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A sensitive HPLC-FLD method combined with multivariate analysis for the determination of amino acids in L-citrulline rich vegetables



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ABSTRACT

The proposed analytical method reports the separation and quantification of 21 amino acids including L-citrulline from fresh vegetables and commercial juices using a C₈ column. Optimal separation conditions for amino acids analysis were obtained with 20 mM sodium acetate (solvent A) and water with organic modifier acetonitrile and methanol (solvent B; 18/50/32 V/V). The ideal pH and column temperature were found to be 5.40 and 35 °C, respectively. The LOD and LOQ values were obtained in the range of 0.02–0.19 ng/mL and 0.04–0.39 ng/mL for all amino acids respectively. Relative standard deviations (RSD) of intraday and interday analysis were found to be <2.7% and 7.9%, respectively. The recovery of amino acids were found to be satisfactory for all the tested crops. The developed method was successfully used for the quantification of amino acids in six fresh vegetable juices including watermelon, cucumber, celery, calabaza squash, zucchini squash, yellow squash and commercial juices. Multivariate analysis was used to determine the significant differences in the amino acids profiles. L-citrulline content was highest in fresh watermelon juice (716.57 ± 24.80 µg/mL) and commercial watermelon lime juice (826.48 ± 34.48 µg/mL). The optimized analytical method is rapid, sensitive, accurate and reproducible for analysis of free amino acids including L-citrulline from different vegetable juices and other food products. To the best of our knowledge, this is the first report to separate OPA derivatives of amino acids using C₈ column from watermelon, cucumber, zucchini squash, yellow squash, calabaza squash, and celery in a HPLC-FLD system.

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1. Introduction

Juices have become a popular option to increase consumption of fruits and vegetables due to various health beneficial properties with unique taste and flavor [1–4]. Free amino acids influence the aroma, taste, and color of juices and are determinants of quality [5–7]. Amino acids are also beneficial for human health and are involved in numerous biological processes. They have functional properties including protein synthesis, cell signaling, cellular metabolism, immune response and some act as antioxidants [8]. For example, L-citrulline is a non-essential amino acid engaged in interorgan metabolism [9]. It is also involved in the production of nitric oxide a signaling molecule and potent vasodilator [10]. The Cucurbitaceae family, has been found to be a source of L-citrulline [11]. Analysis of L-citrulline and other free amino acids in juices is important for the determination of quality and health benefits.

Various analytical techniques, capillary electrophoresis, gas chromatography, qNMR and liquid chromatography have been utilized for the analysis of amino acids in a variety of samples [12,13]. Reversed-phase high pressure liquid chromatography (RP-HPLC) is a commonly used technique in the analysis of amino acids [6,14,15]. Few methods have been reported for the analysis of underivatized amino acids [16,17] and due to the lack of a suitable chromophore the separation of amino acids in biological samples remains challenging [18–20]. Moreover, the separation of amino acids are challenging due to the presence of acidic, basic, and neutral behavior and their critical pairs. The critical pairs are closely eluting amino acid derivatives such as glycine/arginine, alanine/ β -alanine, and methionine/valine [21,22]. Amino acid detection has been performed using fluorescence (FLD), UV, and diode array detector (DAD) by RP-HPLC and is usually carried out after pre-column derivatization [18,23]. Typically used reagents for amino acid derivatization are 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), 9-fluorenylmethyl-chloroformate (FMOC-Cl), phenylisothiocyanate (PITC), dansyl-chloride, and O-phthalaldehyde (OPA) [6,14,18,24]. Derivatization with dansyl-chloride, PITC, and FMOC involve complex and time consuming sample preparations [19]. OPA derivatization involves simple sample preparation and can be carried out rapidly at 25 °C.

Quantification of L-citrulline using HPLC is usually carried out in biological samples such as plasma, urine, and cerebrospinal fluid [25,26]. Few methods have been reported on the analysis of amino acids L-citrulline from cucurbits. Amino acids quantification from watermelon by fluorescence detection using O-phthalaldehyde-N-isobutyryl-L-cysteine (OPA-IBLC) derivatives has been carried out, however L-citrulline was not analyzed and the run time was over 70 min [27]. Dansyl chloride derivatives and HPLC-DAD have also been used for the analysis of L-citrulline in watermelon and other cucurbits including cucumber and squash [28,29]. The rapid determination of L-citrulline from watermelon using FMOC and HPLC-PDA has been reported, however this method was limited to only detecting L-citrulline [16].

To the best of our knowledge, no convenient analytical methods using OPA-derivatives coupled with HPLC-FLD are reported for the routine analysis of free amino acids including L-citrulline from the Cucurbitaceae family using C8 column. The present study reports an optimized HPLC-FLD method using automated precolumn OPA derivatization for the analysis of free amino acids in six vegetables including watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), zucchini squash (*Cucurbita pepo*), yellow squash (*C. pepo*), calabaza squash (*C. pepo*), celery (*Apium graveolens*) and also five commercial juice blends.

2. Materials and methods

2.1. Chemicals and reagents

Amino acid standards, sodium acetate trihydrate, hydrochloric acid, 2-mercaptoethanol, HPLC grade methanol and acetonitrile were obtained from Sigma Aldrich (St. Louis, MO, USA). β -alanine and OPA were purchased from TCI Chemicals (Portland, OR, USA). L-ornithine, glacial acetic acid, Brij-35 and sodium borate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HPLC-grade water (resistivity 18.2 m Ω -cm) was obtained from a Nanopure water purification system (Barnstead, Dubuque, IA).

2.2. Fresh vegetable juice sample preparation

Fresh watermelon (2726 g), cucumber (331 g), zucchini squash (133 g), yellow squash (187 g), calabaza squash (104 g), and celery (531 g) were obtained from a local super market (College station, TX, USA). All vegetables were rinsed thoroughly with tap water followed by nanopure water. Vegetables were chopped and passed through an Omega 8006 Nutrition System HD Juicer (Omega Products, Inc., Harrisburg, PA). The vegetables juices were homogenized, sonicated for 30 min and centrifuged at 7826 \times g for 10 min and supernatants were passed through Whatman filter paper No. 1. The obtained filtrates final volumes were as follows 2400 mL (watermelon), 251 mL (cucumber), 57 mL (zucchini squash), 77 mL (yellow squash), 41 mL (calabaza squash), and 413 mL (celery). Samples were diluted with nanopure water (1:100) then filtered through a 0.40 μ m cellulose syringe filter for HPLC analysis. The samples were used for optimization and validation of the analytical method.

2.3. Commercial juice samples

Commercial juices such as blends of ginger, beetroot and carrot (J-1), carrot and beetroot (J-2), kale and pineapple (J-3), kale, apple and lemon (J-4), and watermelon and lime (J-5) were purchased at a Whole food market (Houston, TX, USA). Samples were kept sealed at 4 °C until analysis. Sample aliquots (1 mL) were diluted with nanopure water J-1 and J-2 (1:20) and J-3 through J-5 (1:10) and centrifuged at 7826 \times g for 10 min and passed through a 0.40 μ m cellulose syringe filter before HPLC analysis. Samples were store at –80 °C until the analysis.

2.4. Preparation of OPA reagent

Derivatization with OPA was carried out using a slight modification of the method according to Wu and Menninger [26]. Briefly, 50 mg of OPA was first dissolved in 1.25 mL of HPLC grade methanol, followed by 11.1 mL of 40 mM sodium borate, 0.5 mL of 3.1% Brij-35 and 50 μ L of 2-mercaptoethanol (ME).

2.5. HPLC-FLD system

Amino acids were separated using a HPLC system comprised of a PerkinElmer Series 200 binary pump and autosampler (Shelton, CT, USA). A Gastorr TG-14 inline degasser (GenTech Scientific Inc., USA) and an Eppendorf TC-50 controller with a CH-30 column heater (Westbury, NY, USA). Detection was carried out by a 1260 Infinity fluorescence detector controlled by an Instant Pilot model G4208A (Agilent Technologies, Santa Clara, CA, USA). The system was aligned by a PE Nelson 900 interface and a PE Nelson 600 Link box.

2.6. Stationary phase and solvent system

Various stationary phases were evaluated to determine optimal amino acids separation. The physicochemical properties of the different stationary phases used for the analysis of amino acids are presented in Table S1. The mobile phase consisted of (A) 20 mM sodium acetate buffer, and (B) acetonitrile/methanol/water (50/32/18). A 28 min separation of the amino acids was achieved by the following gradient program, isocratic 20% B for 4 min, gradually increase from 20% to 32% B within 2 min, 32%–34% B within 4 min, followed by increase from 34% to 38% B (3 min), then linearly increase up to 95% B within 9 min, kept isocratic 5% B for 2 min, then return to initial condition 20% B within 1 min and remain isocratic for 4 min. The flow rate was 0.6 mL/min and the injection volume was 5 μ L. The derivatization procedure was automated; prior to sample injection, aliquots of 100 μ L of OPA were taken up by the autosampler and added to HPLC vials containing juice samples or amino acid standards solution. The OPA-sample mixture was incubated for 2 min at 25 ± 1 °C. The reaction mixture was then immediately analyzed by HPLC-FLD. The excitation and emission of the fluorescence detector were set at 340 nm and 455 nm respectively for the detection of amino acids. The data was processed by TotalChrom version 6.3.2 software.

2.7. Method optimization

Optimization of the HPLC method parameters is essential for reliable and reproducible analysis of amino acids. The peak shape, the retention times, and system pressure is greatly influenced by the pH of mobile phase and temperature, especially during amino acid analysis. Therefore, the effect of different pH and temperatures were evaluated in this study.

2.7.1. Effect of pH

The pH of the mobile phase plays a significant role in the separation of amino acids. The effects of different buffer pH

(5.40, 5.60, and 5.80) on separation and resolution of amino acids were studied. The mobile phase 20 mM sodium acetate was prepared and filtered with 0.45 μ m filter paper and used for HPLC analysis.

2.7.2. Column temperature

Column temperature greatly influenced the retention time (RT), selectivity and resolution of amino acids especially critical pairs. Different column temperatures (25, 30, 35, and 40 °C) were used to study the chromatographic separation of amino acids.

2.8. Validation of developed method

2.8.1. Calibration curve, linearity, LOD and LOQ

Stock solutions of 21 amino acids, L-aspartic acid ($\geq 98\%$), L-glutamic acid ($\geq 99\%$), L-asparagine ($\geq 98\%$), L-histidine ($\geq 98\%$), L-serine ($\geq 99\%$), L-glutamine ($\geq 99\%$), L-citrulline ($\geq 99\%$), L-arginine ($\geq 99\%$), glycine ($\geq 99\%$), L-threonine ($\geq 98\%$), β -alanine ($\geq 99\%$), L-alanine ($\geq 98\%$), L-tyrosine ($\geq 98\%$), L-tryptophan ($\geq 98\%$), L-valine ($\geq 98\%$), L-methionine ($\geq 98\%$), L-phenylalanine ($\geq 98\%$), L-isoleucine ($\geq 98\%$), L-leucine ($\geq 98\%$), L-ornithine ($\geq 99\%$), and L-lysine ($\geq 98\%$) were prepared with 0.1N HCl and nanopure water (1 mg/mL). The stock solutions were further diluted to make a final concentration of 2.5 μ g/mL. The final concentrations were then serially diluted 0.08, 0.16, 0.32, 0.64, 1.28 μ g/mL for the calibration curve. The linearity of the calibration curves of different amino acids were evaluated by injecting 5 μ L of the serially diluted standard solutions. The calibration graph was obtained by plotting the HPLC peak area against their concentrations. The limit of detections (LOD) and limit of quantifications (LOQ) were determined with signal to noise ratios (S/N) of 3 and 10 respectively.

2.8.2. Precision

The precision of the optimized method was evaluated by repeatability (intraday precision) and intermediate precision (inter-day precision) of standard amino acids mixtures and watermelon juice samples. The repeatability of the method was evaluated by the relative standard deviation (% RSD) of the individual peak area obtained from predetermined consecutive injections ($n = 5$) performed on each day over a period of 3 consecutive days. The % RSD of the concentration and retention time of standards and watermelon sample were determined for the all the injections.

2.9. Matrix effect on recovery of amino acids

The influence of different vegetable matrix on extraction and quantitation of amino acids was evaluated. Freshly prepared vegetable juice samples were mixed with 100 μ g/mL and 1000 μ g/mL of standard 21 amino acids. The spiked samples were vortexed for 30 s, homogenized for 2 min, and sonicated for 30 min. Subsequently, samples were centrifuged ($7826 \times g$), filter through Whatman filter paper No. 1 and diluted with nanopure water (1:100). The resulting solutions contained 1 μ g/mL and 10 μ g/mL of each standard amino acids. The same concentrations of individual standard amino acids were injected to HPLC. Samples without standard spiking were used as control for calculating the recovery study.

2.10. Statistical analysis

The results are expressed in means \pm standard error and analyzed by JMP Statistical Discovery™ (SAS) Pro.v.12.0 software package and processed by one-way analysis of variance (ANOVA) to evaluate significant difference ($P \leq 0.05$) and two way analysis of variance at ($P \leq 0.05$) was used to evaluate the effects of buffer pH and temperature on amino acid analysis. Tukey's HSD (honest significant difference) test was used for comparison of sample means. For inter-day and intra-day analysis RSD was calculated according to the formula $RSD = s/\mu \times 100$, s is standard deviation and μ is the average. Principal component analysis (PCA) using MetaboAnalyst 4.0 (Xia Lab @ McGill <http://www.metaboanalyst.ca/>) was conducted to evaluate the effects of temperature and pH on amino acid separation and the changes in amino acids profiles of different vegetable juices.

3. Results and discussion

3.1. HPLC method development

Chromatographic conditions were optimized to separate 21 amino acids in different vegetables using automated pre-column derivatization. Amino acids are weak chromophores to enhance their sensitivity for fluorescent detection pre-column derivatization is required. Precolumn derivatization also increase the hydrophobicity of amino acids which aids in reverse-phase HPLC analysis [30]. The physical and chemical properties of the stationary phase such as pore size, carbon load, specific surface area, particle shape, and size influence peak resolution. The optimal stationary phase was achieved by using Kinetex C₈, Eclipse XDB C₈ and C₁₈. Results show that all the columns gave good peak response but the Eclipse XDB C₈ gave satisfactory peak resolution for amino acids (Fig. S1). Optimization of the buffer pH and column temperature for optimal separation of amino acids was challenging. Various reports show that pH plays a key role in the separation and resolution of amino acids, especially for critical pairs [20,31]. During derivatization of primary amino acids, OPA reacts with the amino group to form an adduct. Fig. S2 presents the HPLC chromatograms of certain amino acids with two or more amino groups showed a single peak similar to other amino acids.

3.2. Effect of pH and temperature

To investigate the combined effect of pH and temperature, an amino acid standard mixture and watermelon juice samples were analyzed at three pH values (5.40, 5.60 and 5.80) and four temperatures (25, 30, 35, and 40 °C). Table 1 represents the effects of buffer pH and temperature on the separation and levels of amino acids present in watermelon juice. In the present study, critical pair 1 consisted of arginine, glycine and threonine, critical pair 2 was alanine, β -alanine, and tyrosine, and critical pair 3 methionine and valine. Results demonstrated that buffer pH and temperature greatly influence amino acid separation especially for critical pairs. For critical pair 1, the best separation was achieved using mobile phase

pH 5.40 at column temperature 35 °C; however, baseline separation of all three was not achieved. Glycine was not detected in watermelon juice however for standard amino acid solution at 40 °C baseline separation between glycine and threonine was observed with almost complete co-elution of arginine and glycine. The second critical pair, L-alanine and β -alanine were well separated at a pH 5.40 for all temperatures. β -alanine and tyrosine showed base line separation at pH 5.60 and 5.80. However, with the increase in temperature, the resolution of alanine and β -alanine significantly reduced. The complete co-elution of both amino acids occurred at temperature 35 °C and 40 °C. Finally, adequate separation of methionine and valine was achieved at a pH 5.40 and pH 5.60. Their resolution also improved as temperature increased.

It was found that column temperature has an effect on the fluorescent signal of OPA derivatives. At higher temperatures OPA derivatives have a reduced fluorescent response. It has also been shown that increasing temperature slightly changes the elution times of the solutes and decreases the FLD signal of amino acids. It may be due to the change in the amino acids elution within the same gradient run cause by the difference in variation of mobile phase composition [32]. A two-way analysis of variance was conducted to evaluate the influence of buffer pH and column temperature on amino acids in watermelon juice. Effects were statistically significant at $P \leq 0.05$. Therefore, buffer pH (5.40, 5.60, and 5.80), temperature (25 °C, 30 °C, 35 °C, and 40 °C) and their interactions significantly affected the separation of amino acids present in watermelon juice.

Li et al. [20] optimized HPLC method using pH range from 4.8 to 6.0 and concluded that pH 5.8 was the best pH for the separation of amino acids. Likewise, Zeng et al. [31] also used pH 5.6 after reviewing the effect of pH 5.0 to 5.8 on amino acids separation. Moreover, previous studies show that fluorescent intensity has been reduced at lower pH [32]. In the present study, good resolutions of 21 amino acids were obtained at pH 5.40 and a temperature 35 °C. Henderson et al. [33] separated standard amino acids including L-citrulline in 26 min using 40 mM sodium phosphate buffer with a flow rate was 2 mL/min, however sample matrix was not used for the separation of amino acids [33]. High concentration of phosphate buffer can cause precipitation in the system during gradient elution with organic solvent, which will generate back pressure [31,34]. Considering these issues, a 10 mM sodium acetate buffer was first studied; however inadequate peak separations and unreliable results were observed (data not shown). This was likely caused by a decrease in buffering capacity at low concentrations in accordance to previous studies [31]. The optimal amino acid separations were observed with a 20 mM sodium acetate buffer concentration.

3.3. Principal component analysis (PCA) of different temperatures and pH

Principle component analysis is an unsupervised technique that assists the comparison of samples [35]. To visualize the effects of temperature and buffer pH on the analysis of amino acids in watermelon juice PCA was used and the results are represented in Fig. 1. For the effect of temperature and pH on the amino acid concentrations calculated from

Table 1 – Effect of column temperatures and different buffer pH (20 mM) on the separation and quantification of amino acids (µg/mL) from watermelon by HPLC-FLD.

	pH 5.40				pH 5.60				pH 5.80			
	25 °C	30 °C	35 °C	40 °C	25 °C	30 °C	35 °C	40 °C	25 °C	30 °C	35 °C	40 °C
Asp	74.85 ± 1.1 ^{ab}	59.09 ± 1.7 ^{bcd}	46.85 ± 0.2 ^{def}	31.11 ± 0.3 ^{fg}	41.63 ± 6.5 ^{ef}	66.36 ± 0.8 ^{abc}	49.94 ± 1.9 ^{dce}	17.99 ± 2.2 ^g	75.94 ± 5.2 ^a	58.96 ± 6.7 ^{bcd}	53.34 ± 1.0 ^{cde}	38.67 ± 1.6 ^{ef}
Glu	10.29 ± 0.2 ^{cd}	9.36 ± 0.1 ^{cd}	9.04 ± 0.2 ^d	8.33 ± 0.2 ^d	12.90 ± 0.5 ^a	11.44 ± 0.2 ^{abc}	9.54 ± 0.3 ^{cd}	10.61 ± 0.4 ^{bcd}	12.54 ± 0.6 ^{ab}	11.54 ± 1.1 ^{abc}	10.26 ± 0.3 ^{cd}	9.01 ± 0.2 ^d
Asn	34.16 ± 0.5 ^a	30.45 ± 0.6 ^{abcd}	27.22 ± 0.0 ^{cdef}	22.70 ± 0.2 ^f	32.65 ± 0.6 ^{ab}	31.61 ± 0.5 ^{abc}	28.47 ± 0.8 ^{bcd}	24.55 ± 0.3 ^{ef}	32.71 ± 1.7 ^{ab}	28.35 ± 2.5 ^{bcd}	30.11 ± 0.4 ^{abcd}	26.62 ± 0.9 ^{def}
His	63.85 ± 0.8 ^a	51.52 ± 1.1 ^c	37.91 ± 0.8 ^e	24.07 ± 0.5 ^{fg}	60.98 ± 0.8 ^{ab}	48.20 ± 0.7 ^{cd}	32.08 ± 0.9 ^{ef}	20.36 ± 0.2 ^g	54.47 ± 3.5 ^{bc}	40.25 ± 3.9 ^{de}	33.90 ± 0.5 ^e	20.13 ± 0.8 ^g
Ser	57.09 ± 0.6 ^a	46.97 ± 1.0 ^{bcd}	37.19 ± 0.3 ^{ef}	26.56 ± 0.3 ^g	52.79 ± 1.5 ^{ab}	50.05 ± 0.7 ^{abc}	40.50 ± 1.2 ^{def}	28.17 ± 0.6 ^g	56.42 ± 3.5 ^a	45.13 ± 4.3 ^{bcd}	41.94 ± 0.6 ^{cde}	32.23 ± 1.0 ^{fg}
Gln	309.19 ± 4.2 ^a	261.89 ± 6.8 ^{abc}	224.54 ± 1.8 ^{cde}	173.35 ± 4.6 ^{ef}	275.67 ± 10.6 ^{abc}	271.15 ± 4.3 ^{abc}	231.55 ± 7.8 ^{cde}	168.78 ± 4.8 ^f	295.69 ± 19.8 ^{ab}	240.17 ± 26.3 ^{cd}	243.87 ± 2.7 ^{bcd}	200.49 ± 7.5 ^{def}
Cit	746.90 ± 9.8 ^a	636.09 ± 12.5 ^{ab}	543.20 ± 4.5 ^{bc}	366.98 ± 37.3 ^d	752.81 ± 9.8 ^a	648.63 ± 10.3 ^{ab}	552.92 ± 16.8 ^{bc}	469.11 ± 12.9 ^{cd}	715.90 ± 49.2 ^a	571.78 ± 62.1 ^{bc}	574.10 ± 8.1 ^{bc}	466.82 ± 14.5 ^{cd}
Arg	611.40 ± 7.6 ^a	545.24 ± 12.7 ^{abc}	498.98 ± 1.5 ^{bcd}	420.64 ± 7.8 ^d	606.00 ± 7.8 ^a	557.47 ± 10.7 ^{abc}	506.72 ± 17.3 ^{abcd}	464.04 ± 9.9 ^{cd}	583.29 ± 39.3 ^{ab}	480.18 ± 51.8 ^{bcd}	520.23 ± 7.2 ^{abcd}	473.53 ± 14.7 ^{cd}
Thr	32.73 ± 1.0 ^a	24.10 ± 0.4 ^b	20.79 ± 0.8 ^{bc}	17.63 ± 0.0 ^{cd}	33.35 ± 0.7 ^a	25.69 ± 1.4 ^b	20.98 ± 0.6 ^{bc}	16.90 ± 0.4 ^d	31.91 ± 2.2 ^a	22.01 ± 1.8 ^{bc}	20.36 ± 0.2 ^{bcd}	15.22 ± 1.3 ^d
Ala	29.35 ± 0.4 ^a	25.09 ± 0.5 ^{ab}	21.38 ± 0.1 ^{bc}	16.62 ± 0.3 ^c	29.15 ± 0.3 ^a	25.62 ± 0.3 ^{ab}	21.73 ± 0.7 ^b	PM	28.36 ± 1.5 ^a	22.89 ± 1.9 ^b	PM	PM
β-ala	14.41 ± 0.2 ^{ab}	14.06 ± 0.3 ^{abc}	15.41 ± 0.4 ^a	PM	14.06 ± 0.2 ^{abc}	14.19 ± 0.3 ^{abc}	13.49 ± 0.2 ^{bc}	PM	15.11 ± 0.5 ^{ab}	12.63 ± 0.7 ^c	PM	PM
Tyr	18.41 ± 0.4 ^a	17.58 ± 0.2 ^{ab}	15.42 ± 0.5 ^{ab}	PM	18.48 ± 0.3 ^a	17.19 ± 0.4 ^{ab}	15.97 ± 1.0 ^{ab}	14.71 ± 0.9 ^b	18.48 ± 0.8 ^a	16.21 ± 1.1 ^{ab}	17.23 ± 0.1 ^{ab}	14.87 ± 0.8 ^b
Met	28.03 ± 0.4 ^a	28.45 ± 0.5 ^a	25.17 ± 0.5 ^{ab}	21.91 ± 0.5 ^b	25.20 ± 0.5 ^{ab}	25.85 ± 0.4 ^{ab}	24.78 ± 0.7 ^{ab}	22.02 ± 0.5 ^b	23.09 ± 1.1 ^{ab}	23.61 ± 2.0 ^b	25.82 ± 0.3 ^{ab}	23.64 ± 0.8 ^b
Val	44.11 ± 0.7 ^a	38.97 ± 0.8 ^{abc}	36.39 ± 0.2 ^c	39.94 ± 0.9 ^c	43.90 ± 0.4 ^{ab}	39.94 ± 0.7 ^{abc}	35.48 ± 1.0 ^c	33.27 ± 1.1 ^c	45.58 ± 2.7 ^a	36.59 ± 3.4 ^c	36.71 ± 0.5 ^{bc}	32.78 ± 1.1 ^c
Trp	58.46 ± 0.8 ^{ab}	32.45 ± 1.0 ^d	29.37 ± 1.3 ^{de}	17.77 ± 0.4 ^f	58.96 ± 0.4 ^a	53.54 ± 0.8 ^b	30.88 ± 1.3 ^{de}	26.52 ± 2.0 ^e	57.85 ± 3.1 ^{ab}	43.69 ± 4.5 ^b	54.21 ± 0.7 ^{ab}	30.32 ± 0.7 ^{de}
Phe	72.38 ± 1.4 ^a	63.68 ± 2.1 ^{ab}	51.58 ± 0.4 ^{cde}	42.79 ± 0.8 ^e	62.39 ± 1.0 ^{abc}	61.27 ± 1.7 ^{abc}	55.02 ± 1.8 ^{bcd}	44.20 ± 0.6 ^e	62.68 ± 3.8 ^{abc}	57.58 ± 5.8 ^{bcd}	53.48 ± 1.3 ^{bcd}	46.56 ± 1.0 ^{de}
Ile	56.21 ± 1.0 ^a	52.15 ± 1.2 ^{ab}	48.69 ± 0.1 ^{abc}	42.81 ± 1.1 ^{bc}	50.73 ± 1.0 ^{abc}	52.64 ± 1.3 ^{ab}	50.33 ± 1.4 ^{abc}	42.14 ± 0.6 ^c	51.80 ± 3.3 ^{ab}	48.11 ± 4.7 ^{abc}	48.97 ± 0.7 ^{abc}	45.55 ± 1.3 ^{bc}
Leu	39.37 ± 0.4 ^a	33.80 ± 0.7 ^{abcd}	29.29 ± 0.4 ^{de}	24.31 ± 0.9 ^{ef}	35.99 ± 1.5 ^{abc}	37.15 ± 0.7 ^{ab}	30.12 ± 0.6 ^{cde}	21.76 ± 0.6 ^f	37.59 ± 2.2 ^{ab}	36.23 ± 3.2 ^{abc}	32.11 ± 0.6 ^{bcd}	24.13 ± 0.4 ^{ef}
Orn	14.46 ± 0.4 ^{de}	14.11 ± 0.5 ^{de}	13.42 ± 0.4 ^e	13.76 ± 1.1 ^{de}	20.69 ± 1.5 ^{ab}	19.31 ± 0.6 ^{abc}	17.92 ± 0.5 ^{abcd}	16.88 ± 0.9 ^{bcd}	21.73 ± 0.9 ^a	16.19 ± 1.3 ^{cde}	17.57 ± 0.7 ^{bcd}	17.06 ± 0.3 ^{bcd}
Lys	50.39 ± 1.0 ^a	44.88 ± 0.8 ^{ab}	40.59 ± 0.4 ^{bc}	33.44 ± 0.7 ^c	52.31 ± 0.9 ^a	48.21 ± 1.0 ^{ab}	41.55 ± 1.2 ^{bc}	35.72 ± 0.6 ^c	51.93 ± 3.4 ^a	35.78 ± 3.5 ^c	41.93 ± 0.9 ^{bc}	36.04 ± 1.0 ^c

Results were expressed as mean ± standard error (n = 4).

Statistical differences were evaluated for the interactive effect of pH and temperature for each amino acid. Same letters signify that the means are not statistically significant at P ≤ 0.05 within the rows. Different letters represent significant differences at P ≤ 0.05 among the rows.

Abbreviations as follows: Asp: aspartic acid, Glu: glutamic acid, Asn: asparagine, His: histidine, Ser: serine, Gln: glutamine, Cit: citrulline, Arg: arginine, Gly: glycine, Thr: threonine, Ala: alanine, β-ala: β-alanine, Tyr: tyrosine, Met: methionine, Val: valine, Trp: tryptophan, Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, and Lys: lysine; PM: peak merged.

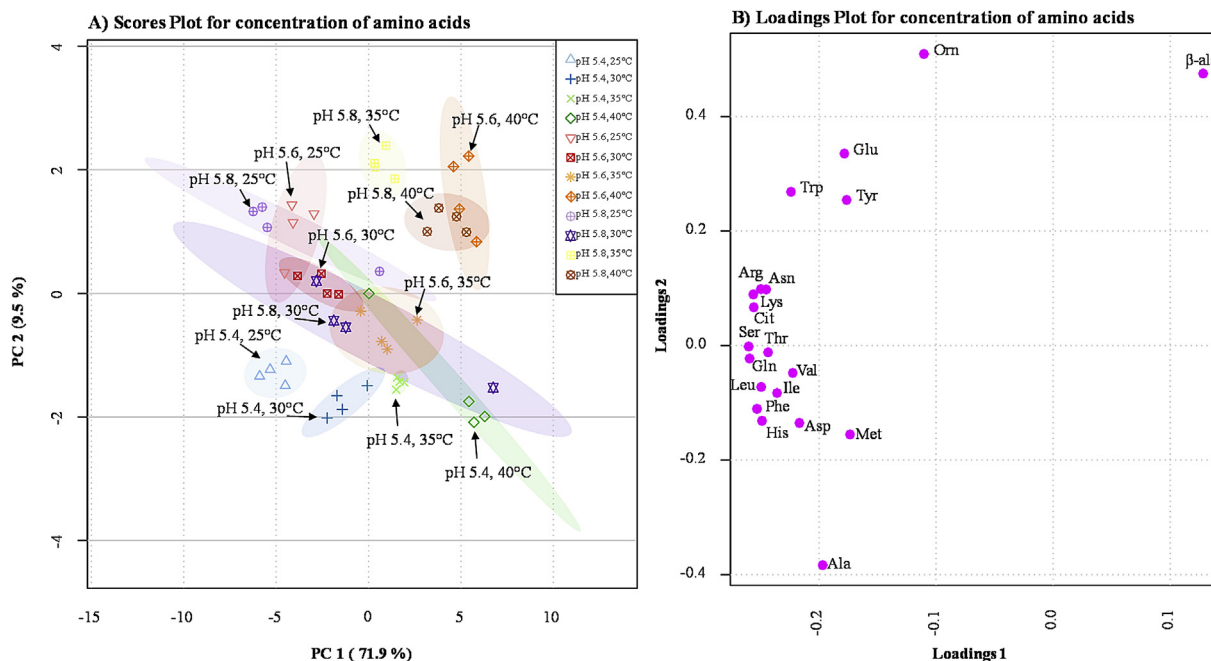


Fig. 1 – Principal component analysis of the effects of different buffer pH (5.40, 5.60, and 5.80) and temperatures (25 °C, 30 °C, 35 °C, and 40 °C) on the amino acids present in watermelon juice. A) score and B) loadings plots for PC 1 and PC 2 of the concentration of amino acids. Explained variances for PC 1 and PC 2 in the score plot are indicated as the percentages in X- and Y- axis, respectively. Abbreviations as follows: Asp: aspartic acid, Glu: glutamic acid, Asn: asparagine, His: histidine, Ser: serine, Gln: glutamine, Cit: citrulline, Arg: arginine, Gly: glycine, Thr: threonine, Ala: alanine, β -ala: β -alanine, Tyr: tyrosine, Met: methionine, Val: valine, Trp: tryptophan, Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, and Lys: lysine.

the area. Fig. 1A and B, the principal components (PC) represented 81.4% of the total variance. PC₁ and PC₂ accounted for 71.9% and 9.5% of the variance, respectively. The results show clustering based on the respective buffer pH and temperature used to analyze the watermelon juice samples. Some of the groups overlapped, however based on PC₁ results suggest the groups separated based on temperature with 25 °C and 30 °C clearly separating from 35 °C to 40 °C. Based on Table 1 most amino acids had a decreased response as temperature increased leading to a lower concentration of amino acids. PC₂ separated the clusters based on pH, the clusters that represented samples run at pH 5.40 separated pH 5.60 and 5.80.

3.4. Method validation

Validation parameters including linearity, regression equation, limit of quantification (LOQ) and limit of detection (LOD) were performed for the developed method (Table S2). Excellent linearity was observed in all 21 amino acid standards by plotting the concentration as a function of peak area obtained from HPLC-FLD analysis (Fig. S3). The linear equation is strongly influenced by the highest concentration in the calibration curve, which affects the slope and the intercept. In the present study, after eliminating the highest concentration (2.5 $\mu\text{g}/\mu\text{L}$) data point from the regression equation, the y-intercepts are less

negative or they become positive (Table S3). Despite having a negative intercept, our calibration curves have excellent linearity (≥ 0.98). The LOQ and LOD were observed by the signal to noise (S/N) ratio.

The results of inter- and intraday variation for concentration and retention times for standards and watermelon are presented in Table S4. Results demonstrated that the %RSD for the standard concentrations was <2.5% for intraday and <8% for interday analysis. The %RSD was below 7% and 9.5% for intraday and interday analysis respectively for watermelon samples. The %RSD for RT was below <2% (n = 5) for intraday and <4% for interday analysis (n = 5). For watermelon juice, the intraday % RSD for RT was lower than 1.1% and for interday analysis was found to be within 3.7%. The results of the matrix effect on the recovery of amino acids from different vegetable juices samples are presented in Table 2. The majority of amino acids recovery was found to be satisfactory. For instance, watermelon, cucumber, celery, Zucchini squash and Yellow squash recovery was found to be 85–115%, 81.5–125.5, 89.7–127.8, 84.4–115.5 and 84.8–103.7% respectively at spiking level of 10 $\mu\text{L}/\text{mL}$. It seems that, juice matrix has no significant effect on the recovery of amino acids may be due to water to solid ratio is too high. In the case of watermelon juice, out of 20 amino acids 16 amino acids recovered >93% whereas all amino acids were recovered fully from cucumber except histidine. A similar trend was observed for rest of the vegetables used in this study.

Table 2 – Effect of matrix on the recovery (%) of amino acids from different vegetable samples.

Amino acids	Watermelon		Cucumber		Celery		Zucchini Squash		Yellow Squash	
	1 µg/mL	10 µg/mL	1 µg/mL	10 µg/mL	1 µg/mL	10 µg/mL	1 µg/mL	10 µg/mL	1 µg/mL	10 µg/mL
Asp	105.1	105.5	95.0	125.5	110.3	120.3	95.6	101.2	98.8	94.8
Glu	101.0	101.5	88.5	117.8	ND	ND	91.6	95.2	111.6	92.8
Asp	89.3	94.9	82.3	125.2	101.6	104.5	83.7	97.2	87.4	98.5
His	113.4	98.9	82.6	81.5	82.6	110.0	85.8	103.3	81.6	84.8
Ser	97.2	101.0	85.2	118.0	100.0	113.1	90.4	96.8	101.0	90.7
Gln	96.3	87.9	84.0	105.2	100.0	103.9	91.6	84.4	110.6	91.1
Cit	94.3	85.9	87.8	116.4	90.0	112.2	94.0	98.9	94.7	89.4
Arg	86.8	85.3	87.4	116.4	87.9	110.3	90.5	91.8	107.3	91.0
Gly	ND	ND	93.3	120.6	104.6	89.7	104.6	106.6	97.6	99.9
Thr	108.5	104.4	88.7	106.7	96.5	85.9	91.7	100.6	103.3	92.2
Ala	97.3	111.1	85.6	124.5	102.9	127.8	95.9	105.6	96.9	101.6
β-Ala	92.2	99.2	81.0	118.0	94.3	113.1	93.3	99.6	92.3	90.1
Tyr	100.6	114.8	87.5	123.7	104.3	115.7	103.7	113.4	91.8	103.7
Met	100.8	97.7	87.8	117.2	108.5	114.1	92.2	98.5	87.7	88.9
Val	100.3	99.4	88.9	113.5	104.1	107.6	92.5	94.6	104.1	93.0
Trp	93.3	87.1	94.5	101.1	138.4	97.5	102.0	85.5	100.0	93.5
Phe	109.6	92.8	81.7	108.6	109.3	104.5	97.4	92.6	96.9	82.3
Iso	101.6	104.6	90.2	116.9	108.3	111.0	98.2	102.3	99.2	95.3
Leu	105.7	100.9	89.2	117.1	109.0	115.6	96.2	99.5	101.0	90.0
Orn	110.5	114.3	88.6	120.1	105.5	119.4	98.3	115.5	91.1	91.9
Lys	110.2	98.8	91.5	112.8	105.8	112.4	97.5	103.4	105.5	84.9

Values were expressed as average percent recovery obtained from the three replications (fresh weight basis).

3.5. Quantification amino acids in fresh vegetable juices

The optimized HPLC-FLD method (pH 5.40 and 35 °C) was used to determine the amino acid profile of six different fresh vegetable juices. Fig. 2 depicts the comparative amino acids chromatograms of the standard mixture, watermelon, cucumber, celery, zucchini squash, yellow squash, and calabaza squash juices. The amino acid content present in the various vegetable juices are presented in Table 3. Results demonstrated that L-citrulline (716.57 ± 24.80 µg/mL) was predominant in watermelon juice followed by arginine (620.84 ± 21.49 µg/mL). The lowest was glutamic acid (10.92 ± 0.23 µg/mL) and glycine was not detected in the present sample. Our results are consistent with others findings that report high levels of L-citrulline in watermelon [36]. Published L-citrulline levels in watermelon vary significantly depending on variety and location with the reported range being 0.5–3.6 mg/g of fresh sample [37,38]. Low levels of glycine in watermelon samples have also been reported [28,39]. Cucumber juice was rich in glutamine (589.04 ± 25.44 µg/mL) but had low tyrosine (14.61 ± 0.44 µg/mL). Glutamine has been previously reported as the primary amino acid present in cucumber [28]. Aspartic acid (626.29 ± 12.62 µg/mL) was the highest amino acid in celery juice but glutamic acid was not detected. The level of glutamine in zucchini squash was the highest among all the juices analyzed (1049.01 ± 39.56 µg/mL). However, yellow squash had the highest levels of asparagine (1128.70 ± 25.00 µg/mL) but lower levels of glutamic acid (15.55 ± 0.81 µg/mL). Calabaza squash juice contained higher levels of glutamine (1006.45 ± 48.84 µg/mL) and low levels of methionine (18.70 ± 1.14 µg/mL). The total amino acids content was highest in zucchini squash juice (4210.89 µg/mL) followed by

yellow squash (3959.08 µg/mL), calabaza squash (3713.58 µg/mL), watermelon (2286.34 µg/mL), and celery (1688.80 µg/mL). Cucumber juice contained the lowest level of amino acids (1622.56 µg/mL).

3.6. L-citrulline content in fresh vegetable juices

L-citrulline is a non-proteogenic and nonessential amino acid that is ubiquitous in mammals, plants, fungi and bacteria [11]. It was first discovered in 1930 in watermelon juice, since then it has been studied for its clinical and therapeutic uses [10,11,40]. It plays various roles in metabolism including the regulation of nitric oxide which affects cardiovascular health and also acts as an antioxidant [10,41]. All vegetables in the present study are a source of L-citrulline, which ranged from 11.48 ± 2.16 to 716.57 ± 24.80 µg/mL. Watermelon juice had the highest level of L-citrulline (716.57 ± 24.80 µg/mL) followed by zucchini squash (115.68 ± 7.82 µg/mL), calabaza squash (70.99 ± 8.16 µg/mL), cucumber (55.66 ± 3.96 µg/mL), yellow squash (46.31 ± 1.53 µg/mL), and celery juice (11.48 ± 2.16 µg/mL). The Cucurbitaceae family is known for accumulating L-citrulline, specially watermelon which has been found to have very high levels [11]. Interestingly, in contrast to a previous report [28] our finding show the presence of L-citrulline in yellow squash.

3.7. Principle component analysis (PCA) of amino acid profiles of vegetable juices

To evaluate the distribution pattern of amino acids in different vegetable juices PCA was performed and the scores and loadings plots are presented in Fig. 3. The results of the score plot (Fig. 3A) demonstrated a clear separation of six clusters of celery,

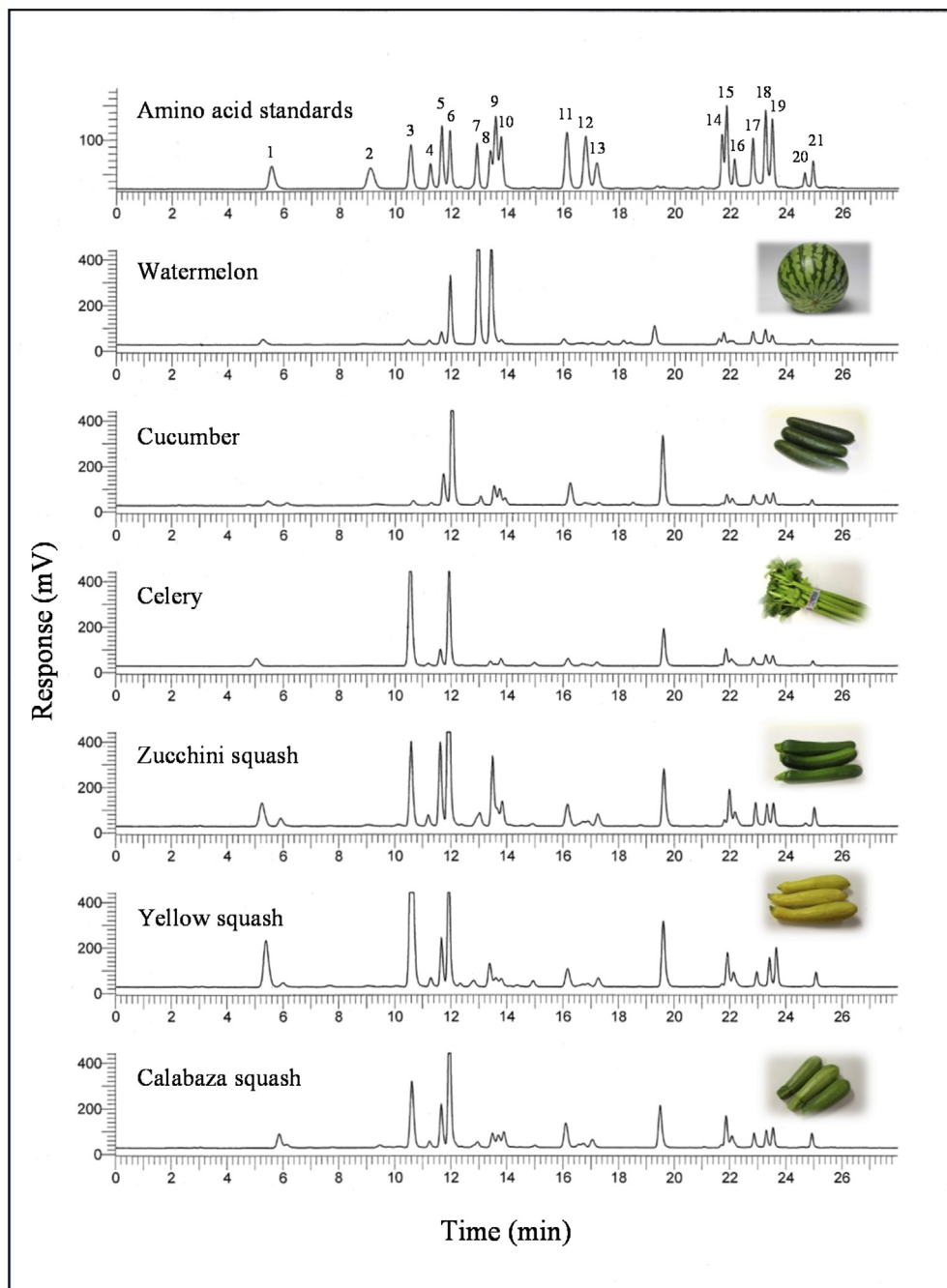


Fig. 2 – Typical HPLC overlay chromatograms of amino acid standard mixture ($0.35 \mu\text{g}/\mu\text{L}$), watermelon, cucumber, celery, zucchini squash, yellow squash, and calabaza squash juices at 35°C using buffer pH 5.40. The amino acid elution order is as follows: (1). L-aspartic acid, (2). L-glutamic acid, (3). L-asparagine, (4). L-histidine, (5). L-serine, (6). L-glutamine, (7). L-citrulline, (8). L-arginine, (9). glycine, (10). L-threonine, (11). L-alanine, (12). β -alanine, (13). L-tyrosine, (14). L-methionine, (15). L-valine, (16). L-tryptophan, (17). L-phenylalanine, (18). L-isoleucine, (19). L-leucine, (20). L-ornithine, and (21). L-lysine.

calabaza squash, cucumber, watermelon, yellow squash, and zucchini squash juices, representing differences in the amino acid profiles associated with the vegetable type. The two uncorrelated PCs account for 77.5% of the total variance. PC₁ showed separation between the three squash juices (calabaza, yellow, and zucchini squash) and watermelon, cucumber, and celery juices. The amount of variation accounted for by PC₁ was 62.3% correlated with the amino acids histidine, serine,

glutamine, β -alanine, tyrosine, valine, and lysine (Fig. 3B). This suggests that these amino acids are determinant of the profiles of squash juices. Based on Table 3 it is evident that these juices had significantly higher levels of these amino acids as compared with watermelon, cucumber and celery juices. PC₂ explained 15.1% of the variation observed, and was strongly correlated with L-citrulline, arginine, and methionine. Watermelon and zucchini squash juices showed significantly higher levels of

Table 3 – Amino acid (µg/mL) content in various fresh vegetable juices analyzed by HPLC-FLD.

Analyte	Watermelon	Cucumber	Celery	Zucchini Squash	Yellow Squash	Calabaza Squash
Asp	64.18 ± 2.0 ^d	48.74 ± 1.5 ^d	84.49 ± 0.7 ^d	244.77 ± 3.6 ^b	539.15 ± 21.3 ^a	157.38 ± 13.9 ^c
Glu	10.92 ± 0.2 ^b	17.03 ± 0.9 ^b	ND	30.60 ± 4.5 ^a	15.55 ± 0.8 ^b	34.48 ± 1.3 ^a
Asn	33.76 ± 1.0 ^c	29.78 ± 0.7 ^c	626.29 ± 12.6 ^b	617.65 ± 58.0 ^b	1128.70 ± 25.0 ^a	715.96 ± 61.6 ^b
His	55.47 ± 1.9 ^c	35.06 ± 2.3 ^c	34.69 ± 1.4 ^c	146.84 ± 10.4 ^a	122.57 ± 9.7 ^{ab}	116.28 ± 8.4 ^b
Ser	51.90 ± 1.7 ^e	115.01 ± 6.9 ^d	64.72 ± 1.1 ^e	307.21 ± 15.7 ^a	183.69 ± 8.1 ^c	246.73 ± 19.1 ^b
Gln	294.20 ± 9.9 ^c	589.04 ± 25.4 ^b	384.50 ± 16.0 ^c	1049.01 ± 39.6 ^a	738.19 ± 67.9 ^b	1006.45 ± 48.8 ^a
Cit	716.57 ± 24.8 ^a	55.66 ± 4.0 ^b	11.48 ± 2.2 ^d	115.68 ± 7.8 ^b	46.31 ± 1.5 ^{cd}	70.99 ± 8.2 ^{bc}
Arg	620.84 ± 21.5 ^a	108.10 ± 11.0 ^{de}	30.34 ± 0.5 ^e	465.05 ± 27.7 ^b	193.25 ± 21.1 ^c	119.18 ± 16.9 ^{cd}
Gly	ND	75.18 ± 8.7 ^a	12.40 ± 0.3 ^c	61.96 ± 4.1 ^{ab}	45.56 ± 2.1 ^b	58.15 ± 2.8 ^{ab}
Thr	26.55 ± 0.8 ^c	37.97 ± 2.7 ^{bc}	37.64 ± 0.7 ^{bc}	117.16 ± 5.5 ^a	49.49 ± 1.4 ^b	110.52 ± 7.6 ^a
Ala	28.10 ± 0.8 ^c	146.91 ± 25.3 ^{ab}	37.59 ± 0.7 ^c	119.07 ± 9.5 ^b	98.46 ± 3.6 ^b	175.75 ± 8.9 ^a
β-ala	14.95 ± 0.2 ^c	20.63 ± 0.6 ^c	18.34 ± 0.4 ^c	39.46 ± 1.1 ^a	32.28 ± 2.1 ^b	36.11 ± 2.3 ^{ab}
Tyr	18.52 ± 0.5 ^c	14.61 ± 0.4 ^c	12.03 ± 0.2 ^c	46.83 ± 3.5 ^a	36.98 ± 1.9 ^b	51.87 ± 2.7 ^a
Met	30.01 ± 0.9 ^a	15.40 ± 1.2 ^b	15.45 ± 3.8 ^b	31.95 ± 3.1 ^a	15.89 ± 1.2 ^b	18.70 ± 1.1 ^b
Val	44.15 ± 1.9 ^c	31.73 ± 3.0 ^c	54.48 ± 1.3 ^c	119.71 ± 10.3 ^{ab}	110.16 ± 6.7 ^b	141.26 ± 8.4 ^a
Trp	30.18 ± 0.8 ^c	57.18 ± 2.9 ^{bc}	71.97 ± 1.3 ^b	138.68 ± 8.1 ^a	131.05 ± 15.3 ^a	141.24 ± 3.9 ^a
Phe	77.17 ± 2.7 ^{bc}	55.00 ± 2.3 ^{cd}	45.98 ± 0.9 ^d	118.61 ± 8.5 ^a	81.04 ± 3.7 ^b	116.29 ± 8.7 ^a
Ile	62.83 ± 2.3 ^b	43.11 ± 2.9 ^b	44.73 ± 1.0 ^b	85.91 ± 8.3 ^a	106.43 ± 5.5 ^a	103.10 ± 7.6 ^a
Leu	43.04 ± 1.5 ^c	47.17 ± 1.6 ^c	44.56 ± 1.3 ^c	96.07 ± 9.5 ^b	144.19 ± 8.7 ^a	109.49 ± 10.0 ^b
Orn	17.69 ± 0.3 ^c	37.64 ± 6.7 ^b	14.23 ± 0.3 ^c	85.23 ± 9.2 ^a	20.62 ± 1.4 ^{bc}	22.62 ± 1.2 ^{bc}
Lys	45.31 ± 1.3 ^c	41.61 ± 1.5 ^c	42.89 ± 0.9 ^c	173.44 ± 16.0 ^a	119.52 ± 6.4 ^b	161.03 ± 7.6 ^a
Total	2286.34	1622.56	1688.8	4210.89	3959.08	3713.58

Data presented is mean ± standard error.

Statistical differences evaluated among vegetables for each amino acid. Letters that are the same signify that the means are not statistically significant at P ≤ 0.05 within the rows. Different letters represent significant differences at P ≤ 0.05 among the rows.

Abbreviations as follows: Asp: aspartic acid, Glu: glutamic acid, Asn: asparagine, His: histidine, Ser: serine, Gln: glutamine, Cit: citrulline, Arg: arginine, Gly: glycine, Thr: threonine, Ala: alanine, β-ala: β-alanine, Tyr: tyrosine, Met: methionine, Val: valine, Trp: tryptophan, Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, and Lys: lysine, ND: not detected.

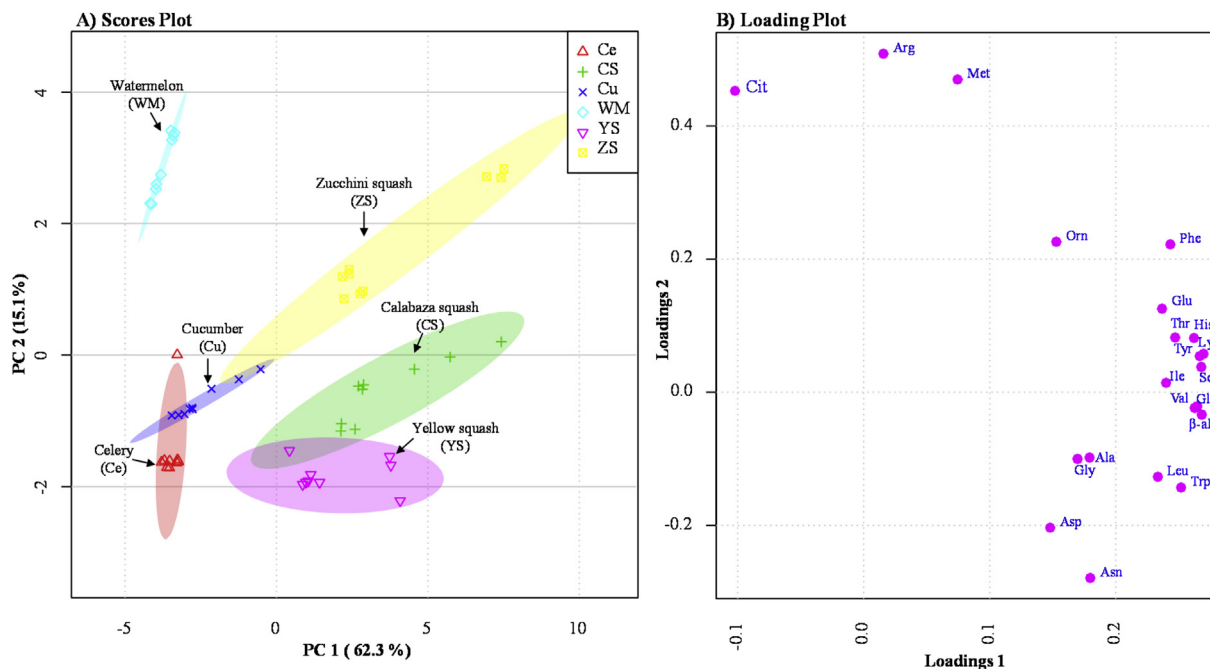


Fig. 3 – Principal component analysis for amino acid data A) score plot of amino acids from celery (Ce), calabaza squash (CS), cucumber (Cu), watermelon (WM), yellow squash (YS), and zucchini squash (ZS) juices. Explained variance for PC 1 and PC 2 are indicated as the percentages in X- and Y- axis respectively, B) loading plot for PC 1 and PC 2 for amino acids from Ce, CS, Cu, WM, YS and ZS juices. Abbreviations as follows: Asp: aspartic acid, Glu: glutamic acid, Asn: asparagine, His: histidine, Ser: serine, Gln: glutamine, Cit: citrulline, Arg: arginine, Gly: glycine, Thr: threonine, Ala: alanine, β-ala: β-alanine, Tyr: tyrosine, Met: methionine, Val: valine, Trp: tryptophan, Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, and Lys: lysine.

Table 4 – Content of amino acids ($\mu\text{g/mL}$) present in five commercial juices.

Amino acids	Commercial Juice Samples ($\mu\text{g/mL}$)				
	J-1	J-2	J-3	J-4	J-5
Asp	140.83 \pm 3.3 ^{bc}	137.67 \pm 5.6 ^{cd}	18.52 \pm 0.6 ^{fg}	70.74 \pm 4.25 ^c	66.88 \pm 8.4 ^{bc}
Glu	13.10 \pm 3.4 ^c	15.01 \pm 1.3 ^f	36.45 \pm 1.3 ^{de}	130.56 \pm 8.07 ^b	51.25 \pm 6.0 ^{bc}
Asn	335.27 \pm 34.0 ^b	293.22 \pm 12.0 ^b	107.45 \pm 3.6 ^a	245.20 \pm 10.00 ^a	71.55 \pm 3.5 ^{bc}
His	49.54 \pm 7.9 ^c	43.81 \pm 0.7 ^{ef}	32.39 \pm 1.0 ^{de}	21.41 \pm 0.21 ^d	48.97 \pm 1.0 ^{bc}
Ser	ND	ND	51.78 \pm 2.0 ^c	61.20 \pm 5.69 ^c	ND
Gln	ND	ND	76.33 \pm 2.3 ^b	147.08 \pm 1.10 ^b	ND
Cit	ND	ND	17.94 \pm 0.1 ^{fg}	ND	826.48 \pm 34.5 ^a
Thr	131.51 \pm 13.3 ^{bc}	111.74 \pm 5.3 ^{cde}	22.67 \pm 0.7 ^f	33.69 \pm 1.82 ^d	32.72 \pm 2.3 ^c
Ala	210.12 \pm 26.3 ^{bc}	187.57 \pm 7.7 ^c	17.34 \pm 0.7 ^{fg}	21.55 \pm 0.84 ^d	72.04 \pm 3.8 ^{bc}
β -ala	ND	ND	40.22 \pm 1.9 ^d	16.20 \pm 0.01 ^d	24.06 \pm 0.1 ^c
Tyr	825.26 \pm 42.0 ^a	677.17 \pm 49.3 ^a	12.18 \pm 1.1 ^{gh}	ND	97.66 \pm 5.0 ^b
Trp	30.35 \pm 4.0 ^c	28.04 \pm 1.1 ^{ef}	9.04 \pm 0.2 ^h	12.87 \pm 0.71 ^d	27.76 \pm 1.1 ^c
Ile	80.77 \pm 8.7 ^c	72.48 \pm 2.2 ^{def}	8.37 \pm 0.3 ^h	15.92 \pm 0.86 ^d	38.80 \pm 3.0 ^c
Leu	76.28 \pm 6.8 ^c	68.46 \pm 1.6 ^{def}	21.40 \pm 0.4 ^f	18.71 \pm 0.20 ^d	23.97 \pm 0.5 ^c
Lys	108.93 \pm 15.9 ^{bc}	66.22 \pm 2.7 ^{def}	31.77 \pm 0.6 ^e	19.46 \pm 2.17 ^d	37.91 \pm 1.4 ^c

Data presented as mean \pm standard error from 4 replications.

The juice composition of J-1: Ginger, beetroot, and carrot, J-2: Carrot and beetroot, J-3: Kale and pineapple, J-4: Kale, apple, and lemon. J-5: Watermelon and lime.

Statistical differences were evaluated within each juice among amino acids. Letters that are the same signify that the means are not statistically significant at $P \leq 0.05$ within the rows. Different letters represent significant differences at $P \leq 0.05$ among the rows.

Abbreviations as follows: Asp: aspartic acid, Glu: glutamic acid, Asn: asparagine, His: histidine, Ser: serine, Gln: glutamine, Cit: citrulline, Arg: arginine, Gly: glycine, Thr: threonine, Ala: alanine, β -ala: β -alanine, Tyr: tyrosine, Met: methionine, Val: valine, Trp: tryptophan, Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, and Lys: lysine, ND: not detected.

these three amino acids Table 3. Watermelon juice had significantly higher levels among all the juices of the amino acid L-citrulline. Based on the score and loadings plot (Fig. 3) watermelon juice clustered separately indicating that L-citrulline is a strong determinant of the amino acid profile of watermelon. The clusters for celery and cucumber juices overlapped, both of these juices had significantly lower levels of amino acids overall.

3.8. Application of developed method for commercial juice analysis

The developed method was further applied for the analysis of commercially available fresh pressed juices (J-1 to J-5). Results of amino acids in commercial juices are presented in Table 4. Results show that commercial juices had lower amino acid content than fresh juices except for Juice 1 (J-1) and Juice 2 (J-2). For overall amino acid content J-1 had the highest amount (2001.95 $\mu\text{g/mL}$) followed by J-2 (1701.38 $\mu\text{g/mL}$), J-5 (1395.98 $\mu\text{g/mL}$), J-4 (814.58 $\mu\text{g/mL}$), and J-3 (503.84 $\mu\text{g/mL}$). J-1, a freshly squeezed juice, comprised of ginger, beetroot, and carrot had tyrosine (825.26 \pm 41.98 $\mu\text{g/mL}$) as the highest amino acid. The lowest amino acid was glutamic acid (13.10 \pm 3.41 $\mu\text{g/mL}$). The same trend was observed with J-2, a freshly squeezed juice of carrot and beetroot. It had 677.17 \pm 49.32 $\mu\text{g/mL}$ of tyrosine and 15.01 \pm 1.25 $\mu\text{g/mL}$ of glutamic acid. J-3, made up of freshly squeezed juices of kale and pineapple, had 107.45 \pm 3.61 $\mu\text{g/mL}$ of asparagine and 8.37 \pm 0.34 $\mu\text{g/mL}$ of isoleucine (lowest level). Other amino acids were below the limit of detection. J-4 (cold-pressed juice) contained kale, apple and lemon had an asparagine level of 245.20 \pm 10.00 $\mu\text{g/mL}$ and 12.87 \pm 0.71 $\mu\text{g/mL}$ tryptophan (lowest). J-5 was a raw cold pressed watermelon-lime mixture. Interestingly, serine, glutamine, arginine,

methionine, valine, phenylalanine, and ornithine were not detected in the commercial watermelon juice in contrast to fresh watermelon juice. Conversely, L-citrulline was found in higher concentration (826.48 \pm 34.48 $\mu\text{g/mL}$) in J-5 than the fresh watermelon juice (716.57 \pm 24.80 $\mu\text{g/mL}$) used in our study. This may be due to the cultivar used for the commercial juice and their growing conditions.

4. Conclusions

The proposed optimized analytical method has high sensitivity and reproducibility for the analysis of amino acids using C₈ stationary phase. Fresh juices from cucurbits, celery and five commercial juices were successfully analyzed for their amino acids composition. Health beneficial L-citrulline was detected in all fresh juices. Commercial watermelon juice contained the highest L-citrulline content followed by fresh watermelon juice. This method has a high potential to for routine analysis of amino acid in various vegetables and fruit juices.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jfda.2019.04.001>.

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