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ORIGINAL ARTICLE

The effect of frozen storage on the phenolic compounds of *Morus nigra* L. (black mulberry) and *Morus alba* L. (white mulberry) fruit

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Abstract – Introduction. *Morus nigra* L. (black mulberry) and *Morus alb*a L. (white mulberry) display high concentrations of health-promoting compounds, particularly phenolics. However, no published studies have addressed the changes in the content of phenolic compounds during frozen storage, a widely used form of preservation of these fruit in the Turkish countryside. This work was undertaken to determine these alterations, if any, in order to assess whether the bioactive properties of the produce may be altered significantly. **Materials and methods.** Black and white mulberry fruit were collected at commercial maturity and frozen at –25 °C for up to 5 months. The content of selected phenolic acids and flavonoids was analysed at harvest on fresh fruit and at monthly intervals on thawed samples by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC/DAD). **Results and discussion.** Phenolic compound levels were higher in black than in white mulberry fruit at harvest. Rutin and chlorogenic acid predominated quantitatively in black mulberry, and decreased along frozen storage even though some fluctuations were observed. Cathechin was the main compound detected in white mulberry, and remained largely stable during the whole experimental period. **Conclusion.** Although the concentration of the investigated phenolics varied to different extents during frozen storage, fruit retained acceptable levels, which suggests that this practice allows preserving satisfactorily the health-promoting properties that characterise these fruit species.

Keywords: Turkey / mulberry / *Morus nigra* / *Morus alba* / frozen storage / phenolics

Résumé – Effets de la congélation sur les composés phénoliques des fruits de *Morus nigra* L. (mûrier noir) et de *Morus alba* L. (mûrier blanc). Introduction. *Morus nigra* L. (mûrier noir) et *Morus alba* L. (mûrier blanc) affichent de fortes concentrations en composés bénéfiques pour la santé, en particulier les composés phénoliques. Cependant, aucune étude publiée n'a abordé les changements de teneur en ces composés phénoliques durant la congélation, méthode de conservation de ces fruits très courante dans la campagne turque. Notre travail a été entrepris pour déterminer les éventuelles modifications liées à la congélation, en particulier les propriétés bioactives du produit. **Matériel et méthodes.** Les fruits des mûriers noirs et des mûriers blancs ont été prélevés à maturité commerciale et congelés à −25 °C pendant cinq mois. Les teneurs en certains acides phénoliques et flavonoïdes d'intérêt spécifique ont été analysées à la récolte sur fruits frais et sur échantillons décongelés à intervalles mensuels, par chromatographie liquide à haute performance à détecteur à barrettes de diodes (HPLC/DAD). **Résultats et discussion.** À la récolte, le niveau des composés phénoliques est plus élevé dans les mûres noires que dans les mûres blanches. Rutine et acide chlorogénique sont quantitativement prédominants sur mûrier noir, et diminuent au cours de la congélation avec certaines fluctuations. La cathéchine est le principal composé qui a été détecté sur mûrier blanc, et est restée stable pendant toute la période expérimentale. **Conclusion.** Bien que la concentration des composés phénoliques étudiés varie à des degrés divers au cours de la congélation, les fruits ont conservé des niveaux acceptables, ce qui suggère que cette pratique de conservation permet de préserver de manière satisfaisante les propriétés bénéfiques pour la santé qui caractérisent ces espèces fruitières.

Mots clés : Turquie / mûrier / *Morus nigra* / *Morus alba* / congélation / composés phénoliques

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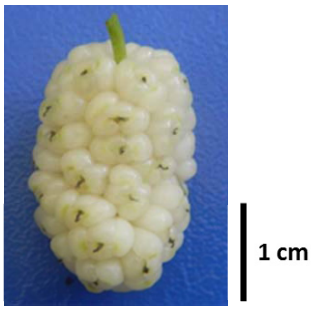

Figure 1. Fruit of white mulberry (*Morus alba* L., clone "Beyaz").

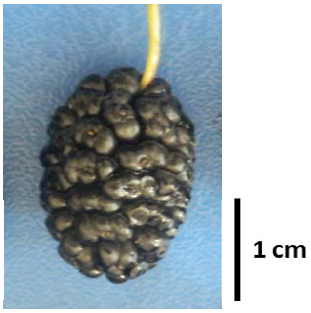

Figure 2. Fruit of black mulberry (*Morus nigra* L., clone "Kara").

1 Introduction

Mulberry (*Morus* sp.) fruit must be picked when fully ripe in order to attain their delicate taste and flavour. Ripe mulberries are very soft and juicy, which causes the fruit to be very prone to mechanical damage and to fungal rots. This means that mulberry fruit are a highly perishable commodity, and shelf life at ambient temperature may be as short as one day. All these aspects considerably limit the commercial exploitation of mulberry species as a fresh produce, although mulberries are used for the confection of a range of processed products, including juices, anti-obesity drinks, sauces, cakes, teas, wines, fruit powder or food colorants [1]. Yet, extremely limited research efforts have been focused on the postharvest physiology of these fruit, or on possible procedures for the extension of their keeping potential. Packaging under modified atmosphere has been reported to reduce significantly weight loss and to improve the overall appearance of mulberries after storage at 3 °C for up to 10 days [2]. Calcium chloride dips, alone or in combination with 1-methylcyclopropene application, have also been recently found to extend the shelf life potential of mulberries [3], but we are not aware of any additional published work on the postharvest quality of these fruit.

According to the Turkish Statistical Institute, mulberry production in Turkey amounted to around 70,000 tons in 2013. These fruits are widely cultivated in the central and the east regions of Turkey, where they are consumed fresh, dried, turned quickly into juices, jams and marmalades, or prepared as local specialties such as *pekmez, pestil* or *cevizli sucuk*. Traditionally, mulberries have also been given a medicinal use in Turkey [4]. This traditional use reflects the ancestral knowledge of the excellent health-promoting properties of these fruit, resulting from their high contents in total phenolics and flavonoids [4–9], which have been shown to display some variation according to the *Morus* genotype considered in each case [10–13].

Most mulberries used for fresh consumption belong to the species *Morus alba* (white mulberry) (*figure 1*) and *M. nigra* (black mulberry) (*figure 2*). Owing to the extremely short shelf life of the fresh fruit, local families either process the produce immediately, or freeze them (particularly black mulberries) for future uses. While allowing for extended preservation and availability of fruit, this practice might compromise the health-promoting properties of the produce. Therefore, we undertook this study with the purpose of assessing the fate of some important phenolic acids and flavonoids throughout frozen storage of mulberry fruit from two different species.

2 Materials and methods

2.1 Fruit material and storage

Mulberry fruit of the species *Morus nigra* L. (clone 'Kara') and *M. alba* L. (clone 'Beyaz') were harvested from a family-led orchard in Alkamer (Iğdır, Turkey) at the commercially ripe stage according to the usual indices in the producing area. Harvest date was July 3, 2013, and fruit was collected from three trees of the same age per species. Defect- and rot-free fruit samples were selected for uniform shape and colour, and transported immediately to the laboratory. For each of both species, samples were grouped into six batches, each of which was comprised of around 300 fruits (approximately 1 kg per batch). One batch per species was analysed immediately after harvest as the fresh control. The five remaining batches were introduced into food-grade polyethylene bags and stored at −25 °C for up to five months. One batch per species was removed monthly from the freezer and allowed to thaw overnight at +4 °C, after which samples remained at ambient temperature during two hours before being analysed.

2.2 Assessment of standard quality at harvest

Standard quality parameters were determined on 30 fruits per each species at harvest. Fruit weight was determined with an electronic balance (0.01 g accuracy). Fruit width and length were measured with a digital calliper (0.01 mm accuracy). Titratable acidity (TA), pH, and soluble solids content (SSC) were assessed in juice pressed from the whole fruit (10 fruits per replicate × 3 replicates). TA was determined in 10 mL fruit juice by diluting in 10 mL distilled water and titrating with 0.1 N NaOH to pH 8.1 [14], and expressed as g malic acid L^{-1}. A digital table refractometer (WAY-2S, Seoul, South Korea) was used for SSC assessment, and data given as °Brix. The pH of fruit juice was determined using a portable pH meter (Jenco Instruments Inc., San Diego, USA).

Table I. Mobile phase gradient programme for HPLC analysis of phenolic extracts obtained from fresh and frozen samples of black and white mulberry fruit (Solution A: Acetic acid / water (2:98, v/v), Solution B: 50% aqueous acetonitrile / 0.5% aqueous acetic acid (1:1, v/v), Solution C: acetonitrile).

Time (min)	Solutions (%)		
	A	B	C
0	95	5	0
5	95	5	0
8	80	20	0
10	78	22	0
17	75	25	0
19	73	27	0
30	60	40	0
35	55	45	0
40	35	65	0
45	0	10	90
50	0	0	100
52	95	5	0
60	95	5	0

2.3 Extraction and analysis of phenolic compounds in fruit samples

Phenolic compounds were extracted according to the method described in [15] with some modifications. Approximately 200 g whole mulberries per species were diced at each analysis date, and 5 g of this starting material were weighted and sonicated for 10 min in 10 mL of 80% (v/v) acetone. The extract was centrifuged at 15,000 *g* and 4 °C for 10 min, and the supernatant was collected. The insoluble material was re-extracted twice in 10 mL of 80% acetone, and the supernatants were pooled. Residual acetone was removed at 37 °C in a rotary evaporator (Heidolph, Schwabach, Germany) under reduced pressure. This procedure was carried out in triplicate per each species and analysis date.

The phenolic extracts were analysed by High Performance Liquid Chromatography (HPLC). The HPLC system included an LC-20 AT pump, a CTO-20A column oven, and a SPD-M20A prominence diode-array detector, and was equipped with a SIL-20A HT auto sampler (Shimadzu Corp., Kyoto, Japan). The LabSolutions LC (Shimadzu) software was used for collecting and processing the data, obtained through reading at 273 and 370 nm. An aliquot (20 µL) of each extract was filtered through a 0.45 µL nylon filter (Millipore Corp, Billerica, USA) before injection Chromatographic separations were performed on an Inertsil®, ODS-3V column (250 mm × 4.6 mm i.d., 5 µm particle size) (GL Sciences, Tokyo, Japan). Column temperature was 40 °C. The mobile phases were (A) acetic acid / water (2:98, v/v), (B) 50% aqueous acetonitrile / 0.5% aqueous acetic acid (1:1, v/v) and (C) acetonitrile, delivered at a flow of 1.2 mL min^{-1} according to a gradient programme as described in *table I*. The total running time per sample was 61 min. Individual phenolic acids (chlorogenic acid, caffeic acid, syringic acid, *o*-coumaric acid, *p*-coumaric acid) and flavonoids (cathechin, myricetin, quercetin, rutin) were quantified from regression curves calculated for authentic standards purchased from Sigma-Aldrich (Steinheim, Germany). All the calibration curves displayed a good linear relationship, with correlation coefficients above 0.999. The identification was carried out based on retention times and UV spectra. Compound concentrations were calculated by comparison of peak areas with those of standards. Concentration data are presented as mg kg^{-1} fresh weight (FW).

Table II. Some physical and chemical quality attributes of black and white mulberry fruit at harvest. Values represent means of three (pH, titratable acidity TA, soluble solid content SSC) or 30 replicates. Mean values followed by a different letter within the same row are significantly different at $P \leqslant 0.05$ (LSD test).

Measured quality attributes	Black mulberry (*Morus nigra* L.)		White mulberry (*Morus alba* L.)	
Weight (g)	3.77	b	4.01	a
Width (mm)	15.61	a	15.32	a
Length (mm)	20.62	b	25.94	a
pH	4.68	b	5.65	a
TA (g malic acid L^{-1})	10.37	a	6.48	b
SSC (°Brix)	18.37	b	20.57	a
SSC/TA ratio	1.77	b	3.17	a

2.4 Statistical analysis

All determinations were done in triplicate. For each species, means were tested for statistical differences among storage periods by analysis of variance, using the SAS System 9.0 software package (SAS Institute, Cary, NC, 2002), followed by the Fisher's least significant difference (LSD) test at $P \leqslant 0.05$.

3 Results and discussion

Some standard quality parameters were determined for black and white mulberries at harvest (*table II*). White mulberries were in average longer and heavier, and less acid as shown by titratable acidity and juice pH values. SSC was also significantly higher in these fruit, thus leading to SSC/TA ratios almost two-fold those in black mulberries. In spite of high sweetness of white mulberries, low acidity causes imbalances in the sweet/sour ratio, which often results in these fruit being perceived as insipid. These indices indicate that fruit were harvested at the usual maturity stage according to the commercial standards in the producing area, and are comparable to the values recorded for mulberry fruit at harvest in other areas of Turkey [4, 9, 16, 17] as well as in other parts of the world [10, 18].

According to the common practice by local families, fruit were kept frozen for several months after harvest. The fate of phenolic compounds throughout this frozen storage was investigated. The evolution of selected phenolic acids is summarised in *figure 3* and *table III*. Data show that the concentrations of chlorogenic, caffeic, syringic, *o*-coumaric and *p*-coumaric acids at harvest were in all cases higher for black than for white mulberries, amounts ranging two- to six-fold those in the latter. The highest content corresponded to chlorogenic acid (35.8 and 5.4 mg kg^{-1} FW in black and white

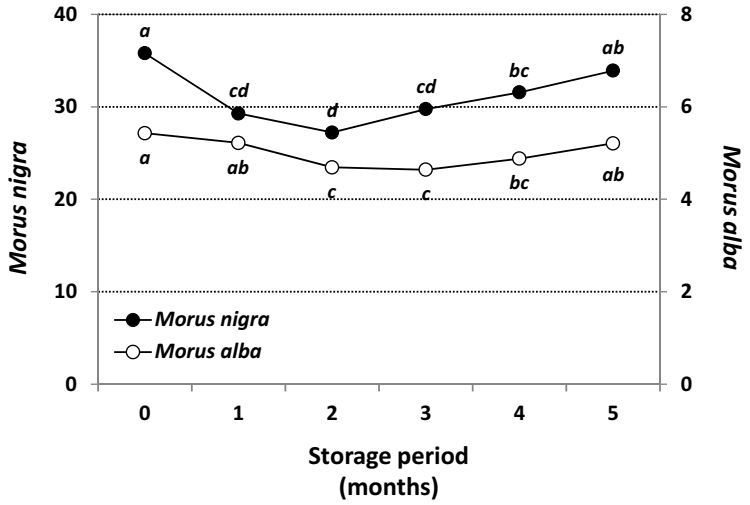

Figure 3. Content of chlorogenic acid (mg kg^{-1} fresh weight) in black (*Morus nigra*) and white (*Morus alba*) mulberry fruit after frozen storage. Values represent means of three replicates. Within each species, different letters represent significant differences along frozen storage $P \leqslant 0.05$ (LSD test).

Table III. Content of selected phenolic acids (mg kg^{-1} FW) in black (*Morus nigra*) and white (*Morus alba*) mulberry fruit after frozen storage at –25 °C. Values represent means of three replicates (ND, non-detectable). Mean values followed by a different letter within the same column are significantly different at $P \leqslant 0.05$ (LSD test).

	Caffeic acid				Syringic acid				*o*-Coumaric acid				*p*-Coumaric acid			
Storage period) (months	*Morus nigra*		*Morus alba*		*Morus nigra*		*Morus alba*		*Morus nigra*		*Morus alba*		*Morus nigra*		*Morus alba*	
0	8.79	a	1.45	a	4.49	a	2.30	b	2.49	a	0.59	bc	0.56	b	ND	
1	6.72	c	1.44	a	3.38	c	2.38	b	1.87	c	0.61	b	0.47	c	ND	
2	7.55	b	1.36	a	4.21	b	1.48	d	1.57	d	0.48	d	0.62	a	ND	
3	7.18	bc	1.42	a	3.42	c	1.63	cd	1.89	c	0.56	c	0.43	cd	ND	
4	6.51	c	1.43	a	3.50	c	1.89	c	1.70	cd	0.55	c	0.37	de	ND	
5	7.18	bc	1.38	a	3.09	d	2.77	a	2.09	b	0.71	a	0.35	e	ND	

mulberries, respectively) (*figure 3*), while those of *p*-coumaric acid were very low (0.56 mg kg^{-1} FW) in black mulberries, and non-detectable in white fruit (*table III*). Chlorogenic acid was also found to be a major phenolic acid in both black and white mulberries [6, 11], whereas *p*-coumaric acid was not detectable in white mulberries regardless of the extraction method employed. However, some quantitative differences with those previous reports were found in this study; for instance, chlorogenic acid concentration was higher in white than in black fruit, while the opposite was observed herein (*table III*), in accordance with reports that the contents of total phenolics and flavonoids were higher in black mulberries [4]. Genotypic characteristics or climatic conditions may underlie these differences, as phenolic-synthesising metabolic pathways are highly responsive to internal and external factors.

In this work frozen storage at –25 °C significantly affected the contents of phenolic acids in both black and white mulberry fruit. Generally speaking, concentrations of phenolic acids were lower in frozen fruit in comparison with freshly harvested samples. However, some fluctuations were observed throughout frozen storage. As an example, the content of chlorogenic acid decreased significantly during the first 3 months of frozen storage in both black and white mulberry fruit followed by a late increase up to the end of the experimental period to similar levels to those at harvest (*figure 3*). Although also displaying some fluctuations along storage, caffeic acid content in black mulberries was lower than that at harvest at all analysis dates, while no significant time-course differences were observed for white mulberries (*table III*). For syringic and *o*-coumaric acids, different trends were observed for each mulberry species: whereas their concentrations decreased significantly in black mulberry with respect to harvest, the opposite was found for white mulberry (*table III*). The content of *p*-coumaric acid in black mulberry showed a transient increase after two months of frozen storage, but declined thereafter to levels well below those at harvest.

The content of selected flavonoids in black and white mulberry fruit after frozen storage at −25 °C was also determined. Similar cathechin levels were found at harvest for both mulberry species considered (*table IV*). However, the time-course evolution was different in each case: while levels decreased significantly during storage of black mulberry, no significant changes were observed for white fruit, with the exception of a transient decrease after the first month. Similarly, although myricetin levels were similar at harvest for both species, their evolution along frozen storage was dissimilar, with declining trends for black mulberries, and a transitory increase after two months for white mulberry samples. Contrarily, and in spite

Table IV. Content of selected flavonoids (mg kg^{-1} FW) in black (*Morus nigra*) and white (*Morus alba*) mulberry fruit after frozen storage at –25 °C. Values represent means of three replicates (ND, non-detectable). Mean values followed by a different letter within the same column are significantly different at $P \leqslant 0.05$ (LSD test).

	Cathechin				Myricetin				Quercetin				Rutin			
Storage period (months)	*Morus nigra*		*Morus alba*		*Morus nigra*		*Morus alba*		*Morus nigra*		*Morus alba*		*Morus nigra*		*Morus alba*	
0	14.02	a	12.85	a	0.64	a	0.41	cd	5.34	ab	2.99	a	95.01	a	ND	
1	11.37	bc	11.58	b	0.43	c	0.37	d	5.18	ab	2.64	b	68.52	d	ND	
2	12.42	ab	12.50	a	0.55	b	0.58	a	5.48	a	2.30	d	78.72	c	ND	
3	12.70	ab	13.00	a	0.52	b	0.51	ab	5.17	ab	2.45	c	88.63	b	ND	
4	8.77	d	12.26	ab	0.37	c	0.36	d	5.39	a	2.65	b	77.21	c	ND	
5	9.64	cd	12.82	a	0.41	c	0.49	bc	5.00	b	2.30	d	86.12	b	ND	

of some fluctuations, quercetin levels in black mulberry fruit were statistically similar at harvest and at the end of the experimental period, whereas they decreased in white mulberries (*table IV*).

Anthocyanins are an important class of bioactive compounds. Quercetin-3-O-rutinoside (rutin) has been found to be particularly potent in this regard, with strong anticlotting activity that allegedly helps preventing heart attacks and strokes [19]. Additional attributed benefits of anthocyanins on human health include anti-inflammatory and chemoprotective properties that reduce the risk of cardiovascular diseases, cancer or cerebral damage. Early descriptions indicated that *Morus nigra* fruit contained uniquely cyanidin-3-O-glucoside, while *Morus alba* contained a complex anthocyanin pattern [20]. More recently, it was reported that mulberry anthocyanins are mainly cyanidin-based, the major types being cyanidin-3-glucoside and cyanidin-3-rutinoside [13]. Yet rutin was found to be a predominant flavonoid in black mulberry in this study (*table IV*), levels at harvest amounting to 95 mg kg^{-1} FW, while no detectable content was observed in white mulberries, in accordance with previous work [7]. Rutin was also found recently to be a a prominent phenolic compound in *Morus nigra* fruit [6], although in that work substantial amounts were detected as well for *Morus alba* samples. Frozen storage caused significant decreases in rutin content in black mulberries regardless of storage period (*table IV*), even though some fluctuations were also observed.

To the best of our knowledge, there have been no published studies on the evolution of individual phenolics during frozen storage of mulberry fruit. Therefore, results are discussed in comparison with other small fruits. Data indicate that all the compounds considered in this work decreased to different extents during frozen storage of black mulberry samples. In a similar study on wild blackberry (*Rubus ulmifolius* Schott), it was found that total anthocyanins and phenolics decreased after 6 months of frozen storage at –24 °C [21]. Significant anthocyanin loss also occurred in blueberry (*Vaccinium corymbosum* L.) fruit after 6 months of frozen storage at –18 °C [22] which was attributed to oxidation and/or condensation reactions with other phenolic compounds. Häkkinen [23] reported that the effects of frozen storage for up to 9 months on the content of flavonols and phenolic acids varied for different berries. For lingonberry (*Vaccinium vitis-idaea* L.) and bilberry (*Vaccinium myrtillus* L.) fruit, it was found that myricetin levels decreased by 30% and 25%, respectively. In contrast, no significant effects on total phenolics were found after frozen storage of raspberries (*Rubus idaeus* L.) at −20 °C [24, 25]. These examples illustrate this wide variation across species, and agree with the contrasting data for black and white mulberries regarding the evolution of particular compounds along frozen storage, specifically syringic and *o*-coumaric acids (*table III*). Myricetin levels in raspberries [23], and total anthocyanins in myrtle berries (*Myrtus communis* L.) [26] were likewise reportedly higher after 6 months of frozen storage. It was accordingly suggested that storage at –20 °C to –35 °C might provide better anthocyanin retention over extended frozen storage of raspberries [27].

For both considered mulberry species, fluctuations in the content of the selected phenolics were observed throughout the experimental time. These fluctuations may be explained by the high reactivity of these molecules. For instance, cellular disruption caused by thawing of the fruit prior to extraction and analysis may increase extraction efficiency [24], or total phenolic content may be enhanced through the formation of antioxidant Maillard reaction products [28], through peroxidase-catalysed oxidations, or through continued production of these compounds [22, 29, 30].

4 Conclusion

In this work, the content of all the investigated phenolic compounds was higher in black than in white mulberries at harvest. Generally speaking, frozen storage at –25 °C significantly affected the levels of these compounds, but dissimilar trends were observed for each species. In both black and white mulberry fruits, fluctuations in the concentration of the selected phenolics were observed throughout the experimental period, which may be attributed to their broad reactivity. However, although some decrease was found in most cases in comparison with values at harvest, the most prominent phenolics (particularly chlorogenic acid and rutin in black mulberry) retained acceptable levels after 5 months of frozen storage.

Since this work was formulated as a preliminary exploration of the effects of the countryside practice of freezing mulberries on the contents of particular phenolics, no additional anthocyanins were investigated. Given the strong effects on human health attributed to these compounds, and the finding that rutin content declined to some extent throughout storage (*table IV*), a detailed analysis of the anthocyanin profile in black mulberry as affected by storage conditions appears advisable.

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