Chemical fingerprinting as nutraceutical quality differentiation tool in *Asimina triloba* L. fruit pulp at different ripening stages: an old species for new health needs

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Summary

The pawpaw (*Asimina triloba* L. Dunal) is a fruit tree belonging to the *Annonaceae* family native throughout eastern United States. An assessment of the chemical composition and antioxidant efficacy may increase its utilization as a functional food. The aim of this preliminary study was to obtain a specific chemical fingerprint to evaluate the single antioxidant class contribution to total fruit phytocomplex. Compared with other fruits, total polyphenolic content and antioxidant activity of pawpaw were equivalent or even superior in some cases: total polyphenolic compounds ranged from 1837.2 $mg \cdot kg^{-1}$ to 2123.8 $mg \cdot kg^{-1}$, expressed as gallic acid equivalents, while antioxidant activity ranged from 3.45 $mmol \cdot kg^{-1}$ to 22.11 $mmol \cdot kg^{-1}$. Most of the compounds presented a strong positive correlation ($R > 0.99$) with antioxidant activity. Correlations between single compounds and antioxidant activity suggest that antioxidant components of pawpaw pulp have an effect on antioxidant capacity. Chemical fingerprint showed the prevalence of catechins (40.2%) and flavonols (33.3%) in pawpaw fruit. Chemical fingerprinting could be an important tool to assess the chemical composition and pawpaw fruit bioactivities, in order to improve the consumption of these fruits and to find out new sources of natural health-promoting compounds, used as functional food ingredients.

Keywords

pawpaw; polyphenolic compounds; vitamin C; antioxidant activity; health-promoting agents

The pawpaw (*Asimina triloba* L. Dunal) is a tree fruit native to the temperate woodlands of the Appalachian region. It belongs to the *Annonaceae* family [1, 2]. The *Annonaceae* includes 120 genera and over 2100 species. *Asimina triloba* is the only temperate species, while the rest of the family thrives in tropical or subtropical climates.

The fruits of pawpaw have nourished wild animals and mankind in eastern North America for thousands of years [3]. The pawpaw is the largest wild fruit native to North America and it grows over most of the eastern half of the United States in a region from East Texas to the Eastern Seaboard and northward to the Great Lakes, with the exceptions of New England, Florida and the Gulf Coast [1, 4, 5].

This fruit should not be confused with the papaya because they are different species. The pawpaw fruit can grow as a singlet, but normally grows in bunches of three or more. The fruit is cylindrical in shape, typically 5 cm to 15 cm long, 3 cm to 8 cm wide, and can weigh up to 450 g with large blackish-brown seeds [6]. During the growing season, the pawpaw has a whitish to light-green colour that turns yellow to brownish at maturity. The ripe fruit is highly aromatic and the banana-like flesh surrounds two rows of large bean-shaped brown seeds [1, 4, 5].

It is a lesser known fruit among consumers, having only a niche use, because of its limited supply and highly perishable nature. Typically, each tree produces a limited number of fruits; once picked, its rapid rate of ripening prevents successful distribution through commercial food chains for fresh-market sales [4]. With respect to its potential for commercialization, the pawpaw exhibits

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challenging post-harvest properties [7]. Pawpaw is a climacteric fruit with its climacteric peak within 3 days after harvest at room temperature. Major deteriorative changes occur shortly thereafter [4, 8]. The changes include increased production of volatile flavour compounds, an increase in soluble solids, softening of the flesh, colour changes and an increase in enzymatic activity. Different factors exacerbate these quick post-harvest changes [9–11]. The pawpaw reveals no reliable external indicator of ripeness such as skin colour, and fruits on the same tree may not ripen at the same time such that "once-over" harvest from the same tree potentially would yield fruits that range from unripe to overripe [7]. Because the stage of fruit ripeness at the time of harvest determines the final quality of ripe fruit, many studies designed to index ripening parameters have been undertaken for commercial fruit [4]. As a result, the researchers implicitly questioned the usefulness of the pawpaw for commercialization without investing in more developmental research [1, 7, 10]. Since the quick ripening nature of the pawpaw has been a deterrent to commercialization, and pawpaw ripening remains a potential issue even today, a different approach to commercialization of the pawpaw would be to identify its qualities that would not depend on its challenging post-harvest properties [1].

With the global functional food and beverage market expected to reach $125 billion by 2015, different sources of phytochemicals are being explored [12]. The rationale for studying fruit crops is their biodiversity and bioconservation [13], as diverse sources of phytochemical advancement [6], considering the sustainability of their production and use [14].

A diet rich in fruits and vegetables has been associated with lower risk of chronic diseases because, in addition to the vitamin and mineral composition, they contains various compounds with health-protective effects, in particular antioxidants. Polyphenols are the most abundant bioactive compounds reaching values of 1 g per day in the diet, approximately 10-times higher than vitamin C intake [15, 16]. Polyphenols quantity and quality in plant foods may vary significantly according to different intrinsic and extrinsic factors such as plant genetics and cultivar, soil composition and growing conditions, maturity stage and post-harvest conditions [17, 18]. In the plant kingdom, antioxidant compounds can range from simple molecules, such as vitamin C and phenolic acids, to highly polymerized compounds, such as tannins. Many different classes of phenolic compounds can be distinguished, flavonoids and phenolic acids being the most abundant in plant materials [19–21]. These secondary plant metabolites with low molecular weight exhibit excellent antioxidant properties. However, particular mechanisms of their action vary depending both on the structure and the chemical environment [22].

There is increasing evidence in the literature indicating that secondary plant metabolites play critical roles in human health and may be nutritionally important because the development of chronic diseases, such as cardiovascular diseases (CVD), cancer, hypertension and type 2 diabetes, involves large production of free radicals leading to oxidative stress [23]. It is estimated that well over 50% of cancer patients use at least one or more complementary and alternative medicine (CAM) therapies as part of their treatment [24]. Among the alternative medicines, products that contain extracts of pawpaw (e.g., Paw Paw Cell-Reg, Graviola Max, Royal Graviola, Graviola Liquid Extract) were reported to exhibit antitumoral efficacy both in animal models and in a limited number of clinical studies [3]. Previous phytochemical studies of *Asimina triloba* led to the isolation of oil, lipids, fatty acids and proteins from the fruits and seeds, tannins, β-sitosterol, caffeic acid, several flavonoids (procyanidin, quercetin, rutin and isoquercitrin) and a number of alkaloids (asiminine, which was reported to be emetic, analobine, which was once used as a medicine, coreximine, anolobine, asimilobine, isocorydine, liriodenine, and norushinsunine) [3, 25].

The best practice of characterizing extracts is by measuring the concentration of the main bioactive compounds, called "markers". Concentrations of the main chemical components is used to characterize the fruit sample [26]. Different bioactive compound classes can be selected to "represent" the fruit extract and may be referred to as the "fingerprint". Indeed, it is accepted that synergistic or additive biological effects of different phytochemicals (phytocomplex), rather than a single compound or a group of compounds, contribute to disease prevention [27, 28]. Comparing the chemical composition of the extract and total antioxidant activity, the influence of a single identified class of compounds on the total phytocomplex can be evaluated.

The aim of this preliminary study was to investigate quality traits and report on the level of potentially antioxidant compounds and their influence on total fruit phytocomplex and antioxidant activity, at different stages of ripeness of pawpaw. This study emphasizes that quality parameters are not enough for full evaluation of the pawpaw fruits, but it is also necessary to consider nutraceutical features. This involves defin-

ing an effective chemical fingerprint to be used as a quality control tool. The importance of vitamin C and other antioxidant compounds such as polyphenols in reducing heart disease, stroke and some cancers make pawpaw a well balanced and desirable food option [29]. As no information is nowadays available on chemical fingerprint of *Asimina triloba* fruits, the results of present study may motivate more health conscientious people to consume pawpaw fruit [30].

The obtained fingerprint may be useful to highlight nutraceutically positive traits of pawpaw fruits in order to improve commercialization of this underutilized species in the fresh market. This information may be also interesting for pawpaw processors for the creation of pawpaw-based, value added functional food ingredients.

MATERIALS AND METHODS

Plant material

Samples of fruits were picked up in a germplasm repository in Val Germanasca, Turin Province (Italy) over a one-year period (2012). On the day the pawpaw fruits (5 kg for each plant) were manually picked from the tree (three plants for each replication) and assigned into different groups representing one of the three maturity levels (underripe with no softening or few soft spots, ripe with uniform softening, and overripe with many soft spots) based on selected qualitative parameters (firmness and total soluble solids), considering also the literature and the experience of the pawpaw growers. As this study was focused on ripening effects on quality parameters, nutraceutical fingerprint and antioxidant activity, only one cultivar was considered. In particular, cultivar Davis, which is open-pollinated, the most important Italian genotype, was picked up. Davis is the most widespread Italian cultivar, not only in collections but, especially in North-West Italy, also in small family-managed farms and nurseries, where *Asimina* is cultivated for non-commercial purposes. All harvested fruits were collected randomly from different genotypes and analysed fresh or after being stored for a few days at 4 °C and 95% relative humidity (*RH*). All samples were labelled with a code (Tab. 1).

Solvents and chemicals

Sodium carbonate, Folin-Ciocalteu phenols reagent, sodium acetate, citric acid, hydrochloric acid, iron(III) chloride hexahydrate, 2,4,6-tripyridyl-s-triazine (TPTZ) and 1,2-phenylenediamine dihydrochloride (OPDA) were purchased from Sigma Aldrich, St. Louis, Missouri, USA. Acetic acid was purchased from Fluka Biochemika, Buchs, Switzerland. Ethylene diamine tetraacetic acid (EDTA) disodium salt was purchased from AMRESCO, Solon, Ohio, USA, while sodium fluoride was purchased from Riedel-de Haen, Seelze, Germany.

Analytical HPLC-grade solvents, methanol and formic acid, were purchased from Sigma Aldrich and Fluka Biochemika, respectively. Potassium dihydrogen phosphate was purchased from Sigma Aldrich, while cetyltrimethylammonium bromide (cetrimide) was purchased from Extrasynthése (Genay, France). Milli–Q ultrapure water was produced by using Sartorius Stedim Biotech Arium (Sartorius, Goettingen, Germany).

All calibration polyphenolic standards were purchased from Sigma Aldrich: caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, hyperoside, isoquercitrin, quercetin, quercitrin, rutin, gallic acid, ellagic acid, catechin and epicatechin. Ascorbic acid and dehydroascorbic acid (DHAA) were purchased from Extrasynthése.

Qualitative analysis

Physical parameters

Average fruit weight, fruit length, fruit width and firmness were considered in 3 replications, each obtained from 10 fruits. Average fruit weight (in grams) was evaluated by Mettler PM460 Delta-Range Electronic Balance (Mettler, Greifensee, Switzerland), while a digital caliper (Traceable Digital Caliper-6"; VWR International, Milano, Italy) was used for measuring the fruit length and width (in millimetres). Firmness (in kilograms per square metre) was measured with a fruit pressure

Tab. 1. Origin and identification codes of the analysed samples.

Identification code	Sample	Maturity level	Place	Province	Region	Date
H1	1st harvesting	under ripe	Val Germanasca	Torino	Piemonte	20/11/2012
H2	2nd harvesting	ripe	Val Germanasca	Torino	Piemonte	26/11/2012
H3	3rd harvesting	overripe	Val Germanasca	Torino	Piemonte	02/12/2012

tester (Zenith FT 327; Zenith, Inverurie, United Kingdom).

Chemical parameters

Total soluble solids (*TSS*), pH and titratable acidity (*TA*) were evaluated in 3 replications, each obtained from 10 fruits.

TSS (in degrees Brix) was recorded with a digital refractometer DBR35 (Tsingtao Unicom-Optics Instruments, Laixi, China).

TA (in milligrams per litre) and pH were determined by titrating 10 ml of pulp juice (made up to 100 ml final volume with Milli-Q water) with a solution of NaOH (0.2 $mol \cdot l^{-1}$), using an automatic titrator (Crison Titromatic 2S; Crison, Alella, Spain).

Spectrophotometric analysis

Total polyphenolic compounds (*TPC*)

For the extraction of *TPC*, 10 g of fruit pulp (3 replications, each obtained from 5 fruits) were put into a 50 ml test tube and 25 ml of extraction solution (500 ml of methanol, 23.8 ml of bi-distilled water and 1.4 ml of 37% HCl) were then added to the weighed samples. After 60 min in the dark, the extracts were homogenized with an Ultra–Turrax homogenizer (T25; IKA Werke, Staufen, Germany) for about 1 min and then centrifuged for 15 min at 50 Hz in an ALC Centrifuge PK 120 (ALC International; Cologno Monzese, Italy). The supernatants were recovered and transferred to small glass tubes and kept frozen at –20 °C for further analysis [31].

The method used for determining *TPC* was based on Folin-Ciocalteu phenol reagent and spectrophotometric determination at 765 nm [31]. A total of 0.5 g of fruit extract and 30 ml of bi-distilled water were added to 2.5 ml of Folin-Ciocalteu reagent and 10 ml of 15% Na_2CO_3. After 2 h in the dark, absorbance was read at 765 nm. The standard calibration curve was plotted using gallic acid at concentrations of 0.02–0.1 $mg \cdot ml^{-1}$. The results were expressed as milligrams of gallic acid equivalents (GAE) per kilogram of fresh weight (FW).

Bioactivity-antioxidant activity (FRAP assay)

Antioxidant activity in the fruit pulp was evaluated by FRAP (ferric reducing antioxidant power) assay [32]. The extracted samples were the same as used in *TPC* determination. The method is based on the reduction of the ferric (Fe^{3+}) TPTZ (2,4,6-tripyridyl-s-triazine) complex to its ferrous form (Fe^{2+}). The FRAP reagent was prepared daily by mixing 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 \cdot 6H_2O$ solution with 25 ml acetate buffer (0.3 $mol \cdot l^{-1}$) and then warmed at 37 °C before using. A volume of 30 μl of sample (15 μl of extract sample and 15 μl of extraction buffer, dilution 1:2) was added to 90 μl of bi-distilled water and 900 μl of FRAP reagent in a 2 ml microtube, and then incubated at 37 °C for 30 min in a GFL Shaking Water Bath 1083 (GFL Gesellschaft Labortechnik, Burgwedel, Germany). Absorbance at 595 nm was recorded with a UV/Vis Spectrophotometer (1600-PC; VWR International). Standard curve was obtained using $FeSO_4 \cdot 7H_2O$ (concentration range: 100–1 000 $\mu mol \cdot l^{-1}$) and results were expressed as millimoles of Fe^{2+} equivalents per kilogram of FW.

Chromatographic analysis

Sample preparation protocols

Polyphenolic compounds

Methanolic extracts (the same as at *TPC*) were filtered through circular pre-injection filters (pore size 0.45 μm, polytetrafluoroethylene membrane (PTFE)) and then stored for a few days at normal atmosphere (NA), 4 °C and 95% *RH*.

Vitamin C

An amount of 10 g of fruit pulp (3 replications, each one of 5 fruits) were put into a 50 ml test tube and 10 ml of extraction solution (0.1 $mol \cdot l^{-1}$ citric acid, 2 $mmol \cdot l^{-1}$ ethylene diamine tetraacetic acid (EDTA) disodium salt and 4 $mmol \cdot l^{-1}$ sodium fluoride in methanol:water 5:95 v/v) were then added. The extracts were homogenized with an Ultra–Turrax T25 for about 1 min and then centrifuged for 10 min at 66 Hz at room temperature in an ALC Centrifuge PK 120. The supernatants were recovered and transferred to a 15 ml test tube through filter cloth and then acidified with 4 $mol \cdot l^{-1}$ HCl to decrease pH of the solution to a value of 2.2–2.4 [33]. Acidified samples were centrifuged for 5 min at 200 Hz at 4 °C with an ALC Multi Speed refrigerated centrifuge PK 121R (ALC International) and the supernatants were then filtered through a membrane microfilter (pore size 0.45 μm, diameter 17 mm, PTFE; Titan 2 HPLC filter). Polyphenolic compounds were absorbed on a C_{18} cartridge for Solid Phase Extraction (Sep-Pak C-18; Waters, Milford, Massachusetts, USA). Then, 250 μl of OPDA solution (18.8 $mmol \cdot l^{-1}$) was added to 750 μl of extracted samples for DHAA derivatization with a fluorophore 3-(1,2-dihydroxyethyl)furo(3,4-b)quinoxalina-1-one (DFQ). After 37 min in the dark, the samples were analysed by HPLC equipped with a diode array detector (DAD) [34].

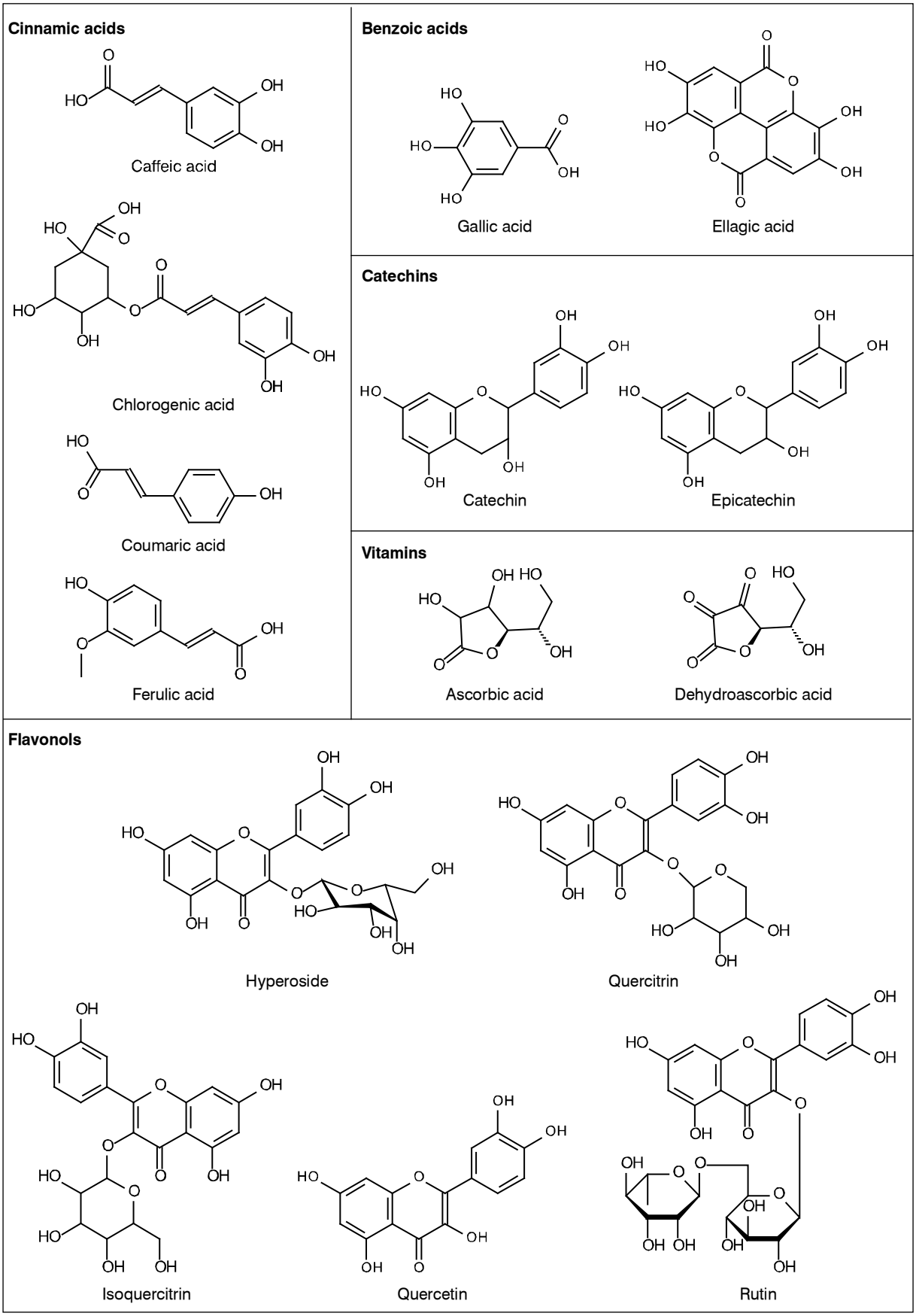

Fig. 1. Chemical structures of the detected antioxidant compounds.

Preparation of standards

Chemical structures of all compounds are shown in Fig. 1. Stock solutions of cinnamic acids and flavonols with a concentration of 1.0 mg·ml^{-1} were prepared in methanol. From these solutions, four calibration standards were prepared by dilution with methanol. Stock solutions of benzoic acids and catechins with concentrations of 1.0 mg·ml^{-1} were prepared in 95% methanol and 5% water; from these solutions, four calibration standards were prepared by dilution with 50% methanol–water. Stock solutions of ascorbic and DHAA with a concentration of 1.0 mg·ml^{-1} were prepared in methanol; from these solutions, four calibration standards were prepared by dilution with methanol.

Apparatus and chromatographic conditions

An Agilent 1200 High Performance Liquid Chromatograph, equipped with a G1311A quaternary pump, a manual injection valve and a 20 μl sample loop, coupled to an Agilent GI315D UV-Vis DAD (Agilent Technologies, Santa Clara, California, USA), was used for the analysis. Three different chromatographic methods were used to analyse the extracted samples, two for polyphenols and one for vitamins.

The first method (A) was used for the analysis of cinnamic acids and flavonols. The separation was achieved on a Zorbax Eclipse XDB–C18 column (4.6 mm × 150 mm, 5 μm, Agilent Technologies), while the mobile phase consisted of methanol and a solution of 40 mmol·l^{-1} potassium dihydrogen phosphate in water. The flow rate was 1.0 ml·min^{-1} (gradient analysis, 60 min) and the detector wavelength was 330 nm [18, 35].

The second method (B) was used for the analysis of benzoic acids and catechins; bioactive molecules were separated on a Zorbax Eclipse XDB–C18 column (4.6 mm × 150 mm, 5 μm), while the mobile phase consisted of a solution of methanol : water : formic acid (5 : 95 : 0.1 v/v/v) and a mix of methanol : formic acid (100 : 0.1 v/v). The flow rate was 1.0 ml·min^{-1} (gradient analysis, 35 min) and the detector wavelengths were 250 nm, 280 nm and 320 nm [18, 36].

The third method (C) was used for the analysis of vitamins: separations of DFQ and ascorbic acid were achieved on a Zorbax Eclipse XDB–C18 column (4.6 mm × 150 mm, 5 μm). The mobile phase was methanol : water (5 : 95 v/v) containing 5 mmol·l^{-1} cetrimide and 50 mmol·l^{-1} potassium dihydrogen phosphate. The flow rate was 0.9 ml·min^{-1} (isocratic analysis, 15 min) and the detector wavelengths were set to 348 nm for DFQ and 261 nm for ascorbic acid detection [34, 37].

Identification and quantification of bioactive compounds

All single compounds were identified in

Tab. 2. Calibration curve equations, linearity, limit of detection and limit of quantification of the used chromatographic methods for each calibration standard.

Class	Standard	Standard identification code	Method	Calibration curve equations (y – peak area; x – concentration)	R^2	*LOD* [mg·l^{-1}]	*LOQ* [mg·l^{-1}]
Cinnamic acids	Caffeic acid	1	A	$y = 10.155x + 13.008$	0.985	1.232	4.107
	Chlorogenic acid	2	A	$y = 7.165x + 95.749$	0.995	0.627	2.091
	Coumaric acid	3	A	$y = 10.904x + 187.144$	0.999	1.037	3.456
	Ferulic acid	4	A	$y = 6.181x - 273.562$	1.000	1.012	3.373
Flavonols	Hyperoside	5	A	$y = 14.315x - 262.753$	1.000	0.549	1.829
	Isoquercitrin	6	A	$y = 11.437x + 100.974$	0.998	0.475	1.585
	Quercetin	7	A	$y = 5.505x - 418.512$	0.996	1.897	6.323
	Quercitrin	8	A	$y = 5.162x - 168.272$	0.996	1.072	3.575
	Rutin	9	A	$y = 8.213x + 105.923$	0.999	0.672	2.241
Benzoic acids	Ellagic acid	10	B	$y = 5.766x + 281.063$	0.988	1.881	6.271
	Gallic acid	11	B	$y = 10.703x + 59.149$	0.998	0.283	0.944
Catechins	Catechin	12	B	$y = 6.567x - 178.554$	0.999	1.755	5.850
	Epicatechin	13	B	$y = 6.104x - 172.263$	0.997	1.749	5.829
Vitamins	Ascorbic acid	14	C	$y = 40.541x - 798.702$	0.998	0.236	0.786
	Dehydroascorbic acid	15	C	$y = 5.844x + 197.332$	0.999	0.836	2.786

R^2 – linearity, *LOD* – limit of detection, *LOQ* – limit of quantification.

samples by comparison of their retention times and UV spectra with those of standards in the same chromatographic conditions. Quantitative determinations were performed using an external standard method. Calibration curves in the 125–1 000 mg·l^{-1} range with good linearity for a four point plot were used to determine the concentration of polyphenolic compounds and vitamins in extracted samples. The linearity for each compound was established by plotting the peak area (y) versus the concentration (x) of each analyte. The t of the three chromatographic methods were defined as the lowest amount of analyte that gives a reproducible peak with a signal-to-noise ratio (S/N) of 3 and 10, respectively. Calibration curve equations, linearity (R^2), *LOD* and *LOQ* for all of the analytes are summarized in Tab. 2.

All samples were analysed in triplicate (three replications for each harvesting time), and all data are given in order to assess the repeatability of the used methods (standard deviation). Accuracy was checked by spiking samples with a solution containing each bioactive compound in a concentration of 10 mg·ml^{-1}. Examples of HPLC/DAD chromatographic profiles are reported in Fig. 2, Fig. 3 and Fig. 4.

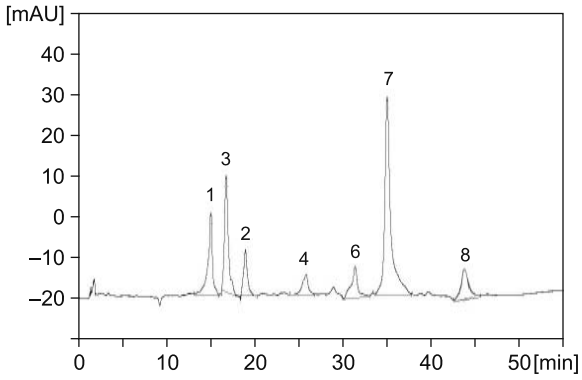

Fig. 2. HPLC/DAD profile, method A.

1 – caffeic acid, 2 – chlorogenic acid, 3 – coumaric acid, 4 – ferulic acid, 6 – isoquercitrin, 7 – quercetin, 8 – quercitrin.

Total antioxidant compound content (*TACC*) were determined as the sum of the most important classes of polyphenolic compounds and vitamins present in the samples. Four polyphenolic classes were considered: benzoic acids (gallic acid and ellagic acid), catechins (catechin and epicatechin), cinnamic acids (caffeic acid, chlorogenic acid, coumaric acid and ferulic acid) and flavonols

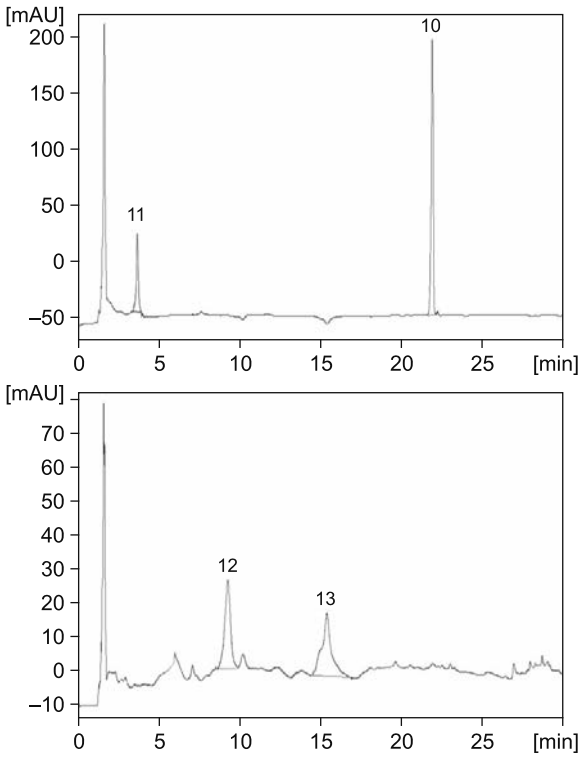

Fig. 3. HPLC/DAD profile, method B.

10 – ellagic acid, 11 – gallic acid, 12 – catechin, 13 – epicatechin.

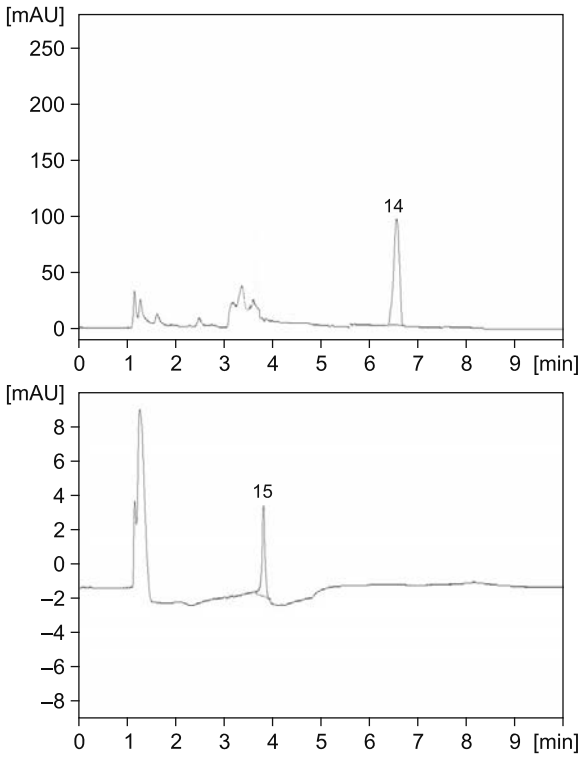

Fig. 4. HPLC/DAD profile, method C.

14 – ascorbic acid, 15 – dehydroascorbic acid.

(hyperoside, isoquercitrin, quercetin, quercitrin and rutin). One vitamin class was considered: vitamin C (ascorbic acid and DHAA). All results were expressed as milligrams per kilogram of FW.

Statistical Analysis

Results were subjected to ANOVA for mean comparison (SPSS 18.0 software, SPSS, Chicago, Illinois, USA) and Tukey's HSD (honestly significant difference) multiple range test ($p < 0.05$). Correlations were evaluated with Pearson coefficient. Principal component analysis (PCA) was performed on the single compoud concentration data.

RESULTS

Qualitative parameters

All results (mean values ± standard deviations) are summarized in Tab. 3. These confirmed the trend of fruit ripening. Significant statistical differences were observed in average fruit weight and firmness and ANOVA test divided samples into two groups: H1 sample on the one hand and H2 plus H3 samples on the other. Fruit length ($F = 2.702$; $p_F = 0.146$) and width ($F = 2.126$; $p_F = 0.200$) did not show differences and ranged from 61.98 mm to 91.16 mm and from 41.27 mm to 49.66 mm, respectively. *F* represented the obtained value of test F for variance comparison and p_F showed its significance. Among chemical qualitative parameters, *TA* ($F = 0.119$; $p_F = 0.890$) did not show significant statistical differences, while they were observed in *TSS* and pH. In particular, *TSS* ranged from 14.87 °Brix to 15.90 °Brix.

Total polyphenolic compounds and bioactivity-antioxidant activity

The content of *TPC* in the analysed extracts is reported in Tab. 4. No statistically significant differences were observed among the analysed sam-

Tab. 3. Physical and chemical quality parameters of pawpaw samples.

Sample	Physical qualitative parameters			
	Weight [g]	Width [mm]	Length [mm]	Firmness [kg·m^{-2}]
H1	124.145 ± 39.485^{b}	49.656 ± 5.240^{a}	91.160 ± 17.709^{a}	85 500.001 ± 4 135.215^{b}
H2	76.440 ± 11.449^{a}	44.308 ± 3.045^{a}	69.417 ± 7.295^{a}	58 833.333 ± 4 956.477^{a}
H3	57.517 ± 6.805^{a}	41.271 ± 3.885^{a}	61.982 ± 9.591^{a}	53 166.677 ± 8 588.752^{a}

Sample	Chemical qualitative parameters		
	Total soluble solids [°Brix]	Titratable acidity [mg·l^{-1}]	pH
H1	14.867 ± 0.058^{a}	7.167 ± 2.428^{a}	5.577 ± 0.067^{a}
H2	15.900 ± 0.100^{c}	7.170 ± 0.442^{a}	5.643 ± 0.038^{a}
H3	15.367 ± 0.058^{b}	6.667 ± 0.499^{a}	5.820 ± 0.026^{b}

Mean values ± standard deviation in each row for the same parameter ($n = 3$) followed by different letters are significantly different as determined by Tukey's HSD test following ANOVA ($p < 0.05$).

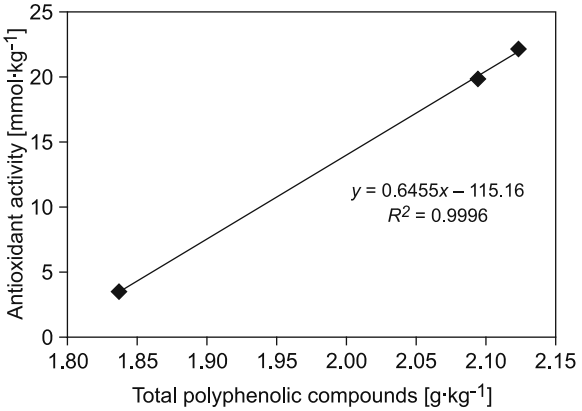

Fig. 5. Correlation between antioxidant activity and total polyphenolic compounds.

Tab. 4. Total polyphenolic compounds and antioxidant activity.

Sample	Total polyphenolic content [mg·kg^{-1}]	Antioxidant activity [mmol·kg^{-1}]
H1	1 837.209 ± 451.916^{a}	3.447 ± 0.334^{a}
H2	2 123.882 ± 346.049^{a}	22.113 ± 1.474^{b}
H3	2 094.736 ± 359.873^{a}	19.847 ± 2.773^{b}

Mean values ± standard deviation of each harvesting time is given ($n = 3$). Different letters for each sample indicate significant differences at $p < 0.05$.
Total phenolic content is expressed as gallic acid equivalents per kilogram of fresh weight. Antioxidant activity is expressed as Fe^{2+} equivalents per kilogram of fresh weight.

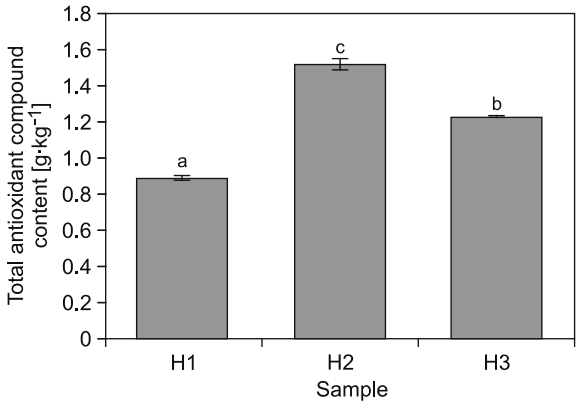

Fig. 6. TACC of pawpaw samples.

Different letters for each sample indicate significant differences at $p < 0.05$.

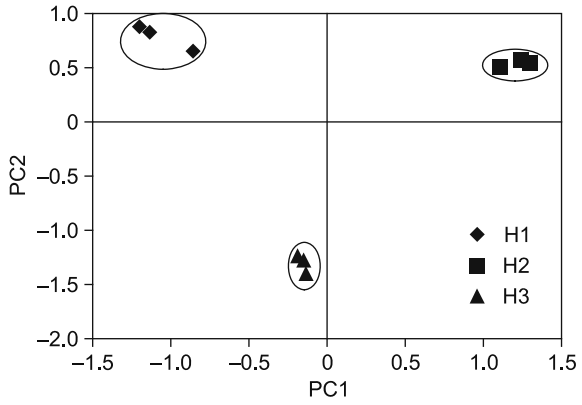

Fig. 7. PCA individual graph of pawpaw samples.

ples ($F = 0.494$; $p_F = 0.633$), with the lowest *TPC* value of 1837.2 mg·kg^{-1} (sample H1) and the highest value of 2123.8 mg·kg^{-1} (sample H2), expressed as GAE. H2 and H3 samples showed higher antioxidant activities than H1 sample. The lowest FRAP value was observed for H1 (3.45 mmol·kg^{-1}) and the highest for H2 (22.11 mmol·kg^{-1}), expressed as Fe^{2+} equivalents (Tab. 4). The correlation between *TPC* and antioxidant activity was positive, with a significant Pearson correlation coefficient ($r = 0.9998$, $p < 0.05$). Equation and regression coefficient are reported in Fig. 5.

Total antioxidant compound content

The *TACC* in the evaluated pawpaw samples is reported in Fig. 6. Statistically significant differences were observed among the analysed samples, with the lowest *TACC* value of 889.6 mg·kg^{-1} (sample H1) and the highest value of 1519.6 mg·kg^{-1} (sample H2). PCA was performed on all samples and it reduced the initial variables (single antioxidant compound concentration) into three principal components (97.4% of total variance) and divided samples to three groups, equal to the initial sample groups, confirming the statistically significant differences of ANOVA test on *TACC* (Fig. 7). PCA variable graph (Fig. 8) showed a correlation between the most of polyphenols-vitamins and PC1 (64.4% of total variance); only coumaric acid presented a correlation with PC2 (24.9% of total variance). Caffeic acid was in an intermediate position between PC1 and PC2.

Single compound profile

All data (mean values ± standard deviations) are reported in Tab. 5 (method A, B and C). Paw-

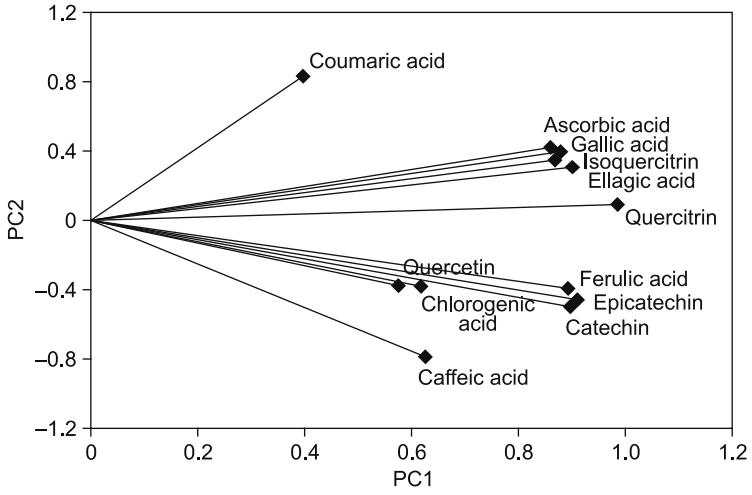

Fig. 8. PCA variable graph of analysed samples.

Tab. 5. Single compound profiles of analysed samples.

METHOD A				
Sample	Cinnamic acids [mg·kg^{-1}]			
	Caffeic acid	Chlorogenic acid	Coumaric acid	Ferulic acid
H1	0.456 ± 0.396^{a}	14.073 ± 1.189^{a}	39.579 ± 0.231^{b}	110.167 ± 0.699^{a}
H2	20.135 ± 1.049^{b}	16.568 ± 2.013^{a}	45.881 ± 0.966^{c}	123.143 ± 1.608^{b}
H3	26.769 ± 0.872^{c}	16.175 ± 0.358^{a}	24.532 ± 0.618^{a}	120.422 ± 1.674^{b}

METHOD A					
Sample	Flavonols [mg·kg^{-1}]				
	Hyperoside	Isoquercitrin	Quercetin	Quercitrin	Rutin
H1	nd	14.948 ± 0.283^{a}	190.321 ± 1.936^{a}	143.392 ± 0.962^{a}	nd
H2	nd	36.200 ± 5.187^{b}	193.158 ± 3.067^{a}	228.978 ± 3.561^{c}	nd
H3	nd	14.263 ± 0.644^{a}	192.874 ± 0.369^{a}	166.430 ± 1.312^{b}	nd

METHOD B					**METHOD C**
Sample	Benzoic acids [mg·kg^{-1}]		Catechins [mg·kg^{-1}]		Vitamins [mg·kg^{-1}]
	Ellagic acid	Gallic acid	Catechin	Epicatechin	Vitamin C
H1	40.596 ± 1.059^{a}	18.577 ± 1.102^{a}	154.654 ± 0.053^{a}	123.091 ± 2.308^{a}	39.740 ± 5.476^{a}
H2	101.105 ± 16.191^{b}	33.215 ± 2.076^{b}	296.584 ± 6.379^{c}	352.152 ± 8.398^{c}	72.499 ± 1.546^{b}
H3	41.806 ± 1.649^{a}	17.158 ± 0.646^{a}	267.976 ± 2.319^{b}	305.590 ± 1.168^{b}	35.042 ± 1.885^{a}

For method A, B and C, different letters for each sample indicate significant differences at $p < 0.05$ ($n = 3$).

Tab. 6. Correlation among antioxidant activity and total polyphenolic compounds, total antioxidant compound content and all single antioxidant compounds.

	Pearson correlation coefficient (*R*)	
	Antioxidant activity	Correlation
TPC	0.9998	positive strong
TACC	0.9333	positive strong
Caffeic acid	0.9372	positive strong
Chlorogenic acid	0.9994	positive strong
Coumaric acid	–0.1205	negative weak
Ferulic acid	0.9961	positive strong
Isoquercitrin	0.5709	positive medium
Quercetin	0.9998	positive strong
Quercitrin	0.7822	positive strong
Ellagic acid	0.6072	positive medium
Gallic acid	0.5271	positive medium
Catechin	0.9968	positive strong
Epicatechin	0.9966	positive strong
Vitamin C	0.4966	positive medium

TPC – total polyphenolic compounds, *TACC* – total antioxidant compound content.

paw samples showed the following antioxidant composition: four cinnamic acids (caffeic acid, chlorogenic acid, coumaric acid, ferulic acid), three flavonols (isoquercitrin, quercetin, quercitrin), two benzoic acid (ellagic acid, gallic acid), two catechins (catechin, epicatechin) and one vitamin (vitamin C expressed as sum of ascorbic acid and DHAA); hyperoside and rutin were not detected. Single bioactive compound content ranged from 0.5 mg·kg^{-1} (caffeic acid, H1 sample) to 352.2 mg·kg^{-1} (epicatechin, H2 sample). Statistically significant differences were observed in all analysed samples: the most important differences were observed in the contents of quercitrin, ellagic acid, catechin, epicatechin and vitamin C. Correlations among antioxidant activity and all single antioxidant compounds are reported in Tab. 6. Only coumaric acid showed a weak negative correlation with antioxidant capacity, while most of the compounds presented a strong positive correlation ($R > 0.99$).

Chemical fingerprinting

Chemical fingerprint of *Asimina triloba* fruits was created. In total, 12 antioxidant compounds were identified by HPLC-DAD. By single bioac-

tive compound profile, health-promoting agents were grouped into different classes to evaluate the single contribution of each class to total fruit phytocomplex composition. Chemical fingerprint showed the prevalence of catechins and flavonols in all analysed samples (mean values were considered): the most important class was catechins (40.2%), followed by flavonols (33.3%), cinnamic acids (15.8%), benzoic acids (6.8%) and vitamins (4.0%), (Tab. 7). Therefore, catechins and flavonols were two major groups of antioxidant compounds in the evaluated pawpaw fruits: catechin contribution ranged from 31.2% in H1 sample to 46.7% in H3 sample, while flavonols contributed to total phytocomplex in a range from 30.2% (H2) to 39.2% (H1).

DISCUSSION

Pawpaw fruits were traditionally consumed by Native Americans, then by European explorers and settlers. Today, pawpaws are consumed by local populations in rural areas. Despite some consumer and professional grower interest, the high perishability of the fruit has been a major factor slowing the development of a larger market for the fruit [29].

Relatively little is known about ripening of pawpaw, and several problems, such as short shelf life and duration of harvesting, hamper pawpaw production: after harvest the fruits have a shelf life of only 2–5 days, though recently it has been established that pawpaw fruit can be stored for one month at 4 °C with little loss in quality [7]. It has been well established that many fruit varieties undergo physical and chemical changes upon ripening that cause greater perishability and reduced shelf life [4]. For this reason, researchers have identified indicators of fruit maturity for many commercial pawpaw varieties [4, 38]. These indicators were used to determine harvest times of fruit with acceptable flavour characteristics and structural integrity. ANOVA results of this study were similar to other works [30, 38], but emphasize that the quality parameters are not sufficient for a fuller evaluation of the pawpaw fruits, especially for a better appreciation of this underutilized species. While previous investigations have resulted in identifying physical and chemical properties associated with ripening, the effects on phenolic content and antioxidant capacity have not been investigated [39]. In addition to defining physical and chemical parameters of pawpaw pulp during ripening, the objectives of this study were to investigate changes in total antioxidant compound content related to antioxidant activity, defining a chemical fingerprint, used as a quality control tool.

Recently, much attention has been paid to understanding the roles of fruits and vegetables in the promotion of human health and prevention of chronic diseases [30]. The chemoprotective properties of fruits have been partly attributed to phenolics such as gallic acid and chlorogenic acid, and the antioxidant compound content was generally found to correlate with antioxidant activity for various types of fruits [40, 41]. Over the years, studies have proven that many compounds commonly found in fruits and vegetables, such as vitamin C, vitamin E, and antioxidant compounds such as carotenoids and phenolics, have a positive effect on lowering the risk of developing cancer and heart disease in people that consume the products on a regular basis [29].

During the past several years, the quest for alternative crops with high nutritional value has increased interest in pawpaw [29]. However, *TPC*, antioxidant activity and most of potential health-promoting agents of pawpaw remain unscrutinized. Despite many reports of commonly available fruits such as blueberry, orange and apple on their *TPC* and antioxidant activity [37, 42, 43], little information is available for currently underused fruits. Pawpaw fruits may contain a significant amount of phytochemicals or even unique compounds that may be health-promoting [29, 30]. The fruits are comparable to apple and orange since they are rich in polyphenolic compounds, vitamin C (30–200 $mg \cdot kg^{-1}$), magnesium (1 000–1 400 $mg \cdot kg^{-1}$), iron (60–80 $mg \cdot kg^{-1}$), copper (2–8 $mg \cdot kg^{-1}$) and manganese (20–30 $mg \cdot kg^{-1}$).

Tab. 7. Contribution of antioxidant classes to the fruit phytocomplex in analysed extracts.

Sample	Cinnamic acids	Flavonols	Benzoic acids	Catechins	Vitamins
H1	18.5%	39.2%	6.7%	31.2%	4.5%
H2	13.5%	30.2%	8.8%	42.7%	4.8%
H3	15.3%	30.4%	4.8%	46.7%	2.9%
Mean value	15.8%	33.2%	6.8%	40.2%	4.0%

Also their antioxidant activity may be comparable to that of the more extensively studied fruits [30]. Moreover, pawpaw fruits are also a good source of potassium (3 000–3 800 $mg \cdot kg^{-1}$) and several essential aminoacids (mean value: 40 $mg \cdot kg^{-1}$ of protein), and they contain significant amounts of riboflavin (0.06–0.15 $mg \cdot kg^{-1}$), niacin (10–12 $mg \cdot kg^{-1}$), calcium (500–800 $mg \cdot kg^{-1}$), phosphorus (400–500 $mg \cdot kg^{-1}$) and zinc (10–12 $mg \cdot kg^{-1}$) [29]. In this research, only antioxidant compounds were considered. Pawpaw fruits are comparable to some important tropical fruits, for example, it has a content of polyphenols that is similar (e.g. caffeic acid and vitamin C) or higher (e.g. ellagic acid, ferulic acid and quercetin) than mango (*Mangifera indica* L.) [44, 45]. The levels of antioxidant compounds is also similar to other tropical fruits, such as guava, papaya, banana and ananas [46–48].

Changes in *TPC* and antioxidant activity of fruits are often associated with ripening of fruits [49]. *TPC* and antioxidant activity tended first to increase (from underripe to ripe) and then to decrease with ripening of fruit (from ripe to overripe) in our study as well as in other studies [30, 39].

In this study, ANOVA test and PCA confirmed *TPC* and antioxidant activity results of other authors [1, 6, 30, 39], but the use of additional analytical methods significantly contributed to improve the knowledge of this species. Certain antioxidant compounds can be used cumulatively as representative standards of a plant sample in quantification [50], as done in this study, but these methods still allow quantification of individual compounds. *TACC* obtained by HPLC can be used for quantification of health-promoting agents. HPLC methods give more information on individual compounds or groups of compounds than *TPC* by Folin-Ciocalteu method [51].

Antioxidant activity and bioactive compound contribution to total fruit phytocomplex were used to highlight nutraceutical/pharmaceutical and medicinal properties. Determination of antioxidant activity was considered an important method to evaluate the nutraceutical properties of pawpaw fruit, as shown in other previous studies [1, 52]. In particular in this study, the correlation between *TPC, TACC* and antioxidant activity was useful to show that the detected single antioxidant compounds were strongly related to some nutraceutical properties, such as the antioxidant capacity.

Moreover, an innovative approach has been applied to evaluate the pawpaw medicinal properties: chemical fingerprint was used to show the contribution of a single bioactive class to the total fruit phytocomplex. Indeed, synergistic or additive biological effects of different bioactive compounds or antioxidant classes could contribute to disease prevention more than a single compound or a group of compounds. In previous studies [28, 53, 54], this approach was only considered in relation to the evaluation of medicinal properties of herbal preparations, while in this research, it was applied to the study of fresh fruit quality.

Few reports on the analysis of phenolic acids (e.g. caffeic acid and its derivatives) by HPLC with diode array or mass detectors have been published [1, 6]. They describe their determination in pawpaw fruits according to single antioxidant compound contents shown in this research. Among other identified classes, flavonols and catechins were also selected for quantitative studies [1]. Based on the obtained results, this study pointed out that the identified antioxidant compounds significantly contributed to the antioxidant activity of these fruits, as shown by correlation among antioxidant activity and all single antioxidant compounds [1]. A high correlation was found between total antioxidant compound content, single compound profile and antioxidant capacity of pawpaw pulp, suggesting that these parameters can be incorporated into methods to estimate the ripeness of pawpaw fruit.

ANOVA and PCA results showed that the composition of *Asimina triloba* fruits from different stages of ripeness was similar but the constituent contents were different. Moreover, comparing the observed chemical composition, a few compounds were not detected in pawpaw fruits. As opposed to quality parameters [30], chromatographic fingerprinting could be applied to differentiation of fruits of *Asimina triloba* in different stages of ripeness and could be useful for authentication and quality control purposes.

This study is only a preliminary research about pawpaw fruit chemical composition, as data were actually completely missing for this species. Genotype is an important variable to define the nutraceutical quality traits, but in case of this research, we only focused on the ripening time effects on antioxidant compositions. This first overview could be extended in further studies with a careful comparison of different pawpaw genotypes, the diversity in total antioxidant compound content and antioxidant activity between pawpaw cultivars [4, 6]. The need for additional screening to identify cultivars with high antioxidant capacity and health-promoting potential can be emphasized.

CONCLUSION

The assessment of the chemical composition and antioxidant activity of *Asimina triloba* at different ripening stages may allow to increase utilization of pawpaw fruit as a functional food. In this study, *TPC, TACC* and antioxidant activity of pawpaw were equivalent, or even superior in some cases, compared with other common fruits. Although *TPC* of pawpaw fruit examined in this study was lower than that of cranberry, red grape or strawberry, it was comparable or superior to other fruits such as peach, apple, orange, banana, pear, pineapple, some blueberry cultivars and grapefruit [37, 41, 42]. Correlations between single compounds and antioxidant activity suggested that antioxidant components of pawpaw pulp have an important effect on antioxidant capacity, as reported for other fruits and vegetables [37, 55]. Chemical fingerprinting could be an important tool to assess the chemical composition and bioactivities of the pawpaw fruits, in order to distinguish the different stages of ripeness, to increase the consumption of the fruits of this underutilized species and find out new sources of natural health-promoting compounds. Finally, this study contributed to development of an effective tool for the pawpaw fruit quality assessment through its chemical fingerprinting.

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Received 30 May 2013; 1st revised 12 July 2013; 2nd revised 13 August 2013; accepted 18 August 2013; published online 17 February 2014.