Analyzing changes in metabolite profile during postharvest ripening in *Achras sapota* fruits: GC-MS based metabolomics approach

Das, S. and De, B.

Phytochemistry and Pharmacognosy Research Laboratory, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India

**Abstract**

*Achras sapota* L. (syn. *Manilkara zapota* (L.) P. Royen) fruits are commonly known as sapodilla. The mature fruits when unripe are not edible. They are allowed to ripen at room temperature when the pulp softens and become edible. The aim of the present investigation was to determine the changes in metabolite composition during postharvest ripening over a span of 10 days following a GC-MS based metabolomics approach. The present metabolic data revealed, for the first time the composition of *A. sapota* fruit metabolites and changes in individual metabolite during postharvest ripening. Total 46 identified metabolites (11 sugars and sugar alcohols, 11 organic acids, 14 amino acids, 5 phenols, 4 fatty acids and 1 inorganic acid) and 20 tentatively identified compounds which showed significant differences during ripening could be detected. On the whole it appears that in spite of decrease in the level of many metabolites, a large number of amino acids and sugar alcohols increased in quantity ameliorating the fruit quality during ripening.

**Keywords**

*Achras sapota*, fruit, postharvest ripening, metabolomics, GC-MS

**Article history**

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**Introduction**

Fruit quality is a direct function of metabolite content, and the compositional analysis of plant metabolites is an established application in metabolomics research (Moco et al., 2007). Organoleptic quality of fruit is a complex trait of fruit quality involving a combination of taste, flavor and aroma (Oms-oliu et al., 2007). The nutritional quality of fruits is correlated closely with the presence of soluble sugars, organic acids, essential fatty acids, amino acids and some major secondary metabolites. These compounds play an important role in balancing fruit quality and nutritional aspects. That is why fruit compositional analysis is of great interest to food chemists (Zhang et al., 2011). Fruit ripening generally influences the level of pigments (e.g. carotenoids and flavonoids), sugars, acids and aroma volatiles to make the fruit more appealing and promotes tissue softening (Giovannoni, 2004; Giovannoni, 2007).

*Achras sapota* L. [syn. *Manilkara zapota* (L.) P. Royen] commonly known as sapodilla, is one of the fruit crops of South Asia. The tree is native to Mexico and tropical America and is now cultivated throughout the tropics (Shui et al., 2004). The mature unripe fruits when harvested are not edible. They are allowed to ripen at room temperature when the pulp softens with change of color from greenish to brown and become edible. In our previous study, inhibitory activities of *A. sapota* fruit against α-glucosidase, α-amylase and angiotensin I- converting enzyme have been reported (Das et al., 2012; Das and De, 2013). The major objective of the present investigation was to monitor the metabolic changes during postharvest ripening. Changes in metabolite profile can be determined by a number of methods e.g. gas chromatography coupled with mass spectrometry (GC-MS), liquid chromatography coupled with mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (NMR). NMR has a smaller dynamic range than mass based technologies for detection of metabolites (Sumner et al., 2003). A GC-MS based metabolomic approach was adopted to study the metabolic changes during the present study.

Metabolic changes during development of important fruits e.g. tomato (*Solanum lycopersicum* L.) (Carrari et al., 2006), strawberry (*Fraggaria Xananassa* Duch.) (Zhang et al., 2011), grape berry (*Vitis vinifera*) (Deluc et al., 2007), black raspberry (*Rubus coreanus* Miquel) (Kim et al., 2011), guava (*Psidium guajava* L.) (Lee et al., 2010) have been reported. But there are very few reports regarding changes in metabolite pattern in fruits during postharvest ripening. Postharvest and pre harvest metabolic changes in peach (*Prunus persica* (L.) Stokes) (Lombardo et al., 2011) and tomato (Oms-oliu et al., 2011) have been discussed. We report here changes in metabolite pattern during postharvest ripening in *A. sapota*.
Materials and Methods

Plant material

Mature, unripe *Achras sapota* fruits (Variety cricket ball) of more or less equal sizes were collected right after their harvest in the morning from Agricultural Field Station of University of Calcutta, Baruipur, India during its peak fruiting season (i.e., the month of April, 2012). The surface of the fruits was cleaned with distilled water just after harvesting. These fruits were then stored at room temperature (day temperature 35°C ± 2; night temperature 25°C ± 1) in the open air from day 0 i.e. the day of harvesting, up to day 9.

Sample preparation

The fruits of *A. sapota* were peeled and the seeds were removed before extraction. The fruit flesh (60 ± 5 mg) (crushed to powder with liquid nitrogen) was extracted with 1 ml of methanol at 60°C for 15 min. Ribitol (20 µl of 200 µg ml⁻¹) was added as an internal standard. The extract was centrifuged at 14,000 rpm for 15 minutes. The supernatant was distributed into eppendorff tubes (4 x 50 µl) and evaporated to dryness. For each biological replication, four fruits were considered. The residue was derivatized with MSTFA [N-methyl-N-(trimethylsilyl)trifluoroacetamide] following the method of Roessner et al. (2006) for GC-MS analyses. The dried residues were re-dissolved in methoxamine hydrochloride in pyridine (30 mg/ml), shaken for 2 h at 37°C. Then MSTFA (40 µl) and retention time alkane mixture [prepared using n-dodecane, n-heptadecane, n-nonadecane, n-docosane, n-octacosane and n-hexatriacontane dissolved in tetrahydrofuran in concentration 0.03%] (10 µl) were added for derivatization.

GC-MS analysis

GC-MS analysis was carried out following the method of Roessner et al. (2006) after little modification. HP-5MS capillary column (Agilent J & W; GC Columns (USA) (length 30 m plus Duraguard 10 m, diameter 0.25 mm narrowbore, film 0.25 µm) was used. The analysis was performed under the following oven temperature programme: injection at 70°C followed by 1 °C min⁻¹ oven temperature ramp to 76°C and then by 6°C min⁻¹ to 300°C and with 10 minute isothermal at 300°C. Helium gas was used as the carrier gas at a flow rate of 1 ml / min (Carrier linear velocity 36.798 cm sec⁻¹). Samples (1 µl) were injected via the split mode onto the GC column. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute GC-MS results and to identify chromatographic peaks distinctly. Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra with entries of mass spectra library NIST 2008, Golm library and with Agilent Fiehn GC / MS Metabolomics library (2008) (Agilent Technologies Inc., Wilmington, USA). The presence of the metabolites was further confirmed by comparing the retention indices relative to n-alkanes (C₁₂ – C₃₆) with those in Golm library. Retention times of some of the metabolites were also compared with that of the standards for confirmation of the metabolites.

Statistical analysis

The relative response ratios of all the metabolites were calculated after normalizing the peak areas of the compounds by sample fresh weight and by the peak area of the internal standard. Data were analyzed by one way ANOVA to describe the significance of variation of each metabolite level between days 0 to 9. p Values of the metabolites ≤ 0.05, were considered significant. Principal component analysis was carried out using SPSS software (Version 16).

Results and Discussions

The mature but unripe fruits, on the day of harvest, were greenish brown in colour with pale yellow flesh, very firm to touch. The flesh was firm and astringent in taste during day 0 and 1. From day 2 onwards the fruits started to ripen. At around days 2-3, the fruits became softer and the colour of the flesh changed to an orange shade, very sweet in taste with a characteristic flavor, perfect stage of ripening to consume this fruit. Flesh of over ripened fruits at around days 8-9 gradually turned from orange to brownish black, tasted sweeter, but with lesser aroma and not very pleasant in taste.

During our study a total of 46 metabolites of known structure comprising 11 sugars and sugar alcohols, 11 organic acids, 14 amino acids, 5 phenols, 4 fatty acids, 1 inorganic acid and 20 tentatively identified compounds, could be detected by GC-MS analysis of the MSTFA derivatized samples during postharvest ripening (Table 1). Relative response ratios of each metabolite were calculated. Such data for each identified metabolite was compared in Figure 1 and Figure 2. Relative response ratios of the metabolites are routinely described (Roessner et al., 2000) for semi quantitative comparison.

Sugars and sugar alcohols are one of the most important contributory determinants for fruit taste.
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(Zhang et al., 2011). Relative response ratios of the sugars and sugar alcohols at different postharvest period are shown (Figure1). Significant decreases in the levels of lactose, melezitose, raffinose and sucrose were noticed. But sugar alcohols like galactinol, glycerol and mannitol were significantly higher during postharvest ripening over that of day 0. Myo-inositol, an important biosynthetic precursor of many cell wall polysaccharides (Oms-oliu et al., 2007), decreased in content. The hydrogenated carbohydrates sugar alcohols (also known as polyols) are used as sugar replacers. They have potential health benefits due to low carcinogenicity, low glycaemia, low insulinaemia, low energy value, as source of substrate for healthy colon and intestinal tolerance (Livesey, 2003).

The phenolic compounds detected in the fruit flesh during postharvest ripening (Figure 1) revealed sharp decline in the concentrations of gallic acid (18 fold) and chlorogenic acid (57 fold) on day 1. The two compounds could not be detected from day 3 onwards. Quinic acid was the only phenolic compound detected right from day 0 to day 9 and a sharp decrease (20 fold) in this metabolite level was observed from day 0 to day 1. Benzoic acid, which could be detected first on day 4, was found in the ripe fruits up to day 9.

Organic acids are important components of fruits that strongly influence the fruit taste. During the study malic acid, lactic acid and succinic acid were detected as the major fruit acids, found at all stages of ripening. On the other hand citric acid, fumaric acid, gluconic acid and oxalic acid were detected only at certain stages of ripening. Maleic acid and glycolic acid were detected only in very unripe fruits (Figure 2). Significant changes in metabolite level of some organic acids were observed during post-harvest
maturation of this fruit. The tricarboxylic acid cycle metabolites detected were citric acid, succinic acid, fumaric acid and malic acid. There was significant increase of citric acid (13 fold) level on day 1. From day 1 to day 2 the content decreased 1.5 fold and was below the detection limit from day 3 onwards. Succinic acid on the other hand gradually increased with ripening. The levels of lactic acid and malic acid decreased significantly. Compound like gluconic acid, believed to be the intermediates of tartaric acid biosynthesis (De bolt et al., 2006) increased during post-harvest ripening stages.

Fatty acids like palmitic acid and stearic acid were present in the fruit flesh at all the stages of ripening. There was a significant decrease in concentrations from day 0 to day 1 in cases of palmitic acid (23 fold) and stearic acid (243 fold). Linoleic acid and oleic acid appeared on the day 0 only (Figure 2).

The variation in the levels of amino acids with different stages of postharvest ripening of this fruit is shown in Figure 2. Glycine, derived from serine, could not be detected on first three days and then detected up to day 9 (except on day 5). Valine and isoleucine, the pyruvate derived amino acids, gradually increased up to day 9. Tricarboxylic acid cycle derived organic acids serve as the precursor of many amino acids. Aspartic acid (day 9) derived from oxaloacetic acid was detected during over ripened stages of the fruit. Proline, which could not be detected on days 0, 1 and 2, was present from day 3 onwards. The emerging evidences show that traditionally classified nonessential amino acids play important roles in gene expression, cell signaling and thus have effect on cell proliferation, differentiation, metabolism, homeostasis and function (Haynes et al., 2009; Stipanuk et al., 2006; Wu, 2010). Both essential and nonessential amino acids should be present in diet to optimize health (Wu, 2010).

Amongst the nonessential amino acids detected in A. sapota fruits, all (aspartate, glutamic acid, glycine and tyrosine) except serine increased in quantity with postharvest ripening. The essential amino acids lysine and threonine decreased whereas isoleucine and valine increased in quantity.

The data set obtained after the GC-MS analysis was subjected to Principal Component Analysis (PCA). Principal component 1 (PC1) and principal component 2 (PC2) score plot segregated three distinct groups separating day 0 (Group I) and day 9 (Group III) distinctly from the rest i.e. days 1-8 (Group II) (Figure 3A). The findings suggest that there is a metabolic change from day 0 to post harvest ripening stages. Loading plot (Figure 3B) analysis suggested that the major metabolites
responsible for such segregation of day 0 are aspartic acid, pyroglutamic acid, tyrosine, valine, proline, galactinol, glycerol, sucrose, raffinose, catechin etc. As a complement to estimate the marker metabolites, significant increase and decrease of metabolites are shown in Figure 4. In spite of decrease in the level of many metabolites, a large number of amino acids and sugar alcohols increased in quantity. The study revealed that metabolic profile changed in *A. sapota* fruits. Further studies are required to be carried out to know the proper biochemical reason for such changes.

**Conclusion**

Metabolomic approaches are increasingly being used to thoroughly study the metabolic composition of plant organs and to characterize the variation in metabolite content (Schauer and Fernie, 2006). Metabolomic approaches to discover the metabolites and metabolic associations correlated with food quality traits are also being used for food crops (Hall et al., 2006; Fernie and Schauer, 2009). The present study used a GC-MS based metabolomics approach to demonstrate how the characteristic metabolic profile of sapodilla fruit varied during the postharvest ripening period. The present data revealed, for the first time, not only the detailed composition of *A. sapota* fruit metabolites but also changes of individual metabolites during postharvest ripening. On the whole it appears that in spite of decrease in the level of many metabolites, a large number of amino acids and sugar alcohols increase in quantity. In conclusion, the obtained results from the GC-MS based metabolic data indicated that *Achras sapota* fruit contains a lot of health beneficial metabolites. On the basis of metabolites that could be identified during the study, it is suggested that the fruit quality is ameliorated during postharvest ripening.

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