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Integration of Three Processes for Production of Two Nutraceuticals and Ethanol Biofuel from Cull Watermelons

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***Abstract* – About 20% of the annual U.S. watermelon crop is rejected for the fresh fruit market because of surface blemishes or atypical shape. This unrealized income after production cost investment amounted to ~$88 million dollars for the 2010 growing season. Watermelon is a rich source of two health-promoting nutraceuticals, lycopene and L-citrulline, as well as containing up to 10% fermentable free sugars. Scalable processes for production of each of the nutraceuticals were integrated together with a fermentation process to convert watermelon sugars to ethanol. Four hundred kg of watermelons carried through the three integrated processes yielded an equivalent of 31g lycopene/tonne (~95% recovery), 1.85 kg citrulline/tonne (~80% recovery), and 30L ethanol/tonne (~85% recovery). Use of these integrated processes or selected processes used individually or in combination may afford watermelon growers and packers the opportunity to recuperate part of their crop losses.**

***Keywords* – Ethanol Biofuel, L-Citrulline, Lycopene, Watermelon.**

I. INTRODUCTION

As well as a refreshing treat, watermelon (*Citrullus lanatus*) has proven to be a health-promoting food. Lycopene, the powerful antioxidant carotenoid that imparts the red color to watermelon, has been shown to be important in prostate health [1], and watermelon has been designated a heart-healthy food by the American Heart Association [2]. Watermelon is also one of the richest natural sources of the amino acid, L-citrulline (cit). L-citrulline is involved in the detoxification of catabolic ammonia and is also a precursor of L-argingine, who with cit, is centrally involved in the production of the circulatory vasodilator, nitric oxide [3]. A recent pilot study demonstrated that intake of cit *via* watermelon juice lowered aortic blood pressure in pre-hypertensive individuals [4].

Red-fleshed watermelon contains 50-120 mg lycopene per kg fresh weight [5] and 1-4 g cit per kg fresh weight [6]-[7]-[8]. Watermelon rind contains 1-3 g cit per kg fresh weight [6]-[7]. Furthermore, the lycopene and cit contents of watermelon are highly dependent on growing conditions [8]-[9]. Watermelon is nominally 60% flesh and 40% rind. Ninety percent of the flesh is juice and about 95% of the rind is juice. The juice of flesh contains 7 to 10% fermentable simple sugars (1-3% glucose, 3-5% fructose, and 2-4% sucrose). The rind contains 3 to 5% of these same fermentable simple sugars.

Watermelon is grown for the fresh fruit and cut-fruit markets, and these will continue to be its primary utilization. However, about 20% of each annual watermelon crop is rejected for fresh fruit marketing because of surface blemishes or misshapen fruit. Although internally sound, the “culls” are left in the field or at the packing shed where, in the latter case, they must be disposed of under strict regulatory guidelines. For the 2010 growing season, culls amounted to 372,000 metric tons (tonnes) of watermelon lost to U.S. growers and packers as a source of revenue post investment in the costs of production [10]. Scalable bench scale processes have been developed for cit production [11] and ethanol production from watermelon juice [12]. With the latter, on-farm biofuel production from cull watermelons has been proposed [12]. An integrated system to allow production of lycopene, cit, and ethanol biofuel offers a potential cost recovery mechanism for packers and fresh cut fruit processors who encounter added disposal costs. The purpose of this manuscript is to outline a method for watermelon lycopene production and to demonstrate the integration of three production processes, with the product of each adding to cost recovery.

II. MATERIAL AND METHODS

A. Watermelon Sources

Watermelons employed in this study and those used in earlier process development studies came from several sources covering five crop years. In 2005 and 2006, watermelons were grown at the Wes Watkins Agricultural Research Center in Lane, OK. In 2007, watermelons came from a commercial field in Hinton, Oklahoma. They were purposely selected because they had been graded “culls” as the result of an anthracnose infection on their rinds. Other sources of watermelons included those from the 2007 crop in Terral, Oklahoma, from the 2008 crop in Louisiana, and from the 2008, 2009, and 2010 crops raised at the Wes Watkins Agricultural Research Center.

Watermelon cultivars included seedless, hybrid seeded, and open-pollinated seeded.

The nominal contents of the flesh and rind of the watermelons used in the integrated process run of Fig. 1 and Table I were as follows. Flesh contained 53 mg lycopene/kg fresh weight, 2.5 g cit/kg fresh weight, and 10% fermentable sugars (glucose, fructose, and sucrose). Rind contained no lycopene, 2.0 g cit/kg fresh weight, and 3% fermentable sugars.

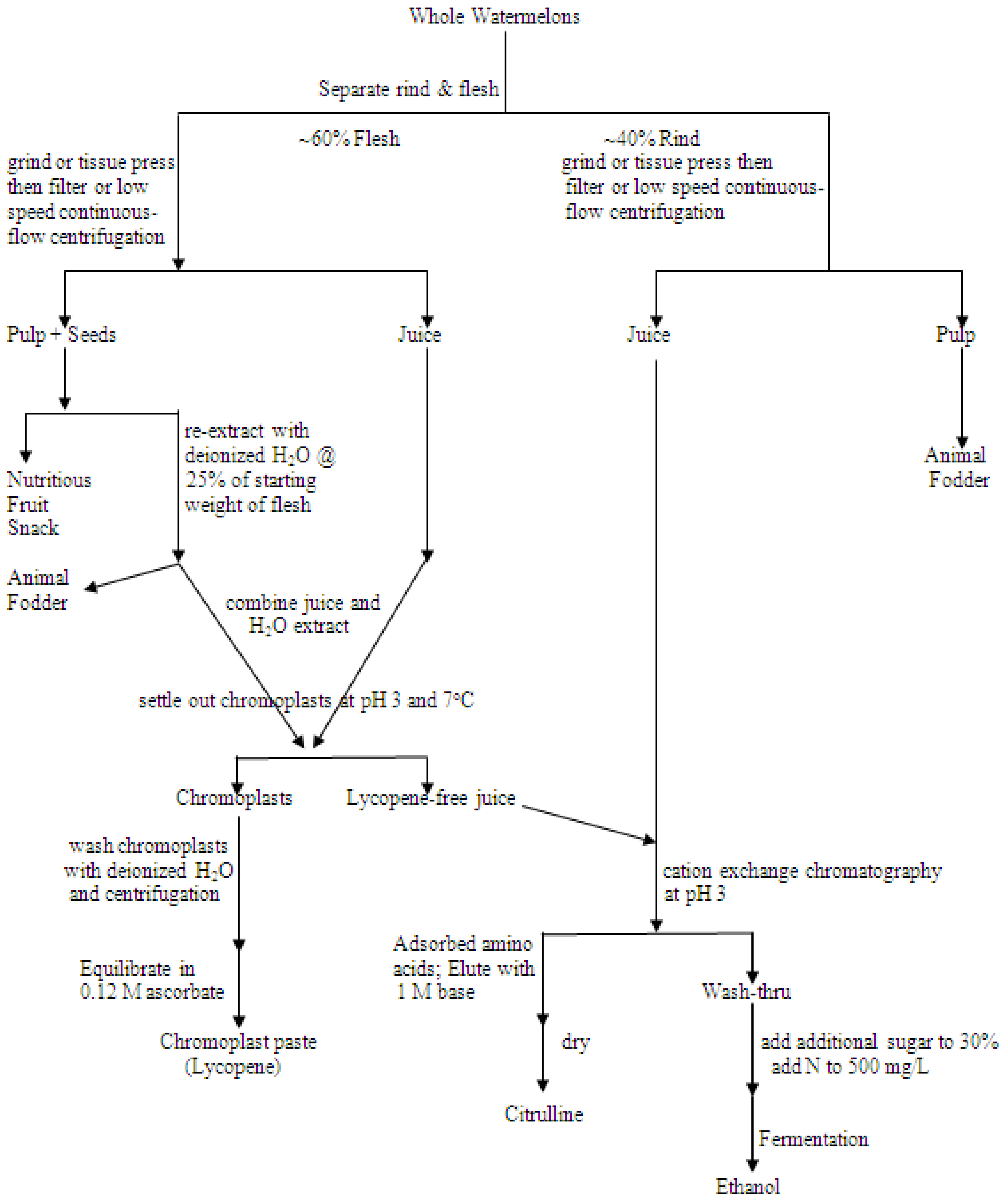

Fig.1. Flow diagram of three processes integrated to produce lycopene, L-citrulline, and ethanol from red-fleshed watermelon.

B. Watermelon Processing for Lycopene

The process for producing watermelon lycopene is outlined as part of Fig. 1. The product of this process is lycopene packaged in the form of watermelon's natural storage vesicles called chromoplasts. The physical and chemical properties of watermelon chromoplasts were described earlier [13]. Watermelon chromoplasts were prepared as follows. Flesh was cut away from rind. Juice was extracted from flesh by either grinding or crushing the fruit. The juice in which the chromoplasts were suspended was separated from pulp and seeds by filtering or pressing through nylon parachute cloth of nominal pore dimensions 35µm x 70µm. Additional chromoplasts could be recovered from the pulp by suspending the pulp in a volume of water equal to about 25% of the starting weight of flesh, stirring for 30 min, and filtering as before. This second filtrate was combined with the original filtrate. About 80% of the total lycopene is recovered in the initial juicing, and about 20% is recovered in the pulp extraction. Chromoplasts were then concentrated from the juice by settling while held at 5-10 ^{o}C. The rate of settling was greatly enhanced by adjusting the pH of the juice to pH 3 with H_2SO_4 or by adding a flocculating agent such as 10 mM Ca^{++}, Mg^{++}, Zn^{++}, or Cu^{++} soluble salts at pH 6.0. Reducing the pH to 3 had the added advantages of inhibiting bacterial growth and having the pH pre-adjusted for cation exchange adsorption of cit and the other amino acids (see below). By utilizing one of these approaches, the chromoplasts in 250 L of juice settled in about 2 to 4 hours. Most of the chromoplast-free juice (~240 L) could be siphoned from the settled chromoplasts. The concentrated chromoplast suspension was then centrifuged at 3,000 to 5,000 x g (relative centrifugal force) for 15 minutes, and the supernatant decanted and added to the rest of the chromoplast-free juice. The chromoplasts were re-suspended in deionized H_2O to about half the volume of the settled chromoplasts and centrifuged again. This process was repeated twice more to thoroughly wash away any soluble contaminants. For long term storage at 7°C, the chromoplasts were then mixed with 0.12 M ascorbate, pH 5.5, to a paste consistency and at a concentration such that the level of lycopene in the chromoplast paste was ~8 mg/g paste.

C. Processing of the lycopene-free juice to obtain citrulline.

The isolation of cit from the chromoplast-free watermelon juice and watermelon rind juice is summarized in Fig. 1 and described in detail in Fish [11]. Briefly, the process consists of adsorbing the free amino acids in the chloroplast-free juice, pH 3, onto a cation exchange resin either by solution batch treatment or column batch treatment. One L of Dowex 50W resin (Dow Chemical Company, Midland, MI) packed in a column has the capacity to bind, and thus remove, the amino acids from ~10 L of watermelon flesh and/or rind juice at pH 3. After washing the amino acid-charged column with water at pH 3, the amino acids were eluted with 1M base such as NH_4OH, NaOH, or KOH. Fractions were monitored either with the ninhydrin reaction for free amino groups (Rosen, 1957) or less accurately but much faster, by monitoring pH of the eluate. Amino acids eluted just ahead of and just behind the leading edge of the pH change from pH 3 to pH 13 (between ~1.5 column volumes and ~3 column volumes). The pH of the amino acid-containing eluate was adjusted to pH 5-6, and dried by lyophilization or spray drying.

D. Fermentation of the free sugars to ethanol.

After the ion exchange step, the amino acid-free juice contained 5-8% readily fermentable sugars with the potential to produce about 25-30 L of ethanol per tonne of whole watermelons. Fermentations were conducted in a BF-110 benchtop modular fermentor system (New Brunswick Scientific Co., Inc., Edison, NJ, USA). The system included a 7.5 L thermostatted glass vessel, a pH/DO controller, a four-pump reagent addition module, an exhaust condenser, a DO probe, and a pH electrode. Fermentation of the cit process byproduct was carried out at pH 5 and 32°C according to the protocol of Fish *et al.* [12]. Supplemental N was added to the juice to a level of 500 mg N/L and additional sucrose was added to a final concentration of 30% total sugars.

E. Analytical methods.

Quantification of lycopene by HPLC was performed as outlined by Craft [14]. Runs were conducted on an Agilent Technologies Model 1100 system fitted with a diode array detector. Separation was carried out on a 3 μm 250 x 4.6 mm C30 column (Waters Corp., Milford, MA) at 25° C. Carotenoids were eluted with the ternary gradient program described by Craft [14].

The method of Sethuraman *et al.* [15] for the determination of physiologic amino acids and biogenic amines by reversed phase HPLC separation of their dabsyl derivatives as modified for fruit and vegetables [16] was employed to quantify watermelon L-citrulline. All reagents and their concentrations were as described by Sethuraman *et al.* [15]. Separation/quantification was with a Varian ProStar ternary solvent HPLC system equipped with an autosampler and diode array detector (Varian, Walnut Creek, CA, USA). A 250 x 4.6 mm i.d. 5□m Luna^{TM} C18 reversed phase column was used (Phenomenex, Torrance, CA, USA) at 50 °C and at a flow rate of 1.0 ml/min. *Alpha*-amino nitrogen in the preparative cation exchange column eluate fractions was quantified by the ninhydrin procedure [17].

Analysis of the fermentation substrates and process products was performed by HPLC analysis on a Varian ProStar ternary solvent system equipped with an autosampler and RI detector (Varian, Walnut Creek, CA, USA). Fermentation substrates, glucose and fructose, and fermentation products, including ethanol, acetate, glycerol, citrate, and lactate, were separated and quantified on an Aminex^{TM} HPX-87H 300mm x 7.8mm column (BioRad, Hercules, CA). Components were eluted with an isocratic system of 5mM H_2SO_4 in H_2O. The flow rate was 0.6 ml/min, and the column temperature was maintained at 50° C. The ethanol and other metabolic products produced by fermentation were quantified by this method [12].

III. RESULTS

A. Watermelon lycopene production

Lycopene processing integrated with the other two processes, is summarized in the diagram of Fig. 1. Flesh and rind from 400 kg of optimally ripe watermelons (~40 watermelons) were separated. This yielded 240 kg flesh and 160 kg rind. The flesh was liquefied in batches with a large blender and filtered through parachute cloth. The retained pulp was re-extracted by stirring with 60 L of deionized water for 30 min and filtering. The filtrate from the second extract was combined with the first filtrate to yield about 250 L of chromoplast-containing filtrate. Concentrated H_2SO_4 was added to the combined filtrates to pH 3. The 250 L were allowed to stand at pH 3 and 7°C overnight. After the first 2-3 hr, most of the chromoplasts (containing lycopene) had settled to the bottom of the holding tank. Approximately 90% (~225L) of slightly pink supernatant was pumped off the settled chromoplasts and saved. The remaining 25 L of settled chromoplasts were centrifuged at 3,000 x g for 15 min to pellet the chromoplasts, and its supernatant was added to that previously pumped off the settled chromoplasts. The chromoplast preparation was washed twice by suspending in ~10 L of deionized water and centrifuging. After the last centrifugation, the pelleted chromoplasts were made into a paste by stirring into 0.12 M ascorbate, pH 5.5 and stored at 7°C. Analysis of the chromoplast preparation by

HPLC yielded a carotenoid composition of 90.2% *trans*-lycopene, 6.7% *cis*-isomers of lycopene, and 3.1% β-carotene. The yield of lycopene (*cis* + *trans*) was 12.5 g in 1.44 kg of chromoplast paste (Table I), and was equivalent to ~95% recovery of the total lycopene. Upon analysis, the chromoplast paste contained approximately 70% water, 2.5% protein, and 0.8% lycopene. Other constituents such as lipids, carbohydrates, and salts made up the balance of the preparation.

B. Watermelon citrulline production

The 160 kg of watermelon rinds were frozen at -20^{o}C for 48 hr, and allowed to thaw. The thawed rind was ground in batches with a large blender and filtered through parachute cloth (Fig.1). This yielded about 125 L of juice. The juice was adjusted to pH 3 with concentrated H_2SO_4 and added to the lycopene-free juice derived from lycopene processing of watermelon flesh. This yielded a total volume of 370 L of cit-containing juice. Fifty liters of the combined juices were pumped through a Dowex 50Wx8 resin bed 23.4 cm in diameter and 11.7 cm high at a flow rate of 0.9 ml/min/cm^2 or a column flow of 0.4 L/min. The column was charged, washed, eluted, and regenerated at approximately this flow rate. The column with adsorbed amino acids was washed with 10 L of water at pH 3, and the water wash discarded. The amino acids were then eluted from the column with 1 M NH_4OH in a final volume of 4 L. Ninhydrin-positive fractions were neutralized, pooled, and analyzed to quantify amino acids. The yield of cit from this run was 108 g which extrapolates to ~800 g cit from the 400 kg of watermelon (Table I). Drying of the neutralized eluate yielded 138 g of dry material which contained 100 g cit. These numbers extrapolate to ~ 1 kg of dried material that contains 740 g of cit processed from the 400 kg of watermelons. This amounts to an 80.6% recovery of cit.

C. Ethanol production

The 370 L of cation exchange column flow-through of watermelon juice contained 6.6% fermentable sugars for a total of 24.3 kg sugars from the watermelons (step 15). To 7 L of the 370 L were added 234 g/L of cane sugar. This gave a final fermentable sugar concentration of 30% (w/v). The cane sugar was added to put the feedstock concentration in a range used commercially. Since all amino acids had been removed from the watermelon juice in the previous cation exchange step, this made the juice N-limited for ethanol production. Therefore, the fermentation medium was supplemented with yeast extract (5.5 g/L), diammonium phosphate (1.5 g/L), Go-Ferm^{TM} (0.625 g/L), and Fermaid K^{TM} (0.25 g/L) to yield 500 mg N/L, of which was 22.5 µmole in *alpha*-amino N /mL. Fermentation was carried out at 32^{o}C, pH 5 and was complete after 70 hr with a level of 11.3% (w/v) ethanol produced. This corresponded to a yield of 0.38 g ethanol per g feedstock sugar and is consistent with a projected production of 11.9 L of ethanol from the sugars of 400 kg of watermelons (Table I). At the fermentation conversion yield of the experiment, this amounts to ~86% recovery of ethanol of that expected from the sugar level in the watermelons.

D. Expected production yields

From the results of Table I that were obtained by processing 400 kg of watermelon by the processes diagramed in Fig. 1, the anticipated production quantities of the three products are 31 g lycopene/tonne watermelons, 1.85 kg L-citrulline/tonne watermelons, and 30 L ethanol biofuel/tonne watermelons. These expected yields are based on the use of both rind and flesh of typical red-fleshed watermelons.

Table I: Process Yields from 400 kg of Red-Fleshed Watermelon

Process Step	**Weight (kg) or Volume (L)**	**Total Lycopene (g)**	**Total Citrulline (g)**	**Fermentable Sugars (kg)**	**Ethanol (L)**
1) Starting material, ~40 watermelons	400 kg				
2) Fresh flesh	240 kg				
3) Fresh rind	160 kg				
4) Juice from watermelon flesh	186 L	9.2	598	18	
5) Pulp from flesh after juice extract	30 kg	3.8		3.0	
6) Water extract of flesh pulp	60 L	3.3		2.8	
7) Watermelon juice + water extrt	250L				
8) Isolated chromoplast paste	1.44 kg	12.5			
9) Juice from 160 kg watermelon rind	125 L		320	3.7	
10) Pulp from 160 kg fresh rind	11 kg				
11) Total cit-containing juice from steps 7 + 9	370 L		906		
12) Portion juice from step 11 for cation exchange	50 L		108		
13) Cation exchange eluate	4 L		800[a]		
14) Dried citrulline	1 kg[b]		740[b]		
15) Cation exchange column flow thru	370 L			24.5[c]	
16) Ethanol (from watermelon only)					11.9[d]

[a] Values from a column run on 50 L of the total 370L of lycopene-free juice extrapolated to what it would be from all 370 L. The actual column yield from 50 L of juice (step 12) was 108 g of citrulline.

[b] Drying of the 4L of column eluate yielded 138 g dry material that contained 100 g of citrulline. These values extrapolate to 1 kg dry material containing 740 g citrulline from 400 kg of watermelon.

[c] The glucose + fructose content (after invertase hydrolysis of sucrose) was 66 g/L in the cation exchange column flow-through.

[d] Value extrapolated from a fermentation run on 7 L of the total 370 L of cation exchange column flow-through.

IV. DISCUSSION

Because of their potential health benefits and because they can be obtained from a natural food source, watermelon lycopene and cit offer the health-conscious consumer the potential for supplements in a natural state similar to that in which they exist in the fruit. Lycopene supplements chemically extracted from tomato must be suspended in oil to improve digestive absorption whereas lycopene packaged in watermelon chromoplasts is available for digestive absorption without additional processing [18]. Currently, the only sources of commercial cit supplements are produced by bacterial fermentation [19].

Cull watermelons can serve as raw material for the two nutraceuticals as well as ethanol biofuel through integration of the three processes discussed herein. Integration of these three processes requires a specific sequence of steps to most efficiently produce the three diverse products. This specific sequence of steps is dictated primarily by the propensity of watermelon chromoplasts to strongly interact with other hydrophobic species. Lycopene-containing chromoplasts of watermelon flesh require removal before cation exchange chromatography because of their propensity to bind to the hydrophobic polystyrene matrix of the cation exchange resin. In fact, the hydrophobic nature of the chromoplast membrane requires processing of the watermelon flesh separate from the rind to prevent binding of the chlorophylls in the rind peel to the chromoplasts which yields a moca-brown-colored chromoplast preparation rather than a vibrant red-colored one. Watermelon chromoplasts must be removed before preparation of cit from flesh, but they can remain in the juice if the juice is employed only for fermentation. Similarly, juice from the complete watermelon can be directly subjected to fermentation. Rind juice can be processed for both cit and ethanol or either individually.

The simple sugars in watermelon juice require no additional processing before fermentation to ethanol. They are, however, not sufficiently concentrated for economical ethanol biofuel production. The juice or process waste stream would have to be concentrated 2.5-3 fold or a supplemental source of sugars, such as molasses, added to it before fermentation would be economically feasible.

Processing of watermelon flesh and rind yields residual solids. One tonne of watermelon would produces about 60 kg of dry pulp solids from flesh and about 20 kg of dry pulp solids from rind. The composition of the solids will be predominantly pectins and hemicelluloses. They may be used as animal fodder or be broken down to constituent pentoses and hexoses and serve as fermentation feedstock. If the pulp from watermelon flesh is not re-extracted after juice removal, it still contains about 30% of the initial lycopene content. For flesh originally containing 60 mg lycopene/kg fresh weight, the pulp after juice removal would contain about 18 mg lycopene/kg fresh weight. Preliminary studies indicate that the flesh pulp at this point can be processed into a nutritious, chewable fruit snack (W. Fish, unpublished results).

In conclusion, the results of this investigation provide a multi-faceted choice for recouping watermelon production investments from the nominal 20 percent of a watermelon crop that cannot be marketed as fresh fruit. Three processes, one for lycopene production (packaged in its natural state), one for L-citrulline production, and one for the conversion of watermelon's simple sugars to ethanol biofuel provide the producer, packer, or processor with processing alternatives that best meet his/her particular circumstances. The three separate processes can be broken out to be used individually to produce a single product, in various combinations to produce two products, or integrated together as demonstrated in this paper to produce three or four products with little waste.

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DISCLAIMER

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He was Professor of Biochemistry at the Medical University of South Carolina from 1970-1986 and Senior Research Associate in Biosciences at Phillips Petroleum Company from 1986-1999. From 1999 to present, he has been a Research Chemist with the Agricultural Research Service branch of the United States Department of Agriculture in Lane, Oklahoma. He has authored or coauthored more than 100 publications in physical biochemistry, enzymology, structure-function in proteins, plant-pathogen interactions, and physical, chemical and biological properties of naturally occurring compounds in fruit and vegetables.

Dr. Fish is a long time member of the American Chemical Society and the American Society for Biochemistry and Molecular Biology. He was the 1986 American Chemical Society’s Outstanding Chemist for South Carolina and was a co-awardee of the Horticulture Society Southern Region’s L.M. Ware Research award in 2004.