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Journal of Chromatography A, 1078 (2005) 196–200
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Determination of citrulline in watermelon rind

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Received 6 March 2005; received in revised form 27 March 2005; accepted 2 May 2005

Abstract

Watermelon (*Citrullus vulgaris* Schrad.) is a natural and rich source of the non-essential amino acid citrulline. Citrulline is used in the nitric oxide system in humans and has potential antioxidant and vasodilatation roles. A method using gas chromatography–mass spectrometry (GC–MS) was developed to separate citrulline from glutamic acid, which co-elute when analyzed by high performance liquid chromatography. Watermelons were analyzed by GC–MS to determine the citrulline content among varieties, types, flesh colors, and tissues. Citrulline content ranged from 3.9 to 28.5 mg/g dry weight (dwt) and was similar between seeded and seedless types (16.6 and 20.3 mg/g dwt, respectively). Red flesh watermelons had slightly less citrulline than the yellow or orange flesh watermelons (7.4, 28.5 and 14.2 mg/g dwt, respectively). Rind contained more citrulline than flesh on a dry weight basis (24.7 and 16.7 mg/g dwt, respectively) but a little less on a fresh weight (fwt) basis (1.3 and 1.9 mg/g fwt, respectively). These results indicate that watermelon rind, an underutilized agricultural waste, offers a source of natural citrulline.
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Keywords: Citrulline; Watermelon; Rind

1. Introduction

Citrulline is a non-essential amino acid first identified from the juice of watermelon, *Citrullus vulgaris* Schrad. [1] and later obtained from tryptic digestion of casein [2]. Citrulline has been isolated in other cucurbitaceous fruits including bitter melon, cucumber, muskmelon, pumpkin, bottle gourd, dishrag gourd, and wax gourd [3]. It has been found in high concentrations in young walnut seedlings but in negligible amounts in the kernels, suggesting its role in nitrogen translocation during germination [4]. Its presence has also been determined in the sap of Japanese white birch [5].

In most mammals, the small intestine is the major source of circulating citrulline which is utilized in the endogenous synthesis of arginine [6]. L-Citrulline given orally to children and adolescents with sickle cell disease resulted in improvement of symptoms, raised plasma arginine levels, and reduced elevated total leukocyte and segmented neutrophil counts to within normal limits [7]. In rats, oral treatment with citrulline malate increased resistance to muscle fatigue elicited with bacterial endotoxins [8]. Therapy with citrulline malate resulted in regression of clinical manifestations of both psychoautonomic syndrome and asthenic symptoms in individuals with chronic arterial hypotension [9]. Citrulline is a co-product of nitric oxide generated from the oxidation of arginine catalyzed by nitric oxide synthase. Nitric oxide (NO) functions as a cellular messenger in the cardiovascular system and is a pivotal vasoprotection molecule. However, a study found that D- or L-citrulline significantly attenuates cardiac contractile dysfunction in the isolated perfused rat heart subjected to ischemia/reperfusion via non-NO-mediated mechanism [10]. Citrulline is also an efficient hydroxyl radical scavenger and is a strong antioxidant [11,12]. Dietary supplements containing citrulline have been

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0021-9673/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.05.009

used to improve sexual stamina and erectile function; however, the mode of action for this activity is still not unknown [13].

Watermelon is the richest known source of citrulline, and it is thought that this amino acid plays an important role in drought tolerance. In wild watermelon collected from the Kalahari desert, citrulline accumulated to as much as 50% of total amino acid content in leaves after watering was withheld for five days [14]. This adaptive ability is thought to be due to an enzyme that is exclusively in the cytosol [15]. Citrulline may protect leaves from drought-induced oxidative stress by acting as a hydroxyl radical scavenger [11].

Although citrulline has been reported in watermelon flesh and rind, the results may somewhat be overestimated because reports were based on colorimetric measurements [16]. Additionally, a comprehensive study of the range of citrulline in domesticated watermelon has not been done. Our overall objective was to determine the range of citrulline content in watermelon varieties, with additional sub-objectives to determine: (1) a satisfactory extraction method for citrulline, (2) if citrulline content differed among diploid (seeded) and triploid (seedless) watermelons, (3) the relative differences among fruit tissues (peel, flesh, rind), and (4) if flesh color (red, yellow, orange, white) affected citrulline content.

2. Materials and methods[1]

2.1. Chemicals

Citrulline was purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) and *N*,*N*-dimethylformamide (DMF) were from Pierce (Rockford, IL, USA). The methanol used was HPLC grade purchased from Fisher Scientific (Fairlawn, NJ, USA).

2.2. Sampling

Watermelon samples were obtained from research plots in Lane, OK, USA and Uvalde, TX, USA, and from commercial growers in Oklahoma, USA, in 2000 and 2001. Melons were cut transversely between blossoms and stem ends. Fruit flesh, rind and peel were separated. Flesh samples were from center, locule and heart. Rind samples were from the white area of the fruit. Peel samples (approximately 2 mm thickness) were removed by potato peeler. Samples were frozen at −80 °C within 2 h of harvest until lyophilized. About 50 g of tissue was lyophilized. Samples used for citrulline analysis consisted of three melons, with about one to two g lyophilized material used per melon. Samples were in duplicate. Percent moisture was determined by weighing samples before and after freeze drying. Statistical comparison of citrulline values for flesh color and for ploidy (diploid versus triploid) was done using SAS, v. 8.0, Analysis of Variance and General Linear Means model. Means were separated using least significant difference (LSD), $P < 0.05$.

2.3. Extraction of citrulline

Three methods of extraction were performed preliminarily to determine an efficient extraction protocol for citrulline. Five hundred milligrams of freeze-dried sample (Trix 313 flesh, ripe) was used for each method. Extraction was performed in duplicate. In the first method, the sample was extracted with 5 mL of MeOH:6 M HCl (9:1) in a sonicating bath for 20 min. The sample was filtered using a glass sintered funnel, and washed twice with 5 mL of extraction solvent. The filtrates were combined and dried in a speedvac (Savant SVC200, Thermo Savant, Holbrook, NY, USA). In the second method, the sample was extracted with 5 mL of 80% MeOH in a sonicating bath for 20 min, followed by heating at 100 °C. The sample was filtered and washed twice with 5 mL of 80% MeOH. The filtrates were combined and dried in a Speedvac. The third method was performed according to the procedure of Woo and Lee [17], with minor modification. Samples in 20 mL screw-capped vials were extracted with 5 mL of 6 M HCl in a sonicating bath for 20 min, N2 gas was then bubbled through samples for 10 min, and the samples were heated at 145 °C for 4 h. Samples were cooled to room temperature, filtered and washed twice with 5 mL of 6 M HCl. The filtrates were combined and dried in a Speedvac. For the extraction recovery study, 0.5 g of samples ($n = 4$) were spiked 1 μg of citrulline in 0.1 M HCl solution. The spiked samples were dried in an oven at 80 °C for 16 h and extracted using method 3.

2.4. Analysis of citrulline by gas chromatography–mass spectrometry (GC–MS)

Dried extracts (1 mg in a GC vial) were treated with 80 μl of MTBSTFA–DMF (60:20), heated at 80 °C for 40 min and allowed to cool to room temperature. The derivatized sample was analyzed by GC–MS [Agilent 6890 Series GC system (Agilent Technologies, Palo Alto, CA, USA), coupled to a JEOL GCMateII mass spectrometer (JEOL USA, Peabody, MA, USA)] using a DB-1 capillary column (J&W Scientific, Folsom, CA, USA) 30 m × 0.25 mm I.D., 0.25 μm film, run under the following GC temperature program: initial 50 °C held for 1 min, raised to 145 °C at 60 °C/min rate and held at this temp for 2 min, then raised to 250 °C at 4 °C/min rate, raised to 300 °C at 7 °C/min rate, and finally held at this temperature for 3 min. The injection port, GC interface, and ionization chamber were maintained at 250, 230, and 120 °C, respectively. The carrier gas was ultra high purity helium at 1 mL/min flow rate. The sample injection volume was 1 μL. The MS detector was a magnetic sector; spectra were acquired in the positive, low resolution, total ion scan mode.

[1] Disclaimer: Mention of trade names or company commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

The retention time of citrulline was 23.1 min. Quantitative determination of citrulline in the samples was performed from a calibration curve of an external standard of citrulline dissolved in 0.1 M HCl ($y = 4{,}650{,}681x + 265{,}271$; $R^2 = 0.9970$, based on four concentration levels in triplicate). Citrulline was expressed on dry and fresh weight basis, using 89% moisture for all varieties except 'Cream of Saskatchewan', where 95% moisture was found.

3. Results and discussion

3.1. Extraction of citrulline

Preliminary studies were performed to determine a satisfactory method for the extraction of citrulline. Extraction methods 1, 2, and 3 provided 6.4, 1.9, and 8.2 μg citrulline, respectively, per mg lyophilized sample. Other digestion methods were performed; namely, using cyanogen bromide, trypsin, endoproteinase ASP-N and leucine aminopeptidase. However, these digestion methods were not as efficient (data not presented) or economical and were not utilized for further extractions. Method 3 appeared to be the most satisfactory method, and was used for extraction of all samples. The extraction recovery from spiked sample was 96.48 ± 0.90% (RSD = 0.93%). The mild acid condition indicates the extraction of only the free citrulline. Earlier studies reported that citrulline in protein required tryptic digestion in dilute ammoniacal solution [2]. Another study suggested that a protein hydrolyzing a peptidic bond exhibiting homology to the enzymes of the ArgE/DapE/Acyl/Cpg2/YscC protein family, which include acetylornithine deacetylase, carboxypeptidase and aminoacylase-1, is involved in the release of free amino acid resulting in accumulation of citrulline in drought stressed wild watermelon [14].

3.2. Analysis of citrulline

Initial studies on the analysis of citrulline by high performance liquid chromatography with evaporative light scatter-

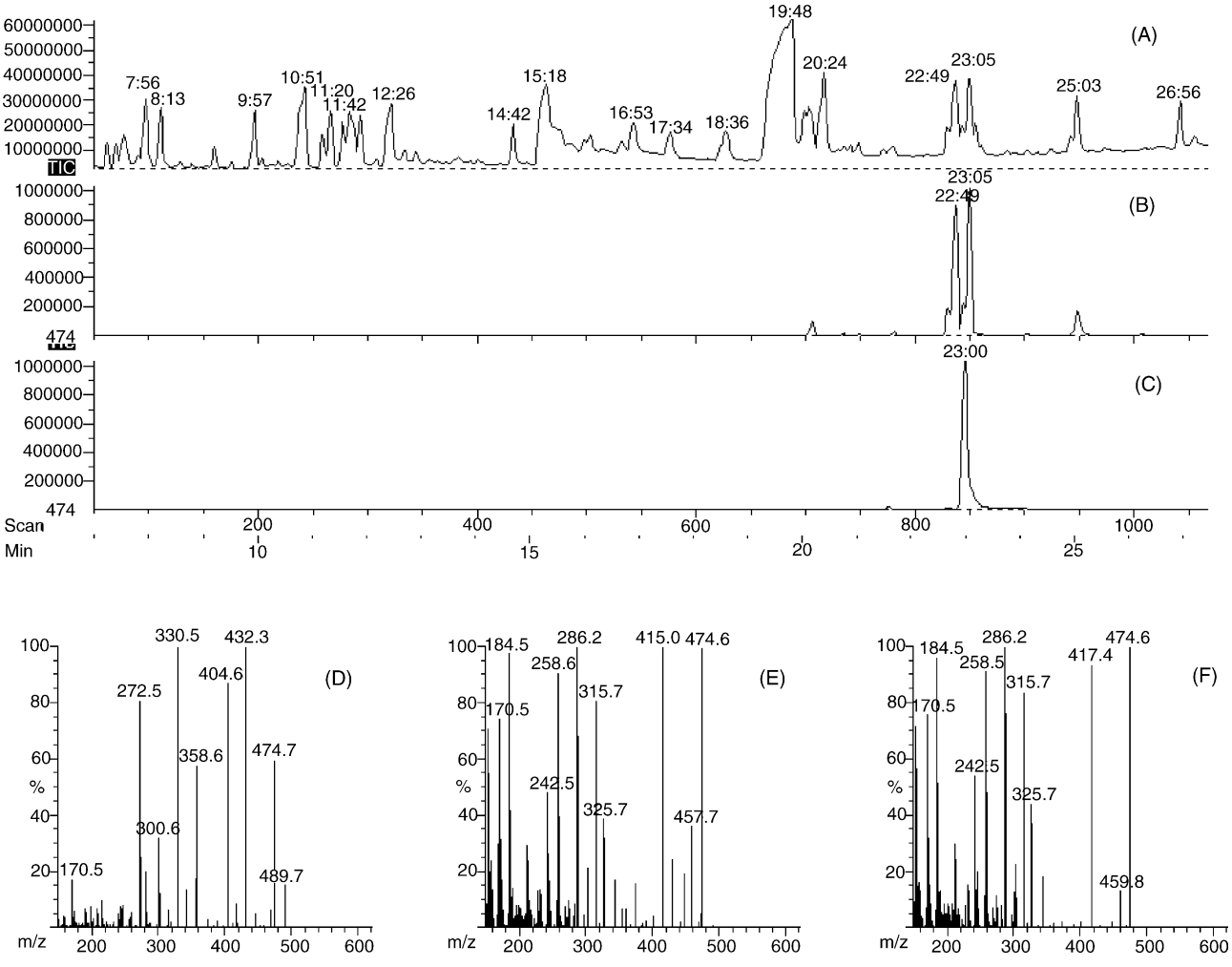

Fig. 1. (A) Total ion chromatogram of an MTBSTFA–DMF-derivatized extract of a watermelon sample, SG rind. Reconstructed ion chromatogram at *m*/*z* 474 of (B) the same sample and (C) MTBSTFA–DMF-derivatized standard citrulline. Mass spectrum, between *m*/*z* 105 and 600 of derivatized (D) sample peak at 22.8 min, corresponding to glutamate, (E) sample peak at 23.1 min, corresponding to citrulline, and (F) standard citrulline.

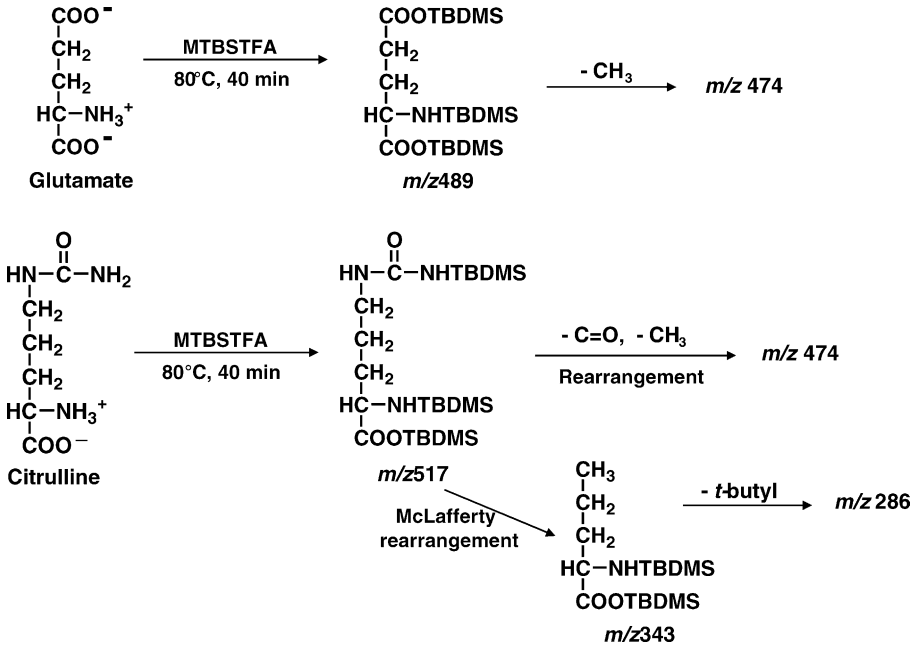

Fig. 2. Fragmentation and major fragment ions of glutamic acid and citrulline.

ing detection did not prove satisfactory. A method utilizing GC–MS was, therefore, employed. Analysis of the watermelon extracts by GC–MS showed two peaks at 22.8 and 23.1 min (Fig. 1A and B) that are very close to the retention time of standard citrulline (23 min, Fig. 1C). These two peaks displayed the fragment ion *m*/*z* 474 (Fig. 1D and E), the molecular ion peak for a MTBSTFA–DMF derivatized standard citrulline (Fig. 1F). The peak for citrulline can be distinguished by the presence of a fragment ion *m*/*z* 286. This arises from a McLafferty rearrangement followed by removal of a *tertiary*-butyl group (Fig. 2). The peak at 22.8 min is that of glutamic acid as identified by comparison with the retention time of a standard (not shown), the molecular ion peak *m*/*z* 489, and reported diagnostic fragmentation ions *m*/*z* 474, 432, 330 [18]. Quantitation of the citrulline peak was performed on the reconstructed ion chromatogram of the peak at 23.1 min.

3.3. Citrulline content of watermelon varieties and tissues

The mesocarp (flesh) of 14 watermelon varieties, consisting of six seeded and eight seedless types, were sampled in duplicate. Citrulline content in flesh was affected by variety (Table 1). Among the flesh samples, the highest level of citrulline (28 mg/g dry weight) was found in the seedless Hazera SW1 and Solid Gold, and the seeded Summer Gold varieties. When averaged, diploid (seeded) had slightly less citrulline than triploid (seedless) melons. This difference was larger on a fresh weight basis, due to the slightly higher dry weight found in seedless melons.

When melons were compared by flesh color, red fleshed melons had less citrulline in flesh or rind than orange or yellow fleshed fruit (Table 2). The citrulline content of the rind was apparently higher on a dry weight basis than that of the flesh when averaged for all colors, but less than the flesh on a fresh weight basis. This difference is because the rind has 95% moisture, compared to 90% moisture in the flesh. In a test done only with the seeded melon Sangria, the peel, rind and flesh were compared. The citrulline content was 6.5,

Table 1
Citrulline content of the flesh of ripe watermelon varieties

Variety	Flesh color	Citrulline content[a] (mg/g)	
		Dry weight	Fresh weight
Seeded			
Cream of Saskatchewan	White	18.2	1.0
Jamboree	Red	25.5	3.1
Sangria	Red	13.3	1.6
Summer Flavor 800	Red	10.1	1.2
Summer Gold	Yellow	28.8	3.6
Tender Sweet Orange	Orange	3.9	0.5
Mean[b]		15.7 b	1.8 b
Seedless			
Hazera 6009	Red	19.0	2.4
Hazera 6007	Red	20.4	2.5
Hazera SW1	Red	28.6	3.5
Orange Sunshine	Orange	24.5	3.0
Scarlet Trio	Red	11.7	1.4
Solid Gold	Yellow	28.3	3.5
Summer Sweet 7167	Red	24.3	3.0
Trix 313	Red	5.7	0.7
Mean[b]		19.2 a	2.4 a

[a] Values represent means of duplicate samples. Dry weight = weight of lyophilized, unextracted sample.

[b] Means of seedless and seeded types were significantly different; $P < 0.05$, least significant difference mean comparison test.

Table 2
Citrulline content of the flesh and rind of watermelons with different flesh colors[a]

Color	Flesh (mg/g dwt)	Rind (mg/g dwt)	Flesh (mg/g fwt)	Rind (mg/g fwt)
Red	7.9 c	15.6 b	1.0 c	0.8 b
Yellow	28.5 a	29.4 a	3.5 a	1.5 a
Orange	14.2 b	28.2 a	1.8 b	1.5 a

[a] Values are means representing a seeded and seedless variety for each color. Red = Trix313 and Summer Flavor 800, Yellow = Summer Gold and Solid Gold, and Orange = Tender Sweet Orange and Orange Sunshine. All melons were from the 2000 harvest season. Means are separated (letter designation) within column (among colors) and between columns (among tissue locations) by least significant difference, $P < 0.05$.

19.0, and 13.4 mg/g dry weight, respectively; and 1.1, 1.0 and 1.5 mg/g fresh weight, respectively. The peel is about 14% dry weight, compared to 5% dry weight in the rind and 10% dry weight of the flesh.

Inatomi et al. [16] found citrulline in all parts of the watermelon fruit, including seeds. They were unable to determine if citrulline was synthesized in the fruit or if citrulline was transported from other parts of the plant.

Half of a watermelon fruit is edible while the other half, consisting of about 35% rind and 15% peel, goes to waste [19]. From this study, it is apparent that the rind contains citrulline in high quantities. This study shows that the watermelon rind is a rich source of an important amino acid and may yield a useful product from an agricultural waste.

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