The ripening and aging of noni fruits (*Morinda citrifolia* L.): microbiological flora and antioxidant compounds

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Abstract: The juice of noni fruit (*Morinda citrifolia* L.) is claimed to be a natural functional beverage with a growing market both in the USA and Europe. It is traditionally produced by keeping harvested fruit in closed containers for several weeks as the fruit senesces or ages. Little is known about the changes that occur in the juice's microbiological, physicochemical, and functional characteristics during this treatment. Traditional processing was simulated in the laboratory, with samples being recovered and analyzed at various time intervals. At first, fermentation occurred and populations of molds, yeasts, and mesophilic bacteria increased significantly. After 2 weeks, microbial growth changed abruptly, stopping for yeasts, molds, and mesophilic bacteria, and decreasing suddenly for lactic bacteria. Analyses of pH, soluble solids, ethanol, and lactic acid in the fruits confirmed the microbial analyses, indicating initial sensitive variations, followed by values remaining comparatively steady during aging. Vitamin C and total phenol contents also remained constant at 300 ± 60 mg and 50 ± 20 mg GAE, respectively, per 100 g of pulp. Antioxidant capacity likewise remained relatively high at 8 ± 1.5 μmol Trolox® g^{-1}. All phenolic compounds, including scopoletin and rutin, varied significantly immediately after harvest but remained more or less steady during aging.

Keywords: *Morinda citrifolia*; polyphenols; ORAC; traditional processing; fruit ripening and aging; microbiological flora

INTRODUCTION

'Noni' is the Hawaiian name for cheese fruit (*Morinda citrifolia* L.), also known as the Indian mulberry. This fruit belongs to the Rubiaceae family and is native to Southeast Asia, Oceania, and tropical Australia, extending from Polynesia to India. It is now grown throughout the tropics and is cultivated on a commercial scale in Latin America, from Mexico to Colombia and Venezuela, including Costa Rica, Panama, Florida Keys, and the West Indies.[1,2]

The noni plant has traditionally been used by Polynesians for medicinal purposes for more than 2000 years. It is claimed to stimulate the immune system and thus fight against bacterial, viral, parasitic, and fungal infections, and even to prevent the formation and proliferation of malignant tumors.[2,3]

The composition of noni fruit has been studied, although not extensively.[4] The presence of certain bioactive compounds has been reported, such as scopoletin, nitric oxide,[5] vitamin C,[6] acetyl derivatives of asperuloside[7], fiber, alkaloids and sterols.[8] It is also claimed to have a high antioxidant capacity.[8] Some *in vitro* and *in vivo* analyses have evidenced anti-inflammatory,[9] analgesic,[10] antitubercular,[11] and anticancer[12] properties.

Although relatively little scientifically reliable information is available on its properties, noni juice is already participating in the growing functional beverage market. Highly efficient marketing has made it readily available, not only in the producing countries, but also in the USA, Japan and Europe.

The fruit is marketed mainly as fresh in the producing countries and as bottled pasteurized juice, either pure or mixed with other juices (usually grape or blackberry), in other countries. The traditional Polynesian process for obtaining noni juice has been

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imitated almost everywhere. It consists of storing the fruits in closed containers for 4–8 weeks, recovering the juice through lixiviation and/or mechanical pressure, pasteurizing, and conditioning.[13] In fact, noni is a climacteric fruit that can be harvested green, after which it continues maturing physiologically, becoming cream-colored. As it ripens, it turns whitish gray to translucent and finally becomes brown as it senesces.[13]

Once the fruits are ripe, their storage until senescence is often described as aging.[13] Traditionally, aging is claimed to facilitate the transport of juice from production areas to consumers, and to facilitate its storage and extraction. It is also supposed to improve the fruit's properties.[13] Nevertheless, the noni has received very limited characterization, comprising a few papers describing the green and ripe fruits. No information is available on the real impact of aging on the fruits' microbiological, physicochemical, and antioxidant properties as they change during traditional processing.

EXPERIMENTAL

Materials

Noni fruits used in this study were obtained from a farm at an experimental plantation established by EARTH University in the humid tropical region of Limón (Costa Rica). The fruits were harvested while still unripe but after they had reached the normal size and had become cream-colored, thus indicating physiological maturity. A total of 90 kg of fruit were washed, disinfected with 200 ppm chlorine, rinsed with clean water and then separated randomly into small lots of exactly 3 kg each corresponding to approximately 13–15 fruits. Fruit lots of 3 kg each were put in 30 food-grade plastic 7L buckets. All buckets were tightly sealed and stored at room temperature ($28 \pm 1\,^{\circ}C$). Three buckets were selected randomly each week for subsequent analysis during the 8 weeks of the experiment. Unripe fruits were analyzed before storage. Ripe fruits were analyzed after only 3 days of storage.

The entire contents of the containers were chopped and homogenized in a mixer. Analyses were then performed for pH, moisture content, total soluble solids (TSS), vitamin C, lactic acid, ethanol, and total plate counts of mesophilic and lactic acid bacteria, molds, and yeasts. For polyphenols and antioxidant capacity the samples were first frozen with liquid nitrogen, lyophilized, ground to powder, and stored before analysis.

Analytical methods

Analyses for pH, moisture content, and TSS followed standard methods.[14] Mesophilic plate counts were determined using aerobic plate count methods,[15] namely the Downes and Ito method[16] for the lactic acid bacteria count and AOAC methods[15] for the mold and yeast counts.

Ethanol and lactic acid contents were assessed using the respective enzymatic kits: Enzytec™ Fluid Ethanol, ID No. 5340[17] and Enzytec™ D-Lactic Acid/L-Lactic Acid, ID No. 1-002-891 (Scil Diagnostics GmbH, Martinsried, Germany).[17] For both analyses, noni pulp was previously centrifuged for 10 min at $8900 \times g$ and the supernatant used to measure ethanol and lactic acid contents.

Ascorbic acid and dehydroascorbic acid contents were determined by high-performance liquid chromatography (HPLC), using the method modified by Kacem *et al*.[18] and Brause *et al*.[19] Total phenolic compounds were determined using a modified Folin–Ciocalteu method.[20,21] Results were expressed as gallic acid equivalents (GAE), measuring absorbance at 760 nm.

All standards and solvents of analytical grade were purchased from Sigma-Aldrich Chimie (Saint-Quentin-Fallavier, France).

Determination of the antioxidant capacity

A lyophilized sample (0.5 g) of noni was submitted to extraction with an aqueous solution of acetone (30:70) for 30 min, in accordance with Ou *et al*.[22] The extract's antioxidant capacity in terms of oxygen radical absorbance capacity (ORAC) was measured by using fluorescein as the peroxyl radical damage indicator. The methodology adopted was developed by Ou *et al*.[22] and adapted to semi-manual measurement by Vaillant *et al*.[23]

Identification of phenolic compounds by thin-layer chromatography

Phenolic compounds were extracted, using 100 mg of freeze-dried material macerated with 2 mL of ethanol at 80% and homogenized in an ultra-sonic bath for 15 min at room temperature. The extract was filtered through Whatman® No. 4 filter paper. All the extracts were separated on cellulose (Merck, Frankfurt, Germany, ref. 1.05552.0001) and silica layers, using thin-layer chromatography (TLC) and ethyl acetate, formic acid, acetic acid, and water at 100:11:11:26 (v/v/v/v). Two-dimensional TLC was also employed, using acetic acid at 2% and methane dichloride, acetic acid, and water at 50:45:15 (v/v/v) as the mobile phases. Standards (scopoletin, alizarin, aucubin, isoquercetin, *1*-kaempferol, kaempferol-3-glucoside, rutin, morin, quercetin, and quercitrin) were prepared in the range of 9–10 mg mL^{-1} of methanol.

Identification and quantification of phenolic compounds by HPLC

Phenolic compounds were identified and quantified using the method described by Dubber and Kanfer.[24] A 500-mg aliquot of lyophilized sample was sonicated at 47 kHz with 25 mL of methanol for 60 min and mixed with a magnetic stirrer for 30 min. The mixture was then filtered through Whatman® No. 4 filter paper, evaporated at low pressure in a Rotavapor®

at 40 °C, and diluted with 2 mL of methanol. The resulting samples were then filtered through disposable 0.45-μm poly(vinylidene difluoride) filters before injection (20 μL). The standards ricinoleic acid, quercetin, rutin, scopoletin, and ursolic acid were used. A 510 Waters separation module and Waters 990 diode array detector (Milford, MA, USA) were used for HPLC analysis, with the detector equipped with a manual injector and column heater. The phenolic compounds were separated at 40 °C on a Merck–Lichspher, 5-μm, C_{18} column with dimensions of 4 × 250 mm, using a one-step linear gradient. Mobile phases A (acetonitrile) and B (0.3% formic acid) ratios changed after 15 min from 15:85 to 25:75. Total run time was 33 min at a flow rate of 700 μL min^{-1}.

Statistical analysis

All values reported are the calculated average value and standard error for triplicate analyses performed on the content of three pails randomly selected each week over 8 weeks. Analysis of variance (Duncan's multiple range test) was used to determine significant differences ($P < 0.05$) for analysis between different storage times, employing the statistical software SPSS (Version 10.0.1, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Microbiological analyses

Figure. 1 shows the results of the microbiological analyses performed on noni undergoing postharvest maturation and aging. Molds, yeasts, and mesophilic and lactic bacteria populations grew exponentially during the first 2 weeks of aging. The molds, yeasts, and mesophilic bacteria then tended to stabilize, whereas the lactic bacteria populations decreased abruptly and became undetectable after 5 weeks.

Overall, the microbial load appeared to be very similar to that observed previously on fully ripe nonis.[25] The stagnation in microbial population growth after almost 15 days may have been caused by chemical changes in the juice that perhaps partially inhibited some of the microorganisms. Indeed, we observed a small decrease in pH and TSS values from fully ripened fruits to fruits starting to senesce (i.e. first week of aging). However, no significant differences were found for pH and dry matter content among the weekly analyses during aging, and only a small decrease in TSS was observed between weeks 1 and 2.

Even if the noni juice still contained sugars (at about 5 g L^{-1} of TSS), ethanol and lactic acid concentrations remained constant after the second week of aging (Table 1). The decrease in the microbial population may have resulted from a change in substrate characteristics, changes in the medium (eg. dissolved gas) and inclusively from the presence of compounds with antimicrobial properties, as reported previously.[26] For example, some organic solvent extracts of noni fruit have an antimicrobial effect on certain bacteria such as *Salmonella*, *Shigella*, *Escherichia coli*, and *Staphylococcus aureus*.[11,27–30]

Changes in contents of phenolic compounds

Thin-layer chromatography (TLC), using commercial standards, of green and ripe fruit extracts led to the identification of only two phenolic compounds: scopoletin (7-hydroxy-6-methoxycoumarin) and rutin (quercetin-3-rutinoside). Three other stains were observed (Fig. 2) but they did not correspond to any of the following standards tested: alizarin, aucubin, isoquercetin, 1-kaempferol, kaempferol-3-glucoside, morin, quercetin, and quercitrin.

Other authors have reported the presence of aucubin[30] and alizarin,[8] but these compounds were not detected in our fruit samples. For quercetin, the gentle extraction method (involving no hydrolysis) may explain why we did not find it in our samples. That is, the rutin is a flavonol glycoside comprised of flavonol quercetin and disaccharide rutinose[31] and is sometimes hydrolyzed. This result was also confirmed by HPLC analysis, as only five separated peaks were observed on the chromatogram (Fig. 3).

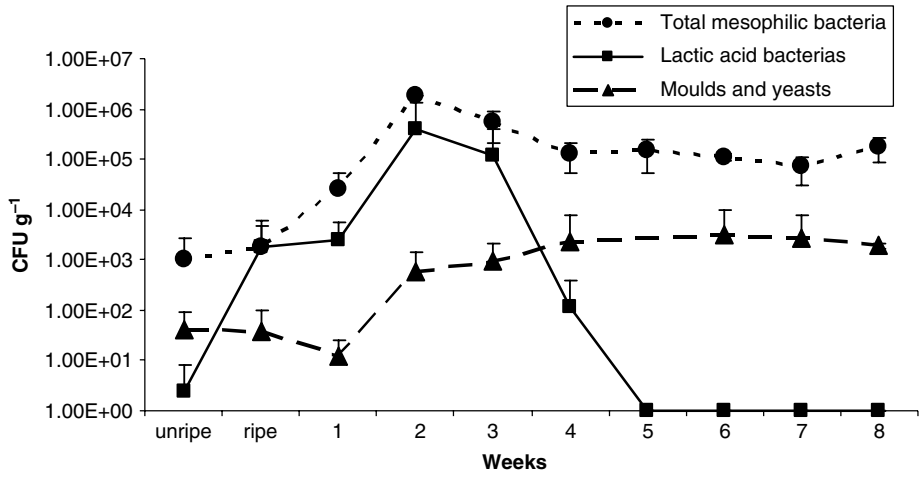

Figure 1. Microbiological analyses of noni fruit during ripening and aging, showing population growth rates of mesophilic and lactic acid bacteria, molds, and yeasts.

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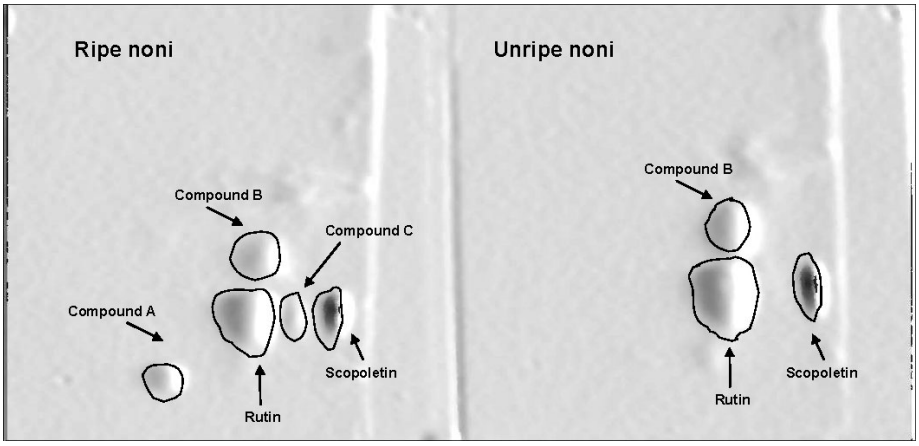

Figure 2. Identification of rutin and scopoletin in ripe and unripe noni fruit with two-dimensional thin-layer chromotography, using acetic acid at 2% and the solvents methane dichloride, acetic acid and water at 50:45:15.

Table 1. Determining the main characteristics of fruits of noni (*Morinda citrifolia* L.) during ripening and aging

Samples	pH	Soluble solids (°Brix)	Moisture (g kg^{-1} pulp)	Ethanol content (mg L^{-1})	Lactic acid content[a] (mg L^{-1})	Phenols content (mg GAE kg^{-1} pulp)	Vitamin C[c] (mg kg^{-1} pulp)	ORAC[d] (μmol Trolox g^{-1} pulp)
Unripe (pale yellow)	4.6 (0.1)^{1}	7.5 (0.3)^{1}	92.0 (0.9)^{1}	–	–	41.4 (4.0)^{1,2}	391 (13)^{1}	7.4 (0.2)^{1,2}
Ripe (translucent gray)	4.0 (0.1)^{2}	7.3 (0.2)^{1}	91.8 (0.2)^{1}	2663 (134)^{1}	658 (23)^{12}	51.1 (1.3)^{1,2}	316 (37)^{1,2}	8.0 (0.4)^{1}
Aging								
Week 1	3.6 (0.1)^{3}	7.0 (0.1)^{1,2}	91.9 (0.5)^{1}	9932 (988)^{2}	694 (41)^{1,2}	43.9 (10.1)^{1,2}	293 (46)^{2}	7.8 (0.8)^{1,2}
Week 2	3.8 (0.1)^{2,3}	5.8 (0.2)^{3,4,5}	92.9 (0.6)^{1}	12054 (92)^{3,4}	1818 (285)^{3,4}	27.5 (6.0)^{1}	278 (31)^{2,4}	6.8 (0.5)^{1,2}
Week 3	3.9 (0.1)^{2,3}	6.5 (0.3)^{2,3}	92.4 (0.6)^{1}	9101 (210)^{2}	2936 (371)^{5}	29.4 (11.2)^{1}	223 (26)^{2,3,4}	7.2 (1.2)^{1,2}
Week 4	4.0 (0.1)^{2}	5.8 (0.2)^{3,4,5}	93.0 (0.5)^{1}	9638 (300)^{2}	2558 (435)^{4,5}	45.3 (7.2)^{1,2}	318 (29)^{1,2}	8.4 (0.4)^{1,2}
Week 5	3.6 (0.1)^{2,3}	6.2 (0.2)^{3,4}	92.9 (0.3)^{1}	13066 (98)^{4}	1067 (32)^{1,2}	50.8 (7.2)^{1,2}	294 (24)^{2}	7.1 (0.3)^{1,2}
Week 6	3.6 (0.2)^{2,3}	5.7 (0.2)^{3,4,5}	91.8 (0.4)^{1}	11366 (288)^{3}	2036 (290)^{3,4}	60.0 (7.2)^{2}	148 (27)^{3}	9.1 (0.6)^{2}
Week 7	3.9 (0.1)^{2,3}	5.2 (0.2)^{5}	91.8 (0.7)^{1}	11075 (182)^{3}	1300 (183)^{4,3}	29.9 (6.1)^{1}	284 (10)^{2,4}	9.4 (0.8)^{1,2}
Week 8	3.6 (0.2)^{3}	5.8 (0.3)^{3,4,5}	92.8 (0.2)^{1}	8813 (210)^{2}	396 (26)^{1}	29.2 (7.5)^{1}	193 (16)^{4,3}	8.7 (0.5)^{1}

Values are the means (standard error) of three samples purchased and analyzed independently. Values within columns followed by the same number are not significantly different at $P < 0.05$ (Duncan's multiple range test).

[a] Sum of L- and D-lactic acids

[c] Sum of ascorbic and dehydroascorbic acid.

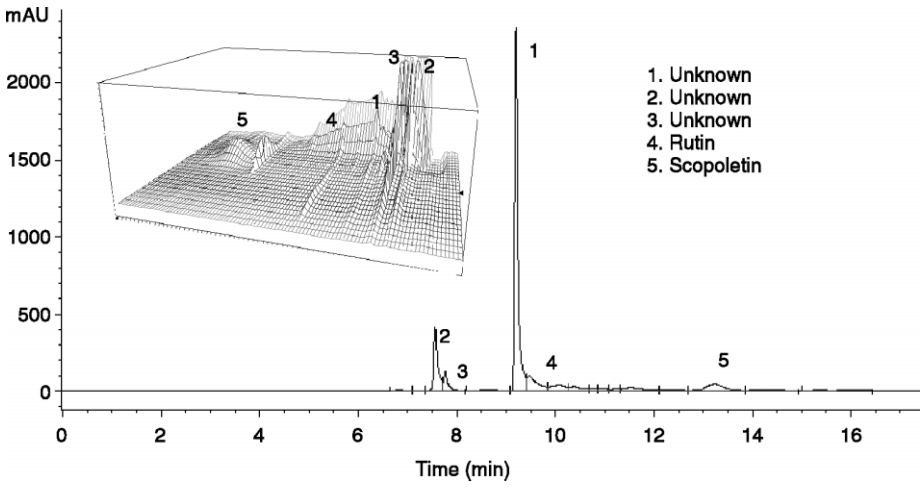

Figure 3. Chromatograms (two- and three-dimensional) from HPLC of methanol extract from ripe noni to identify phenolic compounds.

According to the known standards used, only rutin and scopoletin were identified, whereas the other standards tried (ricinoleic acid, quercetin, rutin, scopoletin, and ursolic acid) did not identify new compounds. Three other phenolic compounds, corresponding to the other peaks in the figure, remain unknown.

The presence of scopoletin in noni fruit was previously reported by several authors,[5,30,32] but not precisely quantified in the fruits. This phenolic compound is uncommon in natural products and is claimed to have good functional and antimicrobial properties,[26,33] anti-inflammatory effects,[34] analgesic properties, antihypertensive effects,[5] and a significant ability to control serotonin levels in the body.[7]

The second phenolic compound identified, rutin, is more common in natural products, and was previously reported in noni by Wang *et al*.[30,35] Again, it was not precisely quantified. Rutin was shown to metabolize into quercetin in the digestive tract. Rutin has high antioxidant properties,[36] with anti-inflammatory effects[37,38] and antimicrobial activity.[39]

The contents of the two identified phenolic compounds were assessed during fruit ripening and aging, using HPLC analysis. Results are shown in Fig. 4. Unripe noni with physiological maturity presented the highest concentration of rutin, which then decreased during ripening but remained almost constant during aging. In contrast, scopoletin content is low in unripe fruits but increases during ripening. It then remains almost steady, with no statistical differences ($P < 0.05$) observed between samples during aging.

Of the three unknown phenolic compounds, compound 1 (Fig. 5) showed considerable increase in concentration during ripening and then remained steady during aging, following the same trends as observed for rutin and scopoletin. Compound 2 showed a significant decrease in concentration during ripening and a similar, almost steady, trend during aging. Compound 3, in contrast to the others, did not show any statistical differences during ripening, although its concentration appeared to increase progressively during aging.

Antioxidant properties

During aging, total phenolic compounds in the noni varied widely from 27 to 60 mg GAE kg^{-1} of juice (Table 1). The concentration of total phenolic compounds is relatively high: comparable with that of lemon, peach, and pineapple,[40] but higher than for tomato, orange juice,[21] banana, litchi and longanberry.[41]

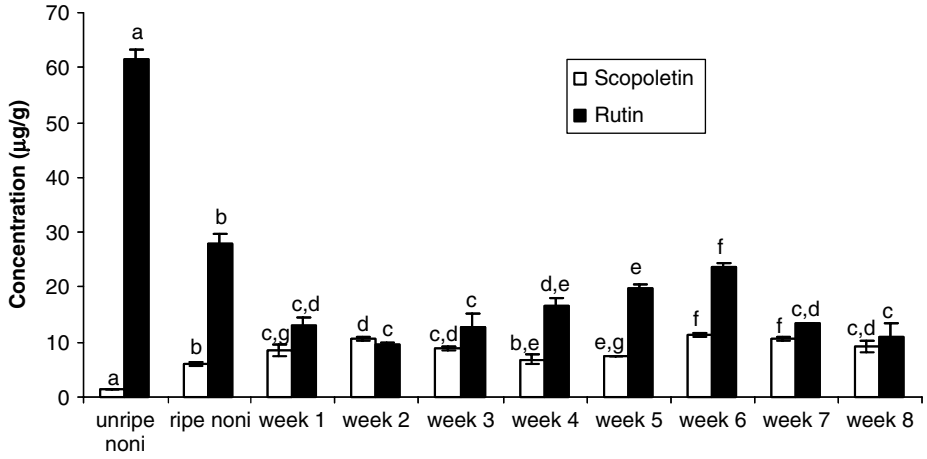

Figure 4. Determination of scopoletin and rutin in noni fruit during ripening and aging. Values refer to means and standard deviations of three samples that were purchased and analyzed independently. Values within columns followed by the same letter are not significantly different at $P < 0.05$ (Duncan's multiple range test).

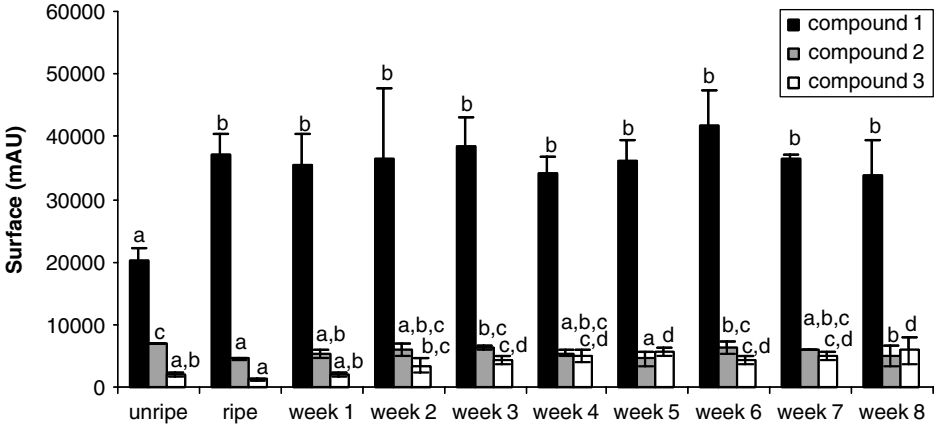

Figure 5. Separation of unknown compounds extracted from noni fruit during ripening and aging. Values refer to means and standard deviations of three samples that were purchased and analyzed independently. Values within columns followed by the same letter are not significantly different at $P < 0.05$ (Duncan's multiple range test).

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As already reported,[6] vitamin C content in noni is high, being almost 10 times that of orange juice[42] and making noni an important source of this micronutrient. Total vitamin C content decreases slightly during ripening, but unexpectedly remains almost constant during aging. Aging occurs at low temperature, and probably in a medium with low content of dissolved oxygen because the buckets were tightly sealed. Actually, even if a decrease in the content of ascorbic acid was observed, it was offset by an increase in dehydroascorbic acid (data not shown), leading to no significant change in total vitamin C content.

Antioxidant capacity varied between 6.8 and 9.4 μmol Trolox® g^{-1} (Table 1) during the entire study period, showing no particular trend. The ORAC values were similar to those found in fruits like grapefruit, banana, apple, plum, orange, grape, and kiwi fruit.[43] Higher antioxidant capacity was detected in ethyl acetate extract from noni fruit,[44] comparable with α-tocopherol and butylated hydroxytoluene. However, in our case, such high levels were not found when analyzing the entire fruit.

All the results obtained prove that traditional processing treatment does not appear to affect the main composition of noni fruits because changes occurred mostly during ripening and imperceptibly during aging. Although microbiological activity was evidenced, it was relatively limited and did not affect significantly the contents of phenolic compounds and vitamin C or the ORAC antioxidant capacity.

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