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Ripening-dependent changes in antioxidant activities and un-targeted phytochemical fingerprinting of mango (*Mangifera Indica* L.) cultivar Safaid Chonsa

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Research Article

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Abbreviations:
RSI-RSV, Ripening stage I-V, UHPLC/MS Ultra high-performance liquid chromatography/ Mass spectrometry; GAE, gallic acid equivalent; DMSO, di methyl sulfoxide; QE, quercetin equivalent; DPPH 1, 1-diphenyl l-2-picryl-hydrazil); RSA, radical scavenging activity; AAE, ascorbic acid equivalent; ABTS 2, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid; OD, optical density; PCA, principal component analysis; ANOVA, analysis variance; LSD, least significant difference; FRAP, Ferric Reducing Antioxidant Power; TEAC, Trolox equivalent antioxidant capacity; EDTA, Ethylenediaminetetraacetic acid

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Abstract

The ripening-dependent changes in antioxidant activities and phytochemical content of mango (*Mangifera indica* L.) cultivar Safaid Chonsa at various ripening stages were evaluated. The ripening time period was divided into five stages (RSI-RSV) and the pulp was subjected to proximate analysis, antioxidant potential, and UHPLC/MS-based non-targeted metabolite fingerprinting. Proximate analyses depicted variations in moisture, dry matter, fat, protein, carbohydrate, and energy parameters. Maximum DPPH activity (51%) was observed at stages III, IV, and V while FRSP increased 31% at RS V as compared to stage I. Total antioxidant capacity and total reducing power potential were maximum (295.7 and 345.71 µg AAE/mg extract, respectively at stage V. Total phenolic content increased from 3.57 µg GAE/mg extract to 5.72 µg GAE/mg extract from stage I to RSIII while 19% increase in total flavonoid content was observed at stage V as compared to stage I. UHPLC/MS analysis showed presence of Aconitic acid, methylisocitric acid, 4-O-methyl gallate, beta-glucogallin, xanthenes, sakebiose, Isobergaptene, Fructoselysine 6-phosphate, Citbismine C, and many others at different ripening stages of chonsa mango extracts. The results conclude that during the mango ripening stages, changes in phytochemical composition have positive correlation with antioxidantive potential. These phytochemicals have nutritional and nutraceutical effects on human health therefore ripening stage should be considered for consumption of mango.

Introduction

Pakistan especially southern Punjab region produces world best mango. The climatic conditions, temperature, humidity, and soil texture are most suitable for growth of most flavoured, best aroma, colour attractive mango production. A number of varieties are cultivated including sindhri, dosehri, chonsa, langra, anwar retol, and many more. Mango (*Mangifera indica* L. of family *Anacardiaceae)* is known due to its flavour, taste, aroma, and nutritional values. As a king of all fruits, likeliness and consumption are worldwide mostly in fresh form and also as processed products. The evergreen mango trees like tropical and subtropical climates. The fruits are harvested at hard green mature stage that ripens quickly at standard conditions. Climacteric respiration and ethylene production are the main parameters to ripen the fruits. During maturation of mangoes, physiochemical changes in pulp tissue especially starch, organic acids, and volatile compounds are main ingredients to develop taste and texture of mangoes. At initial stages of mango ripening, low sugar content is observed that increases with maturity and ripening stages. Other than this degradation of the chlorophyll, breakdown of photosynthetic pigments, hydrolysis of polysaccharides, anthocyanins production, decrease in acidity, accumulation of sugars, organic acids, and carotenoids occur. The ethylene biosynthesis and de novo synthesis of enzymes catalysing ripening specific changes also happen during ripening.

The fruit quality and shelf life are considered critical during the ripening stages.[1] As a result, green mangoes are harvested and it gives significant time for processing and marketing. Ultimately the fully ripened mango ready to eat reaches the consumers.[2] During the ripening process endogenous levels of abscisic acid, indole 3-acetic acid, concentration of carbohydrates, organic acids and other phytochemicals changes that develop flavour, aroma and colour of the fruits.[3] A number of bioactive compounds, such as ascorbic acid, carotenoids, provitamin A, and vitamin C that have dietary roles as antioxidants have been identified. Mango ripening initiates with the production of ethylene that results in physiochemical changes, turning of green colour to yellow, softness of texture, development of flavour and aroma, and so on.[4,5] These changes are due to breakdown of starch and cell wall, accumulation of mono and disaccharides, aromatic organic compounds production, etc.[4]

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The antioxidant capacity has direct correlation with phenolic and flavonoid components.[6] Agronomic conditions, ripening stage, cultivar, post harvest treatment affect the quality and antioxidative potential of mango.[7] Phenolics and flavonoids have been investigated in different varieties of mangos however during ripening what phytochemical changes occur should be investigated. Because these biochemicals have health-promoting properties, boost immune system to fight diseases, and many more.

The ripening process of mango has physiological, enzymatic, and biochemical changes in fruits. These changes define the nutritional and nutraceutical properties of mango fruits. However, there is no precise information on the right stage for mango consumption with highest antioxidant and nutritional properties. In view of this, a study was taken up to investigate effect of ripening stage on proximate analysis, antioxidant activities, and phytochemical profiling of mango (*Mangifera indica L.)* variety Safaid Chonsa at five ripening stages. Proximate analyses include determination of carbohydrate, protein, fibre, fat, and energy content. Total phenolics and flavonoids, total antioxidants, reducing power potential, and free radical scavenging activities were also investigated. To find the variation in metabolic contents at different ripening stages, the extracts were analysed by UHPLC/MS.

Materials and methods

Mango cultivars and ripening process

The green uniform-size mango (*Mangifera indica* L.) fruits (average weight of 150–200 g) of Safaid Chonsa cultivar were harvested from Southern Punjab Multan field. The fruits did not have signs of pathogenic infection or injury. After sanitisation with chlorinated water for 3 min and drying at room temperature, the ripening process was performed as per commercial conditions rather than strict physiological conditions. For this study, fruits were divided into 5 groups of 6 fruits each. The fruits were packed in cardboard boxes with holes. Approximately 0.5 g of calcium carbide in paper bag was also placed inside the box to accelerate the ripening process. Calcium carbide produces acetylene gas that behaves like natural ripening agent, ethylene. Maturity was judged each day by means of visual colour and texture. Chonsa mango changes colour from green to yellow. Based on the visual colour, the ripening was divided into five stages starting from green freshly plucked state to fully ripped. These include RSI, fresh harvested green mature stage; RSII, 10-30% yellow peel colour; RSIII, 40-60%; RSIV, 70–80% and RSV, 100% yellow colour.

Pulp extraction and extract preparation

Mango at each ripening stage was peeled and pulp was cut into small pieces. The fresh pulp was used for proximate analysis while extracts of mango pulp were prepared by homogenisation of 20 g pulp in 100 ml methanol. The mixture was left for 24 hr and then filtered thereafter through Whatman filter paper No. 4. Filtrate was dried at room temperature under continuous air flow. The extract was used for phytochemical analysis and quantification of total phenolic and flavonoid contents and antioxidant activities. For assays, the extract was dissolved in DMSO at 4 mg/ml.

Proximate analysis of pulp

Proximate analysis i.e. moisture content, dry matter, protein content, fat, carbohydrate contents, and ash content of Safaid Chonsa mango pulp was performed according to the Association of Official Analytical Chemists (AOAC, 2000) methods. Moisture content was calculated by taking 5 g of sample in a pre-weight aluminium moisture dish. The dishes were kept in hot air oven at 150°C for 2 hr and weighed again. The moisture content was determined as:

Moisture (%) = (Weight of fresh sample – weight of dry sample)/Weight of fresh sample × 100To measure ash content, 5 g of pulp in silica crucible was heated at 525°C for 5 hr in a muffle furnace. The heating was continued until the weight became constant. The weight of ash was calculated by the following formula:

Ash (%) = (Weight of fresh sample - Weight after ashing / weight of fresh sample)×100

To determine total fat in mango pulp, 5 g pulp was weighed into fat-free cellulose thimbles and placed in SocsPlus condensers. Petroleum ether (50 ml) was refluxed for 1 hr over the sample. Ether was then drained and remained by evaporation. The mass in the silica reflux cups was designated crude fat.

Crude fibre was determined by taking 2 g in beaker and was digested with 2.5 M H_2SO_4 followed by an equal volume of 2.5 M NaOH on a hot plate for 1 h. The sample was centrifuged and the precipitate was dried in muffle furnace at 600°C until constant weight was obtained. Fibre (%) was calculated as follows:

Fibre (%) = ((weight of crucible − weight of crucible containing ash) × 100)/ weight of sample

Protein was determined by Kjeldahl method. Mango pulp (0.5 g) was weighed in a 50 mL Kjeldahl flask and 8 ml concentrated H_2SO_4 was added. A 5 g copper and potassium sulfate mixture was also added as catalyst. Samples were digested until colourless residue was observed. Digested samples were distilled and vapour gas was collected in a conical flask containing mixture of 25 ml of 2% boric acid solution and indicator. The sample was titrated against 0.1 N HCl until a pink colour persisted. Crude protein was calculated as

Crude protein = ((normality of acid × volume of acid used in ml × 15 × 6.25)/weight of sample) × 100

The total carbohydrate was calculated as

Total carbohydrate (%) = 100 – (Moisture (%) + Protein (%) + Fat (%) + Ash (%)).

The gross energy of mango pulp was calculated as

FE (K.Cal/100g) = (%carbohydrate − %fibre) × 4 + (%fat × 9) + (%protein × 4)

Determination of total phenolic content

The total phenolic content in mango pulp was determined by Folin–Ciocalteu reagent[8] with slight modifications. Twenty µl of pulp extract from 4 mg/ml DMSO stock were poured into wells of 96 well plates. 90 µl of Folin–Ciocalteu reagent was added and the plate was incubated for 5 min at room temperature. 90µl sodium carbonate was also added in each well thereafter. Absorbance was determined at 630 nm by microplate reader (Biotech USA, microplate reader Elx 800). Gallic acid was used as standard and the results are expressed as µg gallic acid equivalent per milligram of mango pulp extract (µg GAE/mg extract).

Determination of total flavonoid content

The total flavonoid content in mango pulp was estimated by aluminium chloride colourimetric method described by Ali et al.[8] with some modifications. 20 µl of pulp extract from 4.0 mg/ml in DMSO stock were reacted with 10 µl each of 10 % aluminium chloride and 1.0 M potassium acetate in 96 well plates. 160 µl

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distilled water was added in each well and plates were incubated at room temperature for 30 min. The absorbance was taken at 415 nm using microplate reader. Quercetin was used as a standard and the flavonoid content was calculated as µg equivalents of quercetin per milligram of mango pulp extract (µg QE/mg extract).

DPPH radical scavenging activity

The free radical scavenging capacity of crude mango pulp extracts was determined using 1, 1- diphenyl l- 2-picryl-hydrazil (DPPH) radical discolouration method[9] and ascorbic acid was used as standard. Spectrophotometric analysis was used to measure the percent radical scavenging capacity (%RSA). To determine DPPH radical scavenging activity of mango pulp extract at different ripening stages, 180 µl of methanol solution of DPPH radical in the concentration of 9.2 mg/100 ml was added to separate wells of 96 well plates. Mango pulp extract (20 µl) was then added to each well and incubated at room temperature for 30 min in dark. The absorbance was measured at 517 nm using microplate reader. Ascorbic acid was used as positive control. Scavenging activity in per cent (%RSA) was calculated as

DPPH scavenging effect (%) = (absorbance of negative control - absorbance of sample/absorbance of negative control) × 100

Determination of total antioxidant capacity

Total antioxidant capacity was assessed using a modified method as described by.[9] Activity was performed by mixing 0.1ml mango pulp extract (4 mg/ml DMSO) with a mixture of 1 ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). Ascorbic acid was used as positive control and DMSO was used as negative control. The tubes containing the reaction solution were then capped and incubated in a boiling water bath for 90 min at 95°C. After incubation at high temperature samples were cooled to room temperature and absorbance of the solutions was measured at 695 nm against blank. The antioxidant activity was expressed as the µg ascorbic acid equivalent per mg of mango pulp extract i.e., µg AAE/mg extract.

Estimation of total reducing power estimation

The reducing power of the mango extract was measured by potassium ferricyanide colourimetric assay according to the method described previously.[9] To assess reducing power of the mango pulp extract, 200 µl of sample from 4 mg/ml in DMSO was reacted with 400 µl of 0.2 mol/l pH 6.6 phosphate buffer and 1 % potassium ferricyanide [$K_3Fe(CN)_6$]. The reaction mixture was heated at 50°C for 20 min and 400 µl of 10 % trichloroacetic acid was added. The mixture was centrifuged at 3000 rpm for 10 min and 500 µl upper layer was mixed with 500 µl distilled water and 100 µl, of 0.1 % FeCl3. The absorbance was measured at 700 nm. Ascorbic acid was used as positive control. The reducing power is expressed as µg ascorbic acid equivalent per milligram mango pulp extract (µg AAE/mg extract).

Determination of metal chelating ability

The protocol reported by Wang et al.,[10] was followed to determine metal chelating ability of samples. 20 µl of pulp extract was reacted with 50µl of 2mM $FeCl_2$ in 96 well plate. After incubation for 10 min in dark, 20 µl of 5 mM ferrozine was poured into each well and incubated again for 5-10 min. Absorbance was measured at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as positive control and calculated as

MC ability % = [(Absorbance of Control – Absorbance of sample)/ Absorbance of Control] × 100

Determination of ABTS radical scavenging potential

The mixture of 7mM ABTS and 2.45mM potassium per sulphate (1:1) was kept in dark for 12-18 hr and diluted at 1:2 thereafter. The absorbance was adjusted at 0.7±0.02 at absorbance of 540 nm. To perform assay, 10 µl of samples were reacted with 100 µl above reagent in 96 well micro plates. The plates were incubated in dark at room temperature for 10 min and final absorbance of the reaction mixture was measured at 540 nm.[10]

α-Amylase inhibition assay

The assay was performed following reported protocol.[11] 15 µL phosphate buffer (pH 6.8), 25 µL of α-amylase enzyme (0.14 U/mL), 10 µL of extract (4 mg/mL in DMSO) and 40 µL starch solution (2 mg/mL in potassium phosphate buffer) were periodically added into each well of 96 well plate. The plates were incubated for 30 min at 50°C and 20 µL of 1M HCl, and 90 µL iodine reagent were added into each well. The optical density (OD) was taken at 540 nm. Acarbose was used as positive control at 5-200 µg/mL. The percent α-amylase inhibition was calculated as

Enzyme Inhibition (%) = (OD of Control – OD of sample/OD of control) × 100

Secondary metabolite profiling by UHPLC/MS

Phytochemical profiling of mango extracts was evaluated through UHPLC (Agilent Technologies, Santa Clara, CA, USA) and Agilent 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA) via an electrospray ionisation source was used for the tentative identification and characterisation of the compounds. Agilent Zorbax xdb-C18 at 25°C was used for analysis. Mobile phases 0.1% formic acid in water (A) and acetonitrile (B) at flow rate of 0.5 ml/min was used. Injection volume was 10 µL with run time and post run time of 25 min and 5 min, respectively. The scan was performed from 100–1000 m/z. Peak identification was performed in both negative and positive modes. The mass spectrometry conditions were set, as follows: nitrogen gas temperature 350°C with the flow rate 300 L/hr, sheath gas temperature 250°C with the flow rate 660 L/hr, and nebuliser gas pressure 45 psi. The capillary and nozzle voltage were set at 3.5 kV and 500 V, respectively. The fragmentation voltage was optimised to 125 V. Analysis was performed with a capillary voltage of 3500 V. Data acquisition and analysis were performed using Agilent LC-MS-QTOF Mass Hunter Data Acquisition Software Version B.03.01 (Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis

All the assays were performed in triplicate. The results are reported as mean ± standard error. Moreover, the results were analysed statistically through analysis variance (ANOVA) and the means were analysed by LSD at 0.05% probability. The chromatographic data was further analysed for principal component analysis (PCA) in order to detect the phytocomponents able to differentiate the mango samples of different ripening stages. PCA is multivariate method that is used for visualisation of hidden trends in a data matrix among the variables. Cases plotted in the PCs explain the differences/similarities between the mango ripening stages.

Table 1. Proximate analysis of Safaid Chonsa mango pulp of different ripening stages (RS). The values are mean of triplicates. The small alphabets within the row represent significant difference between the mean by LSD at $p<0.05$

	RS I	RS II	RS III	RS IV	RS V
Moisture (%)	71.77±2.29a	71.39±3.51a	68.43±3.15ab	66.06±3.07b	66.8±2.86b
Dry matter (%)	20.23±1.53c	28.61±1.63b	31.57±2.08ab	33.94±2.38a	33.2±2.09a
Ash (%)	0.41±0.14a	0.42±0.14a	0.41±0.13a	0.39±0.11a	0.43±0.12a
Protein (%)	1.48±0.21ab	1.31±0.20b	1.75±0.44a	1.48±0.34ab	1.75±0.28a
Fat (%)	0.58±0.11a	0.51±0.11ab	0.52±0.09ab	0.5±0.09b	0.6±0.09a
Fibre (%)	1.88±0.18a	1.7±0.16a	1.67±0.16a	1.9±0.17a	1.74±0.16a
Carbohydrates (%)	25.76±2.30b	26.31±3.14b	28.89±2.87ab	31.57±2.95a	30.42±1.94a
Energy K-Cal/100 g	106.66±8.57b	108.27±7.42b	120.56±8.26ab	129.1±7.19a	127.12±6.37a

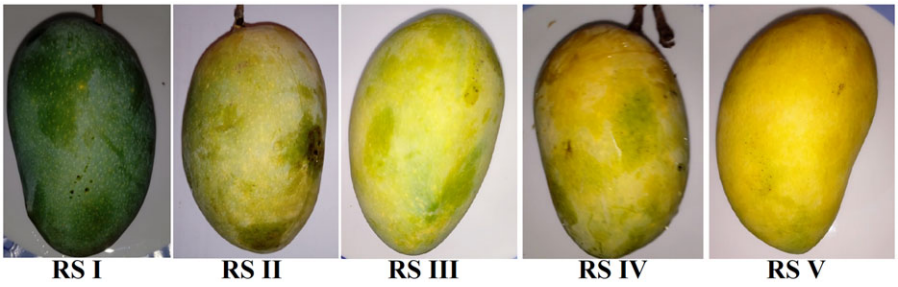

Figure 1. Pictorial representation of Safaid Chonsa mango ripening stages based on colour.

The PCA statistical analysis on phytochemicals was performed by using the OriginPro 9.0 software.

Results and Discussion

The ripening of mango fruit proceeds by alterations in physiological and biochemical processes. These changes result in soft, colourful, edible, and tasty fruits. The colour change from green to yellow of mango is the first indication of fruit ripening.[12] The action of ethylene initiates climacteric increase in respiration that results in yellow pigmentation and other carotenoids production.[13] The intrinsic ethylene production or applied externally have the same effect to reach different ripening stages (Fig. 1). Degradation of starch, change in turgor pressure and cell wall variations determine the degree of fruit softening.

In present investigation of chansa mango, moisture analysis reveals non-significant difference between RSI and RSII that significantly reduced at lateral stages. At RSV total moisture content was 66.8%. Further, the dry matter gradually increased from 20.23% at stage I to 33.94 at stage IV however dry matter was approximately same at stage IV and V (Table 1). The variation in moisture and dry matter is due to respirational, transcriptional and biochemical changes in mango fruits.[14] However the environmental conditions such as temperature, moisture, ethylene production and others determine extent of ripening and time period to attain the ripening stage.[15] Change in fruit solid contents also relates with hydrolytic changes and breakdown of carbohydrates and other complex organic metabolites.[16,17] Due to changes in moisture content and changes in the dry matter variation in ash content in between the stages was also observed though the difference was non-significant variable. These results are in agreement with Raffo et al.[18] and Opara et al.,[19] who reported non-significant variations in ash contents in tomato cultivars.

Total protein content was variable in between the ripening stages of chonsa mango where maximum was observed at stages III and V (1.75% at both). This shows that protein biosynthesis and functioning vary at the different ripening stages and maturity levels (Table 1). This might also be due to production of enzymes to precede the biochemical changes. This also has been reported in mango and other fruits where protein contents varied at ripening stages.[19,20]

Dietary fibres are considered best for the health of gastrointestinal tract. However, may also bind to trace elements while taken in excess. This may lead to deficiencies of other micro nutrients in the body.[21] Just like the case of fibre where 1.88% fibre was observed at RSI and maximum (1.9%) at stage IV. There is non-significant variation in fibre content in mango chonsa cultivar during ripening stages. However crude fibre content from 0.85 to 0.87 % has been reported in different mango varieties.[22,23] Change in total soluble solids is natural phenomenon that correlates with hydrolytic changes in carbohydrates during the ripening process.[17]

A minor variation in total fat content was observed during the chonsa ripening stages. At RSI 0.58% fat was observed that decreased up to 0.5% at RSIV however at stage V, 0.6% fat was observed (Table 1). These variations are due to biochemical changes during the ripening process. The breakdown of carbohydrates and cell wall disintegration may lead to increase in fats at the lateral stages. The pulp becomes more soften at RSV that shows most of the cells have been digested by the internal enzymes therefore maximum fat was observed at RSV. Such variations in fat content during the ripening process have been reported in mango and other fruits.[24,25] It is also presumed that lipids are mobilised and stored in the seeds during the ripening

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process therefore less content are observed in pulp as compared to seeds.[26]

Carbohydrate content gradually increased from 25.76% to 31.57% from RSI to RSIV which slightly decreased at RSV (30.42%). The increase of carbohydrate is due to breakdown of complex carbohydrates and cell walls during the ripening process. The mango ripening leads to sweet in taste pulp production and sweetness is the main characteristic of the chonsa cultivar along with aroma. At stages I and II, while the mangoes were at initial stages of ripening, non-significant change in calories was observed that significantly increased at stage III and stage IV (129.1 Kcal/100g). At the lateral stages high content of carbohydrates is responsible for higher calories however at RSV a minor decline in carbohydrates leading to calorie reduction is due to utilisation of pulp that results in liquidity of the pulp.

Antioxidant analysis of mango pulp

DPPH free radical scavenging activity increased as ripening of chonsa mangoes increased. At stage I, 34.72% DPPH activity was observed 47% increased at stage III and then there was non-significant variation between stages III and V (Fig. 2). Free radical scavenging potential (FRSP) was variable in between the stages. At RSI 32.72 µg TE/mg extract scavenging was observed that slightly varied up to stage IV. While at stage V, FRSP increased 31% as compared to stage I. Metal chelating response was also non-consistent among the stages. At stage I (RSI), 18.12 µg EDTAE/mg extract activity was observed that decreased at stage II and then increased at stage III. Significant decrease (5.75 µg EDTAE/mg extract) was observed at stage IV (Fig. 2).

Among all the methods used to evaluate antioxidant potential, no single method is adequate therefore diverging results are observed in different assays. Furthermore, variable performance of different standards used as positive control changes the results of the samples therefore diversity in assays and standards is most adequate.[27,28] DPPH accepts an electron or hydrogen radical and becomes a stable molecule. Therefore it is used to investigate radical scavenging activity.[29] The DPPH method has been reported for radical scavenging activity in Ataulfo and other mango cultivars.[30,31] They and others reported that this activity is due to presence of phenolic compounds present in mango fruit. Many researchers have reported that DPPH results correlate with total phenolic content.[28,32,33] There is a direct relationship and a linear correlation between polyphenolic contents and free radical scavenging capacity.[34] FRAP assay has also been used to determine antioxidant activity of several mango cultivars.[35,36] However, in FRAP electron-donating substance that does not have antioxidant properties may interact with Fe(III)/Fe(II) and may indicate false values.[36] Therefore variation in FRAP activity was observed. Besides these, the standard used for FRAP also has impact on results calculation as TEAC has no relationship between the FRAP value and the number of electrons.[37]

Total antioxidant activity (TAC) was observed in 131 µg AAE/mg extract that slightly increased at stage II of chonsa mango cultivar. However, significantly increase was observed at stages III, IV, and V (150.6, 254.4, and 295.7 µg AAE/mg extract, respectively). While in case of TRP, there was minor but significant gradual increase from RSI to RSV (Fig. 2). TRP was 345.71 µg AAE/mg extract which increased up to 14.23% till stage V (394.92). Among the several methods for determination of antioxidant activity, each has some limitations.[13] Therefore multiple methods are performed to determine antioxidative activity.[38] In TAC, Mo (VI) reduces to Mo (V) that generates green colour and gives maximum absorption at 695 nm. Oxidation is a natural phenomenon that generates hydroxyl and peroxyl radicals in biological system. Antioxidants quench these radicals. However, presence of excessive radicals damages the DNA, proteins and fatty acid of cell membrane. This may further lead to diseases and cancer.[34] The presence of reductones breaks the free radical chain by donating a hydrogen atom. This property defines correlation between antioxidants and reducing power of extracts.

TPC increased from 3.57 µg GAE/mg extract to 5.72 µg GAE/mg extract from stage I to RSIII and then there was minor decrease at stages IV and V (Fig. 2). However gradually increased from 1.18 µg QE/mg extract to 1.41 µg QE/mg extract from RSI to RSV was observed for TFC. This showed 19% increase in TFC at stage V as compared to stage I. Phenolics are widely distributed in plants and due to their role in antioxidant activity and radical scavenging properties they are considered beneficial for health.[39] Our results are similar to others where total polyphenol content in fully ripe mango flesh is lower than green mature mango flesh.[40] The phenolic contents in this study vary from published results[33] and the variation of the total phenolic content in mango can be attributed to the differences in the cultivar and sources of the materials, as well as ripening stage, soil and climate.[41] The total polyphenolic concentration as well as individual phenolic compounds also varies based on the developmental stage of the fruit.

Robles-Sánchez et al.[28] studied effect of low-temperature storage for 15 days on phenolic content of mature green mango. They reported a decrease in phenolic content with increasing storage time. Gil et al.[42] reported gradual increase in phenolic compounds in mango fruits. The starch also converts into simple saccharides by amylase[43] also reported variations in phenolic contents and antioxidants during treatments and storage. Flavonoids are phenolic compounds, which are very effective antioxidants. Flavonoids account for 60% of total dietary phenolic content.[44] With regards to flavonoids, our results are similar to those reported by Robles-Sánchez et al.[13] where no changes were observed in Ataulfo mangoes. Pinsirodom et al. (2018) concluded that it seems that ripening does not affect the flavonoid content of mango since they were similar in fruits of the four ripening stages. Crozier et al.[45] observed relationship between flavonoids and deteriorative reactions, and these also associate with their long shelf life.[46]

Alpha amylase inhibition of mango pulp

Amylase inhibition analysis of pulp shows that inhibition potential was 6% at stage I that increased up to 40% at stage II. Thereafter inhibition gradually decreased at stage III and IV and at RS V only 2% amylase inhibition was observed (Fig. 3). Amylase is the key enzyme that breakdown the complex carbohydrates in mango into simple sugar. This leads to developing taste of mango fruit. The results show that as the ripening process proceeds, amylase inhibition reduces. This generates sweetness and softness of mango pulp and even increases the liquidity.

Un-targeted phytochemical fingerprinting of mango pulp

Analysis of biomolecules in complex mixture such as plant extracts is critical that demands advanced chromatographic techniques. Such techniques separate the biomolecules on basis of molecular weight that gives peaks at specific retention time. Mass

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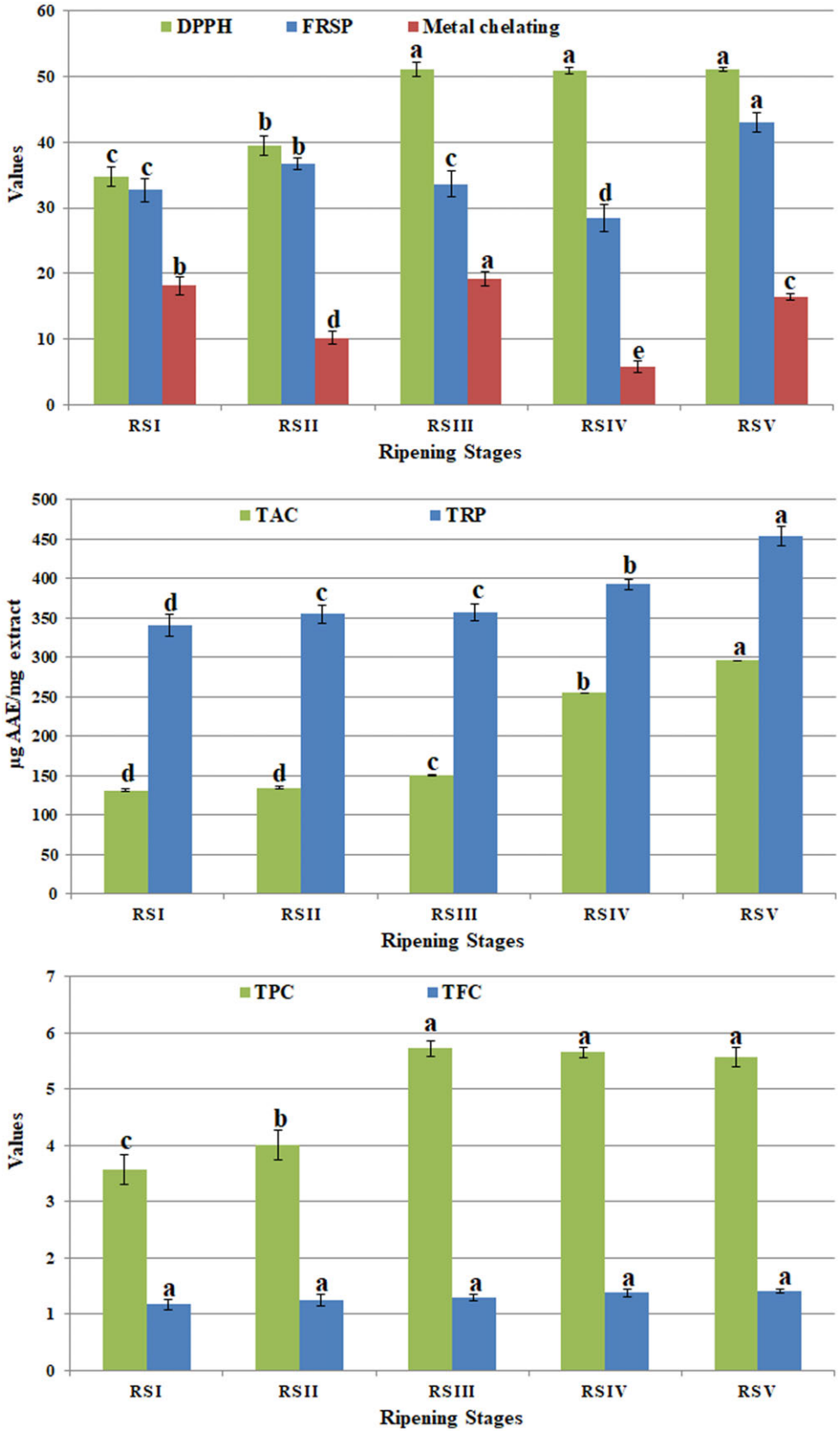

Figure 2. Total phenolic content (TPC µg GAE/mg extract), total flavonoid content (TFC µg QE/mg extract), antioxidative response (total antioxidant capacity (TAC µg AAE/mg extract) and total reducing power (TRP µg AAE/mg extract)), and free radical scavenging activity (% inhibition) The values are mean of triplicates. The small alphabets on bars represent significant difference between the mean by LSD at $p<0.05$.

spectrometry further fragments and ionises the molecules for more precise identification.[47] UHPLC analysis showed presence of different compounds at different ripening stages of chonsa mangoes. The compounds belong to different classes of phytochemicals and their volume (based on the peak area) varied depending upon the stage. High selectivity, sensitivity, and

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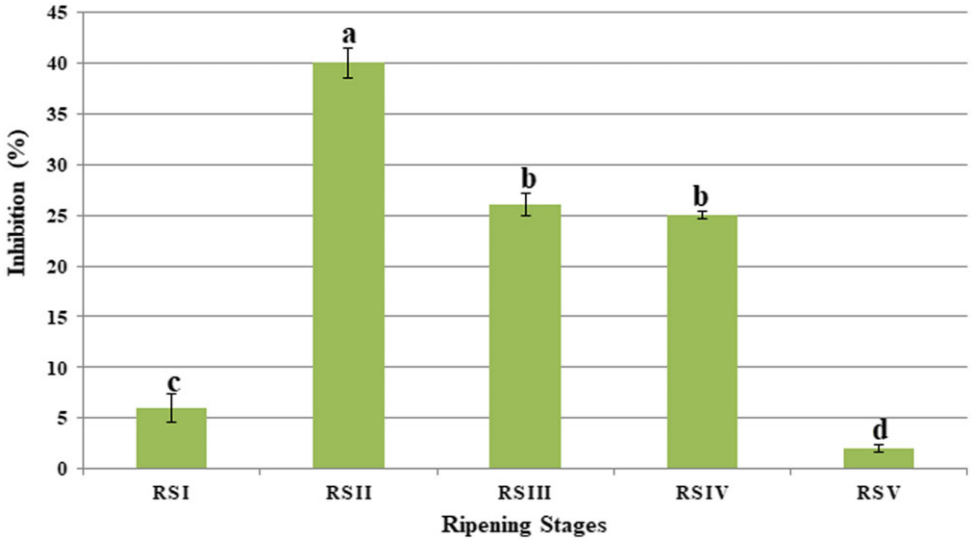

Figure 3. Amylase inhibition activity of pulp extracts of Safaid Chonsa mango at five ripening stages. The values are mean of triplicates. The small alphabets on bars represent significant difference between the mean by LSD at $p<0.05$.

specificity make the UHPLC/MS a powerful tool for identification of biomolecules. Further high resolution and fast separation make it more meaningful for mixture of compounds. UHPLC/MS works through ion chromatograms of targeted and non-targeted components by a full scan. The ion chromatograms are reconstructed again and the fragmentation pattern is interpreted. Finally, compounds are identified by the targeted peaks observations and match with mass-based search, retention times, and MS spectra for elucidation of compound structure.[48–52]

At stage I (RSI) tricarboxylic acids showed maximum peak area (Table 2). Aconitic acid and methylisocitric acid (m/z 173.01 and 205.04, respectively) were detected at 1.222 and 1.183 min, respectively. Other than these polyphenols (4-O-methyl gallate), phenolic acid (beta-glucogallin), xanthenes (theobromine), and glycosyl compound (sakebiose) were also detected. Most of the compounds present at RSI were also present at stage II (RSII) along with many other compounds. Sesquiterpenoids ((6S)-dehydrovomifoliol m/z 221.11), Furanocoumarins (Isobergaptene, m/z 215.03), Hydroxy fatty acids (2-Dehydro-3-deoxy-D-xylonate, m/z 147.03), Oligosaccharides (b-D-Glucuronopyranosyl-(1->3)-a-D-galacturonopyranosyl-(1->2)-L-rhamnose, m/z 515.12) are some of the major components detected in stage II extract.

Hydroxybenzoic acids (2,4,6-Trihydroxybenzoic acid with m/z 169.0147), Carboxylic acid (Fructoselysine 6-phosphate m/z 387.1171), Furanocoumarins (Isobergaptene m/z 215.03) and Xanthines (Theobromine m/z 179.06) are some the components detected at stage III mango ripening extract (Table 2). Stage IV and V showed much diversity of compounds. Some of the major classes/compounds identified in stage IV extract were Fatty acids ((12Z,15S,18S)-15-hydroxy-18-bromo-12,16,17-octadecatrienoic acid m/z 371.1218), Polyphenols (4-O-Methyl-gallate m/z 183.0301), Quinolines and derivatives (Citbismine C m/z 683.2268), Furanocoumarins (Isobergaptene m/z 215.0348), and Tricarboxylic acids (Methylisocitric acid m/z 205.036). 2,4,6-Trihydroxybenzoic acid, 1,2,3,4,6-Pentakis-O-galloyl-beta-D-glucose, Fructoselysine 6-phosphate, Citric acid, Allo-Inositol, 1-O-(8R-hydroxy-8-methyl-3Z,9-decadienoyl)-beta-D-glucopyranose, Citbismine C and some compounds that were identified in RSV extract of chonsa mango pulp.

A number of compounds belonging to Polyphenols, Hydroxybenzoic acids, Carboxylic acid, Xanthines, o-glycosyl compounds, Sesquiterpenoids, Saponin, Pyridinemonocarboxylic acid, Glyoxylic acid, Hydroxy fatty acids, polyols, Steroids, Oligosaccharides, etc were identified in chonsa mango pulp (Table 2). The variation in composition of phytochemicals is due to different ripening stages. Tan et al.[53] also reported that mango pulp has diversity of phytochemicals and this diversity is also based on variety differences.

The phytochemicals identified in chonsa mango pulp have diverse health benefits. As nutraceuticals, these compounds strengthen the body's immune system to eliminate disease-causing agents. They also have ability to fight during the disease for fast recovery and even after disease recovery. These compounds also rebuild the body for normal functions. For example, Gallates, polyphenolic compounds, are widely present in plants *(Genus Acer)* and many others. These compounds have pharmacological roles as nutraceutical, disease prevention, and disease treatment. A number of studies have reported their role in breast cancer therapy, diabetic ischaemia, enzyme inhibitor, antiproliferative activity, ovarian cancer, anti-inflammatory, anti-oxidation, oxidative stress-induced cytotoxicity, antimicrobial, neuraminidase inhibitory, anti-atherosclerosis, neuroprotection, and many other.[54–68]

Xantheose also known as theobromine are heterocyclic alkaloid and bitter in taste. Theobromine has been prone to mitigate age-related cognitive decline, facilitate adipocyte browning, remedy against neurodegenerative disorders, augment lipid metabolism, anti-inflammatory properties.[69–71]

Ripe mangos have significant amount of simple sugars, starch, pectins, cellulose and hemicellulose. Ripe mango pulp contains 15 percent soluble sugars mainly fructose and glucose. Therefore high intake is not recommended as it may induce pro-obesity or pre-diabetic effects.[72] Sakebiose that is also known as nigerose is unfermentable sugar. It also contributes to sweetness of mango pulp along with other carbohydrates. It is presumed that it is potential replacement for traditional sugar, therefore, can be used in metabolic-related diseases such as obesity.[73]

Isobergaptene and other bergapten derivatives have anti-inflammatory, antidiabetes, antimicrobial, anticancer, and neuro-protection properties. Bergapten can cross the blood–brain barrier and therefore has greater bioavailability and has potential to treat brain disease.[74] Studies in cell cultures and animal models have

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Table 2. List of Compounds and their characteristics identified in Safaid Chonsa mango pulp of ripening stage I-V (RS I-V) by UHPLC/MS

m/z	Name	Formula	RT	Actual Mass	Ions	Height	Volume	Hits	Score (MFE)	Ripening Stage
179.0578	Theobromine	$C_7H_8N_4O_2$	0.732	180.0651	11	1718870	7893164	3	100	RSI
387.1172	Fructoselysine 6-phosphate	$C_{12}H_{25}N_2O_{10}P$	0.737	388.1244	4	890742	3653138	1	80.1	RSI
683.2278	Nigerose (Sakebiose)	$C_{12}H_{22}O_{11}$	0.744	342.1187	9	973776	10741726	10	100	RSI
331.0687	beta-Glucogallin	$C_{13}H_{16}O_{10}$	0.977	332.0759	4	508475	2444232	9	100	RSI
205.0363	Methylisocitric acid	$C_7H_{10}O_7$	1.183	206.0435	4	1215706	7164253	5	95.8	RSI
173.0099	Aconitic acid	$C_6H_6O_6$	1.222	174.0171	4	522351	2178424	4	100	RSI
183.0301	4-O-Methyl-gallate	$C_8H_8O_5$	3.385	184.0374	8	496448	4394528	4	100	RSI
119.0363	Purine	$C_5H_4N_4$	0.724	120.0437	3	145290	442996	1	100	RS II
387.1172	Fructoselysine 6-phosphate	$C_{12}H_{25}N_2O_{10}P$	0.735	388.1244	4	878794	3261300	1	100	RS II
215.0347	Isobergaptene	$C_{12}H_8O_4$	0.737	216.0399	4	417175	1731625	6	100	RS II
295.069	Flunixin	$C_{14}H_{11}F_3N_2O_2$	0.759	296.0764	4	111560	378537	2	100	RS II
515.126	b-D-Glucuronopyranosyl-(1->3)-a-D-galacturonopyranosyl-(1->2)-L-rhamnose	$C_{18}H_{28}O_{17}$	0.877	516.1332	8	373050	2120186	2	100	RS II
179.0574	Theobromine	$C_7H_8N_4O_2$	0.934	180.0648	4	115298	540978	3	80	RS II
129.0199	Glutaconic acid	$C_5H_6O_4$	0.986	130.0272	2	95510	308064	9	100	RS II
147.0306	2-Dehydro-3-deoxy-D-xylonate	$C_5H_8O_5$	0.99	148.038	4	329323	1112923	10	100	RS II
215.0188	5-Carboxymethyl-2-hydroxymuconate	$C_8H_8O_7$	0.995	216.0261	2	66911	277867	3	100	RS II
179.0568	allo-Inositol	$C_6H_{12}O_6$	1.053	180.064	4	85684	774621	10	80	RS II
169.0144	2,4,6-Trihydroxybenzoic acid	$C_7H_6O_5$	1.179	170.0217	4	133817	493680	3	100	RS II
205.036	Methylisocitric acid	$C_7H_{10}O_7$	1.199	206.0433	4	2230551	10242125	5	100	RS II
183.0303	4-O-Methyl-gallate	$C_8H_8O_5$	3.397	184.0375	7	500907	4507792	4	100	RS II
165.0193	4-Hydroxyphenylglyoxylate	$C_8H_6O_4$	5.475	166.0266	3	65391	710880	7	100	RS II
469.053	1,2,3,4,6-Pentakis-O-galloyl-beta-D-glucose	$C_{41}H_{32}O_{26}$	8.708	940.1206	8	80162	528635	1	100	RS II
221.1186	(6S)-dehydrovomifoliol	$C_{13}H_{18}O_3$	11.418	222.1258	4	60385	323997	5	100	RS II
345.2805	24-Nor-5β-chol-22-ene-3α,6α-diol	$C_{23}H_{38}O_2$	18.488	346.2878	11	59674	517013	10	100	RS II
517.3184	Perulactone	$C_{30}H_{46}O_7$	19.409	518.3253	4	109134	1020572	6	100	RS II
179.0577	Theobromine	$C_7H_8N_4O_2$	0.727	180.0651	7	1564382	5272879	3	100	RS III
387.1171	Fructoselysine 6-phosphate	$C_{12}H_{25}N_2O_{10}P$	0.732	388.1243	4	958803	3887842	1	85.8	RS III
215.0347	Isobergaptene	$C_{12}H_8O_4$	0.734	216.0399	4	440138	1756851	6	100	RS III
683.2277	Nigerose (Sakebiose)	$C_{12}H_{22}O_{11}$	0.739	342.1187	9	1198746	12743782	10	100	RS III
147.0306	2-Dehydro-3-deoxy-D-xylonate	$C_5H_8O_5$	0.99	148.038	4	215187	766880	10	100	RS III
169.0147	2,4,6-Trihydroxybenzoic acid	$C_7H_6O_5$	1.175	170.022	5	223336	846283	3	100	RS III

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Table 2. (*Continued*)

205.0362	Methylisocitric acid	$C_7H_{10}O_7$	1.185	206.0434	4	616712	2782904	5	100	RS III
173.0094	Aconitic acid	$C_6H_6O_6$	1.217	174.0165	4	309956	1297427	4	100	RS III
183.0302	4-O-Methyl-gallate	$C_8H_8O_5$	3.393	184.0375	8	562395	5127667	4	100	RS III
119.0363	Purine	$C_5H_4N_4$	0.722	120.0437	4	130193	405922	1	80	RS IV
215.0348	Isobergaptene	$C_{12}H_8O_4$	0.736	216.04	4	544532	2469166	6	100	RS IV
683.2268	Citbismine C	$C_{37}H_{36}N_2O_{11}$	0.738	684.2337	5	730241	2847682	1	96.6	RS IV
165.042	1-Methylxanthine	$C_6H_6N_4O_2$	0.741	166.0493	3	139057	452234	5	100	RS IV
135.0306	Hypoxanthine	$C_5H_4N_4O$	0.745	136.038	3	127626	476391	5	86	RS IV
371.1218	(12Z,15S,18S)-15-hydroxy-18-bromo-12,16,17-octadecatrienoic acid	$C_{18}H_{29}BrO_3$	0.746	372.1292	4	722008	1790039	2	88.6	RS IV
473.1514	D-Galactopyranosyl-(1->3)-D-galactopyranosyl-(1->3)-L-arabinose	$C_{17}H_{30}O_{15}$	0.783	474.1367	5	315893	1505544	3	100	RS IV
179.0577	Theobromine	$C_7H_8N_4O_2$	0.914	180.0651	4	173119	622177	3	100	RS IV
169.0149	2,4,6-Trihydroxybenzoic acid	$C_7H_6O_5$	1.176	170.0222	5	367790	1551327	3	100	RS IV
205.036	Methylisocitric acid	$C_7H_{10}O_7$	1.184	206.0432	2	223108	1024134	5	100	RS IV
493.121	6-O-Galloylsucrose	$C_{19}H_{26}O_{15}$	1.433	494.1282	6	131778	1377148	5	96.4	RS IV
183.0301	4-O-Methyl-gallate	$C_8H_8O_5$	3.383	184.0374	8	1465245	15467166	4	100	RS IV
469.0534	1,2,3,4,6-Pentakis-O-galloyl-beta-D-glucose	$C_{41}H_{32}O_{26}$	8.706	940.1213	10	223173	1561954	1	100	RS IV
359.1721	1-O-(8R-hydroxy-8-methyl-3Z,9-decadienoyl)-beta-D-glucopyranose	$C_{17}H_{28}O_8$	9.566	360.1793	4	204238	764004	8	100	RS IV
179.0576	Theobromine	$C_7H_8N_4O_2$	0.728	180.065	12	1344069	6689028	3	100	RS V
387.117	Fructoselysine 6-phosphate	$C_{12}H_{25}N_2O_{10}P$	0.732	388.1241	4	735661	3116982	1	80	RS V
683.2273	Citbismine C	$C_{37}H_{36}N_2O_{11}$	0.74	684.2343	5	615315	2725820	1	100	RS V
371.122	(12Z,15S,18S)-15-hydroxy-18-bromo-12,16,17-octadecatrienoic acid	$C_{18}H_{29}BrO_3$	0.744	372.1295	4	707844	1602042	2	100	RS V
191.0207	Citric acid	$C_6H_8O_7$	0.869	192.0279	4	2734445	8076438	10	100	RS V
179.0568	allo-Inositol	$C_6H_{12}O_6$	0.915	180.0641	7	103179	529118	10	80.9	RS V
169.0145	2,4,6-Trihydroxybenzoic acid	$C_7H_6O_5$	1.147	170.0218	4	265977	940563	3	100	RS V
205.0354	Methylisocitric acid	$C_7H_{10}O_7$	1.148	206.0427	3	112018	536028	5	100	RS V
183.0301	4-O-Methyl-gallate	$C_8H_8O_5$	2.983	184.0374	8	1036544	8989026	4	100	RS V
469.0525	1,2,3,4,6-Pentakis-O-galloyl-beta-D-glucose	$C_{41}H_{32}O_{26}$	8.71	940.1198	9	92352	618273	1	100	RS V
359.1719	1-O-(8R-hydroxy-8-methyl-3Z,9-decadienoyl)-beta-D-glucopyranose	$C_{17}H_{28}O_8$	9.555	360.1792	4	326222	1158069	8	100	RS V
359.1717	1-O-(8R-hydroxy-8-methyl-3Z,9-decadienoyl)-beta-D-glucopyranose	$C_{17}H_{28}O_8$	9.732	360.1789	4	95816	355072	8	100	RS V

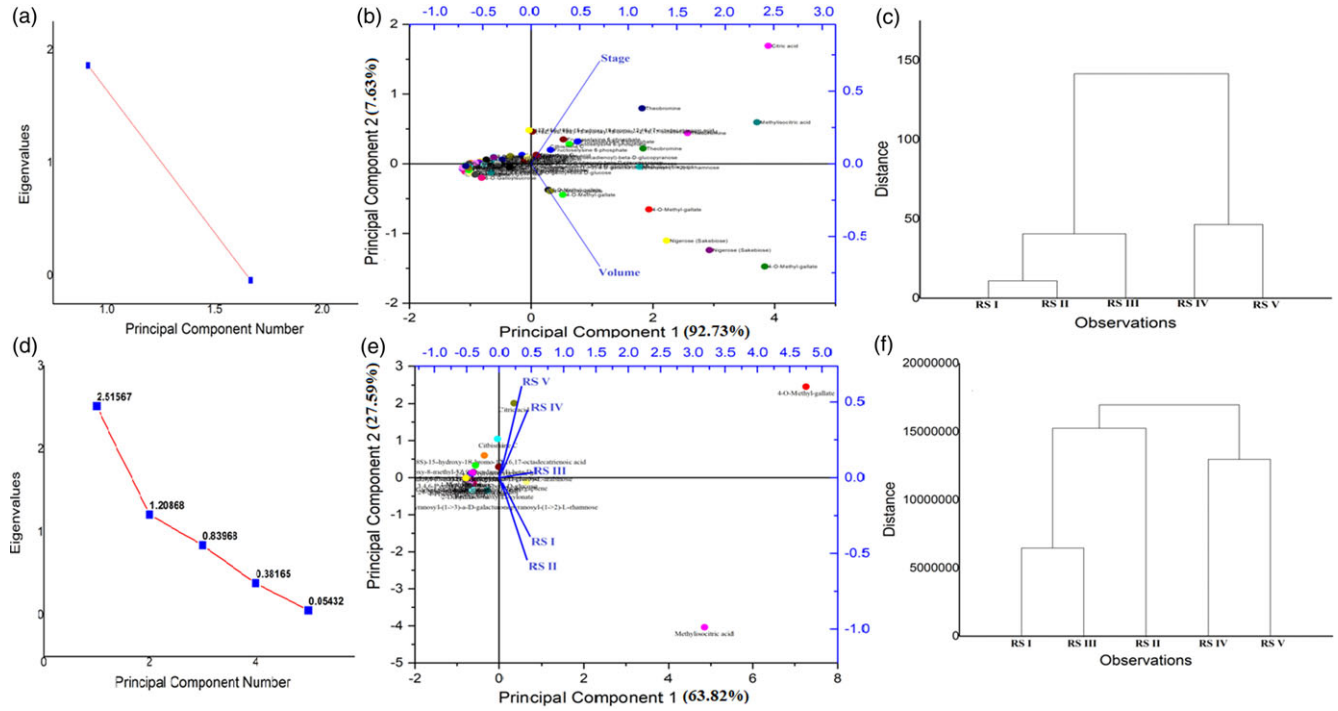

Figure 4. Principle component analysis (PCA) and hierarchal analysis of phytochemical data of Safaid Chonsa mango pulp. 4 A&B Scree plot and Biplot analysis of phytochemical data for variation in between stages and volume of phytocomponents analysed by UHPLC/MS. 4C hierarchal analysis based on antioxidative activities including total phenolic and flavonoid contents, 4 D&E Scree plot and Biplot analysis of phytochemical data for variation in between the ripening stages based on phytocomponents analysed by UHPLC/MS. 4F hierarchal analysis based on phytocomponents analysed by UHPLC/MS.

shown anticancer, photochemotherapy, antimicrobial, hypolipemic, anti-inflammatory, and phototoxicity properties.[75]

Citbismine also known as acridones belongs to Quinolines and derivatives. These compounds have a great potential as anticancer and multidrug resistance inhibitors. They have also shown antipsoriatic, antiprotozoal, acetylcholinesterase inhibitor, antiviral, antimalarial, antimicrobial, and anti-inflammatory activities.[76,77] Allo-Inositol and other inositol derivatives have many physiological processes, including endocrine modulation. Deficiency of these compounds may result in issues in endocrine system and other metabolic diseases such as deficiency decrease biosynthesis, reduce dietary intake, inhibit uptake of nutritional components by gut and cellular system, increase catabolism and/or excretion, and alter microbiota.[78–80]

Phenolic acids either hydroxybenzoic or hydroxycinnamic acid and their derivatives are commonly present in mango pulp. However type and concentration of phenolic or polyphenolic acids vary depending on the variety, plant age, ripening strategy, geographical location, and others.[81] The accountable antioxidant properties of fruits are mostly due to the presence of phenolic acids and their derivatives.[82] Phenolic acids protect the human against various diseases and manage health prospects. Mango pulp contains phenols, polyphenols, and benzoic acid and their derivatives are the main constituents of mango pulp. However, type and concentration vary depending upon, type, location, ripening stage, etc.[83] Ediriweera et al.[84] also identified hydroxybenzoic acid derivatives such as hydroxybenzoic acid, caffeic acid, ferulic acid etc. in the pulp of mango fruit. Hu et al.[85] identified 34 different phenolic acid derivatives in mango pulp using UPLCESI-QTOFMS. Ramirez et al.[86] detected magniferin and its derivatives as main constituents of pulp of Tommy Atkins and Pica varieties and others.[84,87]

A loading plot and a score plot are the two basic components of PCA. The loading plots identify variables responsible for variances. While scree plot show the distance between samples define relationship and gives quantitative value for a variance. The PCA plot shows that the phytochemicals are distributed onto the calculated PCs. This shows that there is diversity of phytochemicals and there is variation in correlation in between the stages and concentration (volume) of phytochemicals (Fig. 4a and b). A number of components were not detected in between the stages or they are scattered according to the positive or negative relation. Principal component analysis (PCA) is often the first choice for phytochemical relationships in samples.[88–90] The plot describes there is change of phytochemicals during the ripening stages of dusehri mangoes. The stage I (RSI) and stage II (RSII) have strong interaction while RS IV and V interact with each other (Fig. 4c and d). RS III falls in between that shows that there is sudden change in the phytochemicals. A number of components are present at all ripening stages however the correlation among them varies. Negative correlation describes that the components have differences in concentration as the ripening stage varies. This shows that although numbers of phytochemicals are present in mango, their correlation with the stage varies depending upon the concentration/presence of that component. Further hierarchal analysis shows that stages I and II (RS I & II) interact with each other while RS IV and RS V interact with each other in both antioxidative activities and phytochemical analysis (Fig. 4e and f). Stage III relays in between these two pairs that predict this is the turning point in between different stages.

Conclusion

The present study showed that Safaid Chonsa variety of mango changes its colour and other proximate parameters while ripen.

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During the ripening process, the mango pulp changes its antioxidative potential and free radical quenching capacity that are due to variations in total phenolics and flavonoid components. Non-targeted phytochemical analysis reveals that during the ripening process a number of phytochemicals varies. These variations are due to biochemical process that goes on during the ripening stages. Some molecules are consistently present from stage I to stage V though their concentrations vary. While some chemicals were detected at particular stage. Such variations are due to enzymatic functioning during the ripening process that is involved in synthesis pathways or might also be due to breakdown of conjugative molecules. However, these phytochemicals have beneficial role for the human health either as nutrient or nutraceutical.

Availability of data and material. All the relevant data are reported in the manuscript.

Authors contributions. Aniqa performed the experiments and wrote the manuscript. AM performed statistical analysis. ZFR supervised the work and proofread the manuscript.

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Competing interests. All authors declare no conflict of interest.

Consent to participate. Manuscript does not contain human-related data therefore consent to participate is not required.

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