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A metabolomics approach to identify and quantify the phytochemicals in watermelons by quantitative ^{1}HNMR

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ABSTRACT

Watermelon (*Citrullus vulgaris*) contains many health-promoting compounds, such as ascorbic acid, carotenoids, phenolic acids and amino acids including L-citrulline, arginine, and glutathione. Reported HPLC method for quantification of L-citrulline and sugars in watermelon involves, time-consuming sample preparation, post-column color development and detection with fluorescence and refractive index detectors. The present study describes development of a method to identify and quantify amino acids and sugars simultaneously from watermelon samples using quantitative proton NMR. Lyophilized watermelon samples (30–50 mg) were extracted with deuterium oxide (D_2O) by sonication and the centrifuged extract was directly used for quantification and identification with ^{1}HNMR. An external coaxial insert containing a 65 μL of 0.012% 3-(trimethylsilyl) propionic-(2,2,3,3-d_4) acid sodium salt (TSP-d_4) in D_2O was used as a quantitative reference. The levels of L-citrulline and sugars were measured in less than 6 min. This rapid quantitation method was validated for specificity, linearity, accuracy, precision, reproducibility, and robustness. The limit of detection for L-citrulline was 38 μg/mL and the limit of quantification was 71 μg/mL; for sugars, the limits were 59–94 μg/mL and 120 μg/mL, respectively. This method can be used widely for confirmation and rapid quantitation of multiple compounds in large number of biological or breeding samples for routine analysis.

1. Introduction

Watermelon (*Citrullus vulgaris*) is an important fruit crop with an annual production of approximately 90 million tons. Watermelon is largely consumed as refreshing summer fruit throughout the world and also provides many health benefits [1]. The consumption of fruits and vegetables in appropriate quantities seems to be beneficial in prevention of cardiovascular disease [2,3], cancer [4], ischemic stroke [5], pulmonary disease, cataracts, and hypertension [6], and can help improve bone health [7]. Watermelon provides a wide range of dietary antioxidants such as carotenoids (lycopene and β-carotene), polyphenolics, micronutrients, vitamins (A, B, C, and E) and specific amino acids (citrulline and arginine). Lycopene has demonstrated antioxidant activity and a potential role in prevention of prostate cancer [8,9]. L-citrulline is a non-protein amino acid that was first identified from watermelon [10]. L-citrulline is emerged as an important amino acid both as a product of the NO cycle and as a precursor for arginine [11]. It is also an essential amino acid for young mammals and adults with trauma, burn injury, massive small bowel resection, and renal failure [12]. Conversion of L-citrulline to arginine by the enzyme peptidyl arginine deiminase liberates NO; peptidyl arginine deiminase also converts arginine back into L-citrulline [13].

Efforts to breed watermelon varieties with improved amounts of L-citrulline in combination with consumer-pleasing levels of sugar will require rapid methods for simultaneous quantification of these phytochemicals. However, reported methods have limited accuracy and require time-consuming separation and detection steps. For example, the reported spectrophotometric method for quantification of L-citrulline has limitations in terms of accuracy and detection levels. This method involves the reaction with oxime (3-hydroxyimino 2-butanone) in the presence of strong acids [14] to obtain a colored product. However, this reaction is not specific for L-citrulline. Other studies have reported HPLC methods for the determination of L-citrulline and homocitrulline by post-column color development using o-phthalaldehyde and N-(1-naphthyl)-ethylenediamine [15], but these treatments are tedious and also detection with visible light is less sensitive compared with detection in the ultraviolet region. Moreover, the resulting color may not remain stable during HPLC analysis. An additional study

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Table 1
Assignment of characteristic proton signals to metabolites identified by ^{1}H NMR spectra obtained at 400 MHz in D_2O.

Metabolite	Proton signals from authenticated standards	Proton signal used for quantitation/identification
L-Isoleucine	3.55 (d, 3.6 Hz), 1.89 (m), 1.35 (m), 1.15 (m), 0.89 (d, 7 Hz), 0.84 (t, 7.5 Hz)	0.89(d, 7Hz)
Valine	3.48, (d, 4.4 Hz), 2.18 (m), 0.96(d, 7.1 Hz), 0.86 (d, 7.1 Hz)	0.96 (d, 7.1 Hz)
Threonine	4.18 (m), 3.51 (d, 4.2 Hz), 1.28 (d,6.5 Hz)	1.28(d, 6.5 Hz)
L-Citrulline	3.68 (t, 1 H), 3.05 (t, 2 H), 1.81 (m, 2 H), 1.48 (m, 2 H)	3.05 (t, 2 H)
Arginine	3.99 (t), 3.12 (t), 1.85 (m), 1.66 (m)	1.66 (m)
Glutamic acid	3.66 (t, 6.2 Hz), 2.35 (m), 2.22 (m)	2.35 (m)
Acetate	2.09 (s)	2.09 (s)
L-Methionine	3.75 (t, 6.3 Hz), 2.52 (t, 7.4), 2.07 (m), 2.01 (s)	2.07 (m)
Citrate	2.92 (dd, 1.9,15.8 Hz), 2.75 (dd, 1.95, 15.8 Hz)	2.92 (dd, 1.9,15.8 Hz), 2.75 (dd, 1.95, 15.8 Hz)
Lysine	3.65 (t), 2.92 (bt), 1.79 (m), 1.6 (m), 1.35 (m)	2.92 (bt)
Glucose	5.11(d), 4.51 (d), 3.77 (dd), 3.6 (dd),3.4 (dd), 3.3 (dd), 3.11 (t)	5.11 (d)
Fructose	3.98 (d), 3.88 (dd), 3.78 (dd) 3.67 (d), 3.59 (dd), 3.43 (dd)	3.98 (d)
Tyrosine	7.10 (d, 8 Hz), 6.89 (d, 8 Hz), 3.81 (dd, 5.3, 7.7 Hz), 3.1 (dd, 15, 5.3 Hz), 2.95 (dd, 14.9, 7.7 Hz)	7.10 (d, 8 Hz), 6.89 (d, 8 Hz),
Ascorbate	4.85 (s), 3.98 (dd) 3.65 (d)	4.85 (s)
Sucrose	5.3 (d), 4.11 (d), 3.92 (t), 3.7 (d), 3.65 (t), 3.45 (dd), 3.35 (t)	5.3 (d)
Fumarate	6.75 (s)	6.75 (s)
Phenylalanine	7.42 (m), 7.36 (m), 7.32 (d), 3.98 (dd), 3.19 (m)	7.42 (m), 7.36 (m), 7.32 (d)
Tryptophan	7.72 (d), 7.53 (d), 7.31 (s), 7.27 (m), 7.19 (m), 4.05 (dd), 3.47 (dd), 3.29 (dd)	7.72 (d), 7.53 (d), 7.31 (s), 7.27 (m)

reported a precise RP-HPLC method for the simultaneous determination of twelve molecules participating in a metabolic cycle [16]. After pre-column derivatization with ortho phthalaldehyde containing 3-mercaptopropionic acid, the fluorescent derivatives were separated by gradient elution and detected using fluorescence detector [16]. This method is sensitive and accurate, but will be tedious and protracted for the analysis of large numbers of samples.

Nuclear magnetic resonance (NMR) spectroscopy is a well-known analytical technique for elucidating the structure of small molecules and macromolecules [17]. ^{1}H NMR spectroscopy has been used for quantitative analysis since 1963 for determining the intra-molecular proton ratios in pure organic substances [18]. In natural products research, quantitative proton nuclear magnetic resonance (qHNMR) has emerged as one of the most reliable and suitable techniques for comprehensive qualitative and quantitative analysis. The main advantage of qNMR compared to other analytical methods is the primary ratio measurement, since the peak area in qNMR is proportional to the number of nuclei (CH, CH_2, and CH_3) giving rise to the signal. With qNMR, the quantitation of the compounds present in a complex sample can be performed in a single, rapid, non-destructive measurement. Sample preparation for qNMR is simple, non-tedious and the uncertainty in quantification is minimal. NMR spectroscopy has additional advantages, such as the ability to determine molecular structures from complex sample matrix and also possible to quantitatively analyze multiple metabolites simultaneously from a mixture. For example, nonselective ^{1}H NMR-based metabolomics and multivariate analysis by partial least-squares regression was used to obtain a model to evaluate sensory quality rankings of watermelon samples [19]. However, this paper did not describe the quantitation of L-citrulline and sugars from watermelon.

The main objective of the present study was to develop a simple and versatile quantitative NMR method for the simultaneous quantification of L-citrulline and sugars in watermelons.

2. Materials and methods

2.1. Plant materials

Watermelon (*C. vulgaris*) varieties such as Petite Treat and Jamboree were harvested at Texas AgriLife Research Center (Uvalde, TX). Small and big watermelons of Petite Treat were also obtained from the local supermarket for comparison. Seeds were removed manually and the edible part was juiced, freeze-dried, and stored at −20 °C until analysis.

2.2. Chemicals

Solvents used for the analysis were all HPLC-grade and were obtained from Fisher Scientific (Houston, TX, USA). Ultrapure water (NANOpure, Barnstead, Dubuque, IA, USA) 18 MΩ cm was used for liquid chromatography. L-arginine and L-citrulline were purchased from Sigma-Aldrich, (St. Louis, MO, USA). Other chemicals and reagents used in this study were purchased form Fisher Scientific, (Somerville, NJ, USA). $TSPd_4$ (3-(trimethylsilyl) propionic acid-D_4 sodium salt, 98 atom %D), D_2O (99.9 atom %D) were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA).

2.3. Sample preparation

2.3.1. For qNMR, HPLC and LC–MS analysis

To identify and quantify metabolites from watermelon samples, 3–4 fruits of each variety were juiced and lyophilized. A known amount (30–50 mg) of freeze-dried juice powder was taken in a 4-mL amber vial, and metabolites were extracted with 2 mL of deuterium oxide (D_2O) by sonication for 1 h with occasional mixing. The sample was centrifuged for 15 min at 10,000 rpm and reside was re-extracted with 500 μL of D_2O as mentioned above. The combined extracts were passed through a 0.45 μm filter to remove insoluble particles and this sample was used for identification of metabolites by ^{1}H qNMR and LC–MS and sugars by HPLC.

2.3.2. References for NMR

^{1}H NMR spectra were recorded for the 18 reference standards including amino acids, organic acids, and sugars listed in Table 1 for assignment of signals. The assignments were aided by comparison with chemical shift values in the literature [20,21]. Each amino acid was dissolved in D_2O at a concentration of 2 mg/mL and 525 μL was used along with the internal standard TSP-d_4. 1D ^{1}H NMR spectra of this mixture were also acquired for the cross validation and identification of each signal.

2.3.3. L-citrulline by HPLC

A known quantity (0.4–0.6 g) of freeze-dried watermelon powder was used for sample preparation according to our published protocols [22] with slight modifications. The samples were extracted with 10 mL of MeOH and 0.5 mL of 1N HCl, by mixing