + quercetin' content explained 89-96% of the interference, with quercetin being the main compound responsible in both yellow and red onions, whereas anthocyanins accounted for only around 7% of the interference in red onions (yellow onions contain no anthocyanins).

Interferences from quercetin and six other phenolic compounds naturally present in onion bulbs were examined by HPLC. Analysis of their absorption spectra revealed that quercetin, myricetin, kaempferol, and rutin (the latter in minimum amounts) absorb at 420 nm and could, therefore, interfere with pyruvate determinations using the SW method whereas catechin, ECG, and EGCG had no absorption at this wavelength (see Fig. 1 in Beretta et al. 2016). At 515 nm (AB method), none of the phenolic compounds analyzed absorbed light. Although myricetin, kaempferol and rutin absorbed light at 420 nm and could, therefore, be considered as potential interfering compounds in the SW method, their concentrations in onion bulbs of all colors was very low (see Fig. 2 in Beretta et al. 2016). Thus, their contribution to the interference was minimally. In yellow and red onions, the over-abundance of quercetin, relative to other 420 nm light-absorbing polyphenols, explains why the former is the main interfering compound in the SW procedure. Conversely, traces amounts of quercetin and myricetin, and very low content of kaempferol and rutin, were generally found in white onions, explaining why no significant differences between the pyruvate methods were found with white onions (Table 1).

Reproducibility and linearity

The method of AB (CV=0.88%) was less variable and, thus, more reproducible than the SW method (CV=4.97%). Also, linearity was improved, as indicated by the broader range of pyruvate concentrations over which the standard curve remained linear for the AB method (0–

0.40 mM) compared to the SW method (0–0.20 mM), and by the better adjustment of pyruvate standard solutions to a linear model in the AB procedure (r^2=0.94) as compared to SW (r^2=0.55). Traditionally, the SW procedure has been the most widely used method for estimating onion pyruvate levels. The present study demonstrated that, using the SW procedure, always led to an over-estimation of pyruvate levels in yellow and red onions, and that such a source of variation could be significant, as observed in ca. 42% of colored onion samples analyzed, and substantial, reaching ca. 65% over-estimation in some (Table 1). Our data indicate strongly that quercetin is the main compound interfering with pyruvate determinations when using the SW procedure in both yellow and red onions, with anthocyanins contributing minimally to the interference. Although we identified three other onion phenolic compounds, besides quercetin and anthocyanins, which absorb light at 420 nm and might therefore interfere with SW pyruvate determination, their concentrations in the onions bulbs of all colors was, generally, very low, meaning their contribution to the interference was minimally. This study provides evidence on the mechanistic basis underlying differences between SW and AB methods for the indirect assessment of onion flavor and nutraceutical value.

Acknowledgments

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Appendix A. Supplementary data:

Table S1. List of publications using the SW procedure for estimating onion pyruvate content.

Abbreviations used

AB: Anthon and Barrett (2003); SW: Schwimmer and Weston (1961); ACSOs: S-alk(en)yl-L-cysteine sulfoxides; TSs: thiosulfinates; fw: fresh weight; CV: coefficient of variation;, w/v: weight/volume, DNPH: 2,4–dititrophenylhidrazine.

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Figure captions

Figure 1. Differences between pyruvate levels obtained by the SW and AB procedures for different onion colors in three growing seasons. Differences were calculated as SW – AB.

Figure 2. Light absorbance of onion extracts due to compounds different from pyruvate, at wavelengths typically used for pyruvate analysis in the SW (420 nm) and AB (515 nm) methods. Asterisks denote statistically different absorbance values between methods for each cultivar ($P<0.05$). Numbers above each bar indicate the percentage that each absorbance value represents

relative to the total absorbance obtained in the standard pyruvate analyses (i.e., with the addition of DNPH) for each method and cultivar.

Figure 3. Absorption spectra for white, yellow and red onion extracts, as well as for the flavonoid quercetin, with and without the addition of NaOH in concentrations used by the SW (0.6N) and AB (1.5N) methods. Vertical lines indicate absorbance wavelengths used by SW (420 nm) and AB (515 nm) methods.

Figure 4. Absorbance at 420 nm of a white (cv. Refinta 20), a yellow (cv. Valcatorce), and a red (cv. Morada1) onion extract, and of the white onion extract additioned with quercetin and/or anthocyanins to a final concentration equivalent to that found in the yellow and red onion extracts, respectively. Bars with no common letters differ; $P < 0.05$.

Conflict of interest statement

The authors of this study declare no conflict of interest.

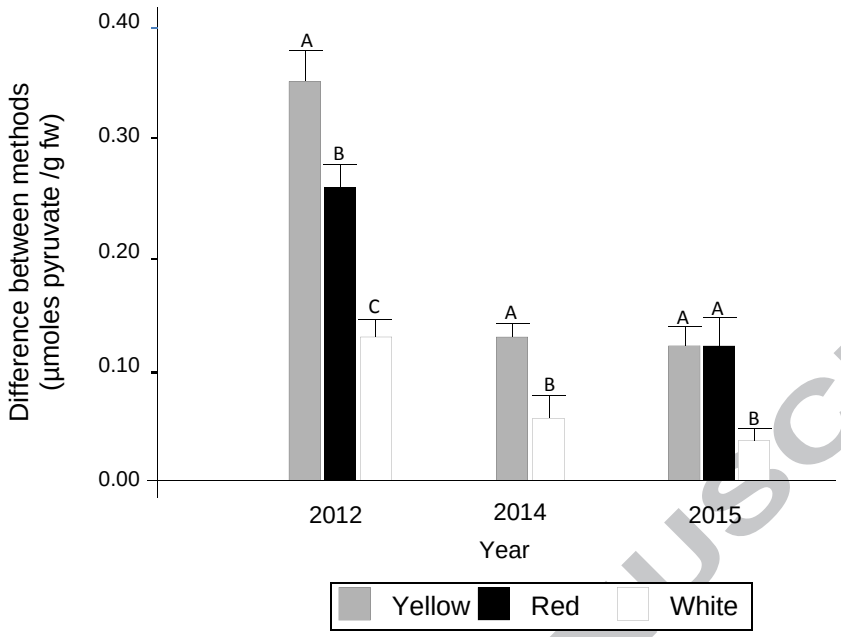
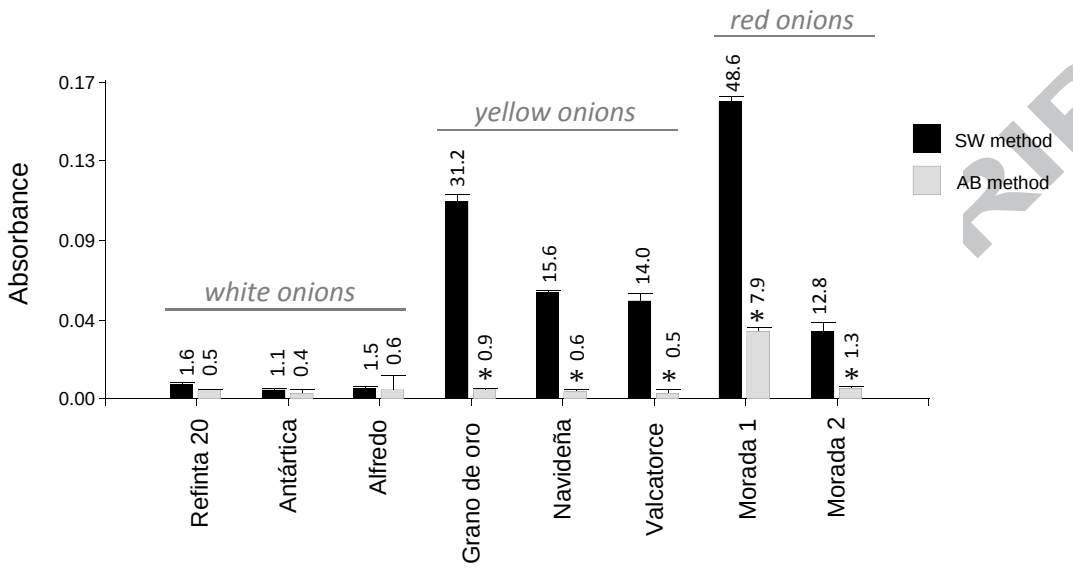


Figure 1.



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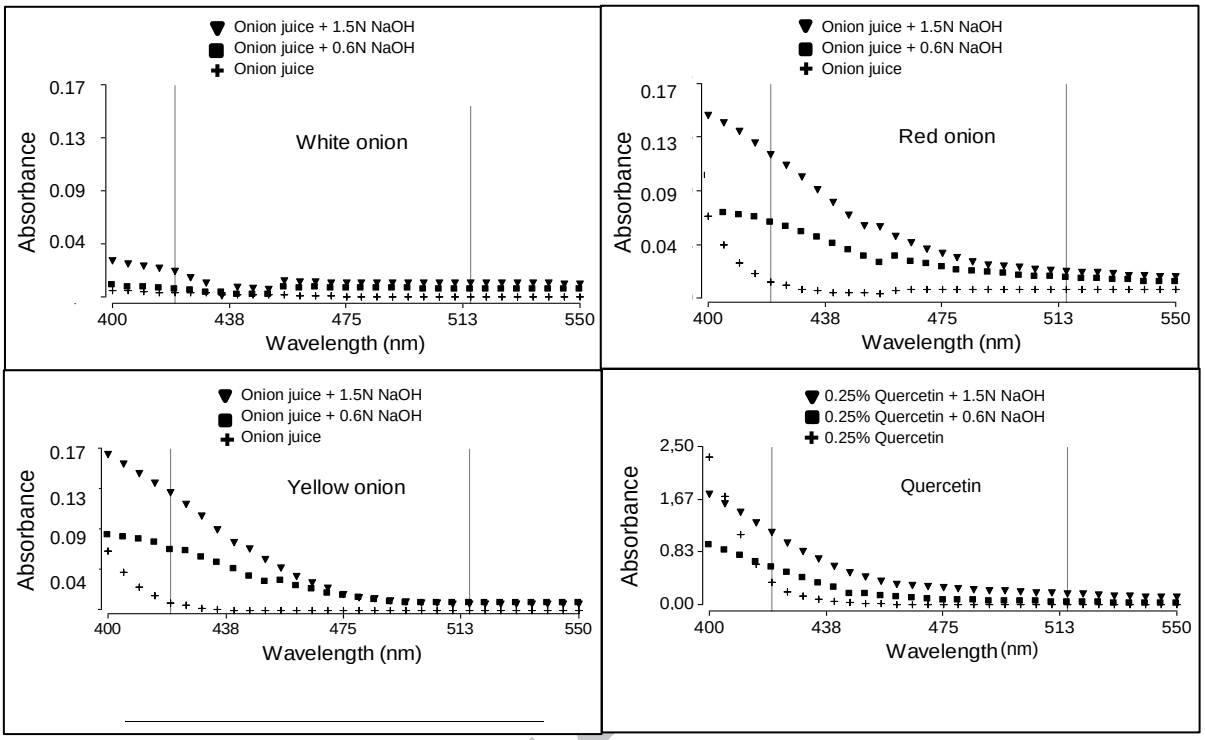

Figure 3

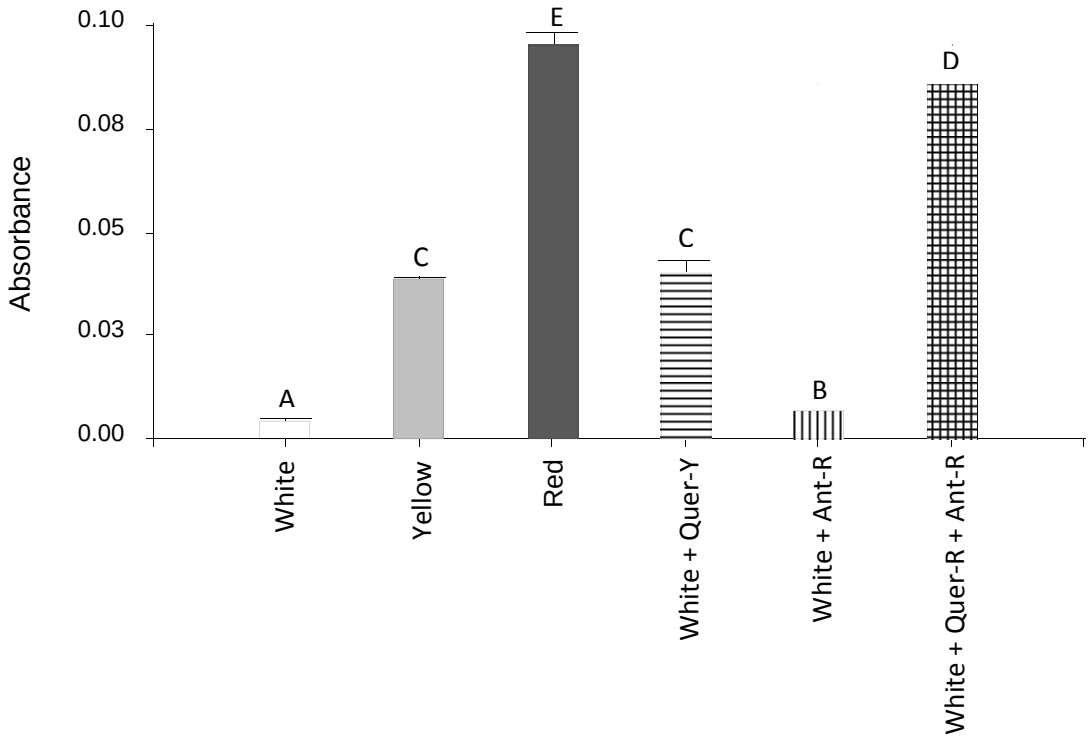

Figure 4

Table 1. Comparison of bulb pyruvate levels determined by the SW and AB procedures for eleven onion cultivars in three growing seasons.

			Bulb pyruvate concentration (µmoles/g fw)											
			2012				2014				2015			
Cultivar	Bulb color*	Photoperiod requirement §	SW	AB	Diff. (SW-AB)	% RD	SW	AB	Diff. (SW-AB)	% RD	SW	AB	Diff. (SW-AB)	% RD
Refinta 20	white	long day	13.50 ± 0.65	12.30 ± 1.06	1.20	10.24	13.0 ± 0.20	11.70 ± 0.50	1.40	11.93	10.18 ± 0.41	9.82 ± 0.60	0.36	3.67
Antártica	white	long day	12.40 ± 0.91	11.10 ± 0.85	1.30	12.02	8.00 ± 0.29	7.00 ± 1.22	10.20	14.52	9.04 ± 2.02	8.60 ± 1.81	0.44	5.13
Alfredo	white	intermediate day	11.70 ± 0.08	10.60 ± 0.09	1.20	11.09	9.75 ± 1.01	9.03 ± 0.84	0.72	7.98	9.06 ± 0.17	8.83 ± 0.17	0.22	2.59
Cobriza	yellow	long day	12.40 ± 1.01	9.30 ± 0.61	**3.10**	**33.50**	7.49 ± 0.45	5.91 ± 0.33	**1.57**	**26.64**	9.27 ± 1.15	7.97 ± 0.42	1.29	16.29
Grano de oro	yellow	long day	10.50 ± 1.02	7.20 ± 0.97	**3.20**	**45.35**	6.56 ± 0.46	5.91 ± 0.57	0.64	10.92	6.64 ± 0.89	5.75 ± 0.67	0.89	15.61
Valcatorce	yellow	long day	11.80 ± 0.61	7.40 ± 0.62	**4.40**	**61.04**	8.89 ± 1.62	7.25 ± 1.41	1.63	22.59	7.98 ± 0.42	6.71 ± 0.50	**1.27**	**18.90**
Valuno	yellow	long day	11.20 ± 0.26	6.80 ± 0.68	**4.50**	**65.64**	6.74 ± 0.68	5.73 ± 0.69	0.10	17.55	6.41 ± 0.56	5.87 ± 0.58	0.53	9.05
Navideña	yellow	intermediate day	11.50 ± 1.01	9.50 ± 0.86	2.00	21.31	6.03 ± 0.47	5.10 ± 0.23	0.93	18.25	5.64 ± 0.23	4.78 ± 0.29	**0.86**	**17.98**
Angaco	yellow	short day	10.22 ± 0.93	7.90 ± 0.69	**2.30**	**29.65**	5.66 ± 0.72	4.14 ± 0.28	**1.51**	**36.41**	6.75 ± 0.08	4.52 ± 0.03	2.23	49.33
Morada 1	red	nd	9.40 ± 0.55	6.60 ± 0.11	**2.80**	**43.07**	nd	nd	nd	nd	9.19 ± 1.43	7.64 ± 1.10	1.55	20.24
Morada 2	red	nd	8.50 ± 1.34	6.20 ± 1.04	2.30	37.08	nd	nd	nd	nd	6.54 ± 0.51	5.73 ± 0.32	0.81	14.19

Values are mean ± SD of three biological replicates. Significant differences (P≤0.05) between the Schwimmer and Weston (SW) and Anthon and Barret (AB) procedures are depicted in bold.
% RD: Percent Relative Difference, as calculated by the formula (SW-AB) x 100 / AB.
*Refers to colors of both, outer dry scales and inner scales. § Classification based on the photoperiod requirement for bulbification (long day: 14 hours, intermediate: 13 hours, short day: 12 hours). nd: no data available.

Highlights

- Two spectrophotometry-based procedures for onion pyruvate analysis were compared
- The most widespread method always over-estimated pyruvate levels in colored onions
- Quercetin and anthocyanins were responsible for nearly all of pyruvate over-estimations
- The pyruvate method is important for inferring onion flavor and functional value