Phenolic Content of Strawberry Spreads during Processing and Storage

Mirta Kadivec, Špela Može Bornšek, Tomaž Polak,* Lea Demšar, Janez Hribar, and Tomaž Požrl

Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

ABSTRACT: This study provides a comprehensive and systematic evaluation of phenolics in strawberry spreads processed according to different industrial procedures and stored under several storage conditions for up to 19 weeks. Total phenolics were determined spectrophotometrically, and individual phenolics were determined by combined liquid chromatography and mass spectrometry: six anthocyanins, four phenolic acids, two flavonols, one flavanol, and one flavone. During storage, the phenolics were modified. The total anthocyanins, vanillic acid, kaempferol, and luteolin decreased, while salicylic and gallic acids increased. Total phenolics, cyanidin 3-(6″-succinyl-glucoside) (here observed for the first time), protocatechuic acid, quercetin, and catechin remained stable. The best phenolic retention was observed in spreads stored at 4 °C. Therefore, the proposed storage process (use of a cold chain) indicates good retention of phenolics in strawberry spreads, which maintain high nutritional and sensorial quality.

KEYWORDS: *strawberry spread, storage, phenolics, anthocyanins, phenolic acids, flavanols, flavonols, flavones, liquid chromatography−mass spectrometry*

■ INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is widely consumed, both as fresh fruit and as an ingredient in processed foods, such as spreads, jams, syrups, alcoholic and nonalcoholic beverages, teas, sweets, and dairy products. Strawberry fruit are a very rich source of bioactive compounds, which include vitamins C and E, and phenolic compounds.[1,2] The beneficial effects of strawberry fruit on human health have been attributed to their high levels of a wide variety of phytochemicals, of which the phenolics constitute the greatest proportion, and they contribute to both the sensorial attributes and the nutritional value of strawberry fruit.[3] Furthermore, several studies have shown that strawberry fruit generally have high levels of antioxidant capacity, which is again linked to the levels of phenolic compounds in the fruit.[1,2,4,5]

Phenolic compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in the plant kingdom. The major phenolics in strawberry fruit have been described as the 3-glucosides of pelargonidin and cyanidin (anthocyanins), followed by quercetin and kaempferol (flavonols), catechin (flavanols), hydrolyzable tannins (ellagitannins and gallotannins), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and condensed tannins (proanthocyanidins). The nutritional contributions and phytochemical compositions of the strawberry have been extensively reviewed very recently.[3]

The first characteristic of any food that is noted is its color, and this predetermines the expectation of both flavor and quality. The color of strawberry fruit is a consequence of the presence of anthocyanins that belong to the flavonoid category. They are widespread, water-soluble plant pigments that produce red, blue and purple colors in fruit and flowers.[6] In strawberries, the anthocyanins are the best known and quantitatively the most important phenolics.[3] Despite their low bioavailability, anthocyanins have intracellular antioxidant activity if applied at very low concentrations (nM range), thereby providing a long-sought

rationale for their health-protecting effects, in spite of their unfavorable pharmacokinetic properties.[7] Their color is pH dependent, red at pH <2 and changing to blue and finally colorless as the pH increases.[3] Chemically they are glycosylated anthocyanidins with the hydroxy and methoxy substitution of ring B. The hydroxylation pattern can directly affect color hue and their stability. Thus, the anthocyanins with more hydroxyl groups can contribute more blue, whereas the degree of methylation can increase the red.[8]

However, anthocyanins are known to be unstable compounds. During food processing and storage, they are subjected to numerous chemical and enzymatic reactions that can lead to their loss or to the modification of their chemical form. The mechanisms of these reactions are very complex and are still not totally explored, especially in food matrices. It is known that anthocyanins can be polymerized or degraded. With other phenolics, as with the phenolic acids, the flavonols, procyanidins and anthocyanins have been shown to form colored polymeric compounds. Depending on enzyme activities (e.g., polyphenol oxidase) and physical and chemical factors (temperature, light, pH, oxygen, ascorbic acid, sugars, protein, sulfites, metal ions, anthocyanin concentration), these can be degraded to their corresponding phenolic acids and aldehydes.[9−11] For example, to prevent discoloration, ascorbic acid is commonly added to fruit products, such as spreads, jams and juices. This ascorbic acid has been shown to have protective effects on the anthocyanins, particularly against condensation reactions.[9]

In recent years, the increasing demand for dietary compounds with functional properties and considerable potential beneficial

effects on human health, such as phenolics,[12−14] has focused interest on fruit as a natural source of these compounds. Many consumers expect products with a number of positive nutritional characteristics and low caloric intake. Such products are fruit spreads with a high proportion of fruit, in which the fruit is equally subjected to longer thermal treatment and storage. Thus, phenolics from fruit are subjected to some changes that will have an influence on their content and composition.[12,15] Some questions remain open as to whether spreads can still be good sources of these bioactive compounds, what kind of changes they might undergo during the manufacture and storage, and what is the most appropriate spread procedure to keep the phenolics well preserved.

Thus, the aim of our study was to follow the phenolic contents in strawberry spreads through processing and storage. We processed strawberry spreads according to different industrial procedures and stored them under several storage conditions for up to 19 weeks. The total phenolics were determined spectrophotometrically, with the individual phenolics of anthocyanins, phenolic acids, flavonols, flavanols and flavones determined by liquid chromatography (LC)−tandem mass spectrometry (MS/MS) and LC−MS. The results obtained were analyzed statistically.

■ MATERIALS AND METHODS

Chemicals and Reagents. Methanol and formic acid were from Merck (Darmstadt, Germany) and acetonitrile from J.T. Baker (Deventer, The Netherlands). All of the solvents used were of HPLC purity. Standards: pelargonidin 3-glucoside was from Extrasynthese (Genay Cedex, France), protocatechuic acid, vanillic acid, salicylic acid, gallic acid, quercetin, kaempferol, catechin and luteolin were from Sigma Aldrich (Steinheim, Germany). Aqueous solutions were prepared using Milli-Q water (Millipore, Bedford, MA).

Ingredients and Spread Preparation. Ascorbic acid was from Shandgong Luwei Pharmaceutical Co. (Zibo, China), citric acid from Anhui BBCA Biochemical Co. (Bengbu, China), pectin from Danisco (Copenhagen, Denmark), cherry red natural colorant from Etol (Celje, Slovenia) and sugar (sucrose) from Mercator (Ljubljana, Slovenia). Strawberries (*Fragaria × ananassa* Duch.) of cultivar Senga Sengana were cultivated by Bosnaplod (Brčko, Bosnia and Herzegovina). They were kept frozen at −20 °C for about 4 months before they were purchased in a local store (Kamnik, Slovenia); they were then stored at −20 °C until further use. The spreads were prepared according to an industrial process system.

Processing and Storage Conditions. Seven different spread varieties were investigated, with all carried out as six repetitions. As listed in Table 1, these were the spread varieties of: A, control; B, pectin supplementation; C, sugar supplementation; D, storage at 4 °C; E, storage under N_2; F, natural colorant supplementation; and G, storage in daylight. Before spread preparation, the strawberries were thawed in a microwave oven for 3 min. The thawed fruit material (800 g) was

blended in a UMC5 Electronic mechanical blender (Stephan Machinery, Hameln, Germany) for 90 s (20 s at 300 rpm, 20 s at 600 rpm, 20 s at 900 rpm, and finally 30 s at 1200 rpm). During the last period, sugar (sucrose) (285 or 385 g for variety C), citric acid (3.5 g) and ascorbic acid (1.5 g) were added gradually, followed by an additional 15 s of blending. The prepared material was then heated to 95 °C under vacuum for 13 min, while stirring for 10 s at 300 rpm every 2 min. After that, the spread was heated for another 10 min, without vacuum. The pulp was stirred for another 10 s and pectin (7 or 14 g) and sugar (15 g) were mixed in, followed by a final 30 s stirring. Then, the spreads were hot-filled into 150 g glass jars immediately. The total soluble content (°Brix) of the fresh spreads was also determined, using an automatic digital refractometer RX-5000CX (Atago, Tokyo, Japan) (Table 1).

Extraction. The extraction method was as reported previously.[16] Briefly, 5 g of each spread was homogenized in 15 mL of ice-cold deoxygenated methanol. The homogenates were extracted for 1.5 h by shaking at room temperature and then centrifuged at 1700*g* for 10 min. Finally, the supernatants were pooled and stored at −20 °C until analysis.

Total Phenolics Determination. Total phenolics were determined by the Folin−Ciocalteu method. Briefly, 250 μL of Folin−Ciocalteu reagent was added to 300 μL of 10-fold diluted extract, 1 mL of 10% Na_2CO_3 and 3.45 mL of Milli-Q water. After 60 min at room temperature, the samples were filtered to 0.45 μm and the absorbance of the solution was measured at 700 nm, against a blank prepared with methanol instead of the extract. The total phenolic contents were expressed as gallic acid equivalents, according to a linear calibration curve, with a correlation coefficient of 0.9849.

Solid Phase Extraction. The extracts were cleaned up as follows: each 2 mL extract was dried in a rotating vacuum evaporator, then resuspended in 3 mL of Milli-Q water, loaded onto a 100 mg of Strata-X cartridge (Phenomenex, Torrance, CA) that had previously been conditioned with 3 mL of pure methanol and 3 mL of Milli-Q water. After washing the cartridges with 3 mL of 15% methanol, the retained phenolic fractions were eluted with 3 mL of pure methanol. The eluates were evaporated and the dried samples were resuspended in 1 mL of 3% formic acid and methanol (97:3; v:v).

HPLC Analyses. The individual phenolics (anthocyanins, phenolic acids, flavonols, flavanols, flavones) were determined by LC−MS using a method described previously.[16] The HPLC−MS system consisted of an Agilent 1100 binary pump (G1312A) and an autosampler (G1330B) coupled to a Micromass Quattro Micro mass spectrometer equipped with an electrospray ionizer source (Waters, Milford, MA). Reversed-phase HPLC separation was carried out using a 150 × 2.00 mm i.d., 3 μm, Gemini C18 column, protected by a 4.0 × 2.0 mm i.d. Gemini C18 Security Guard cartridge (Phenomenex, Torrance, CA). All of the compounds were identified and quantitated on the basis of their retention times, MS spectra, and molecular-ion identification.

LC−MS/MS Determination of Anthocyanins. The mass spectrometer was operated in positive-ion mode with the following operating parameters: capillary voltage, 3.0 kV; cone voltage, 20 V; extractor, 5 V. The source temperature was 100 °C and the desolvation temperature was 350 °C. The cone gas flow was set at the 40 L/h, the desolvation gas flow at 400 L/h, and the collision energy at 30 V.

Table 1. Supplements and Storage Conditions of Each of the Spread Varieties Used[a]

spread variety	experimental design	soluble solids (°Brix)	sugar (%)	pectin (%)	natural colorant (%)	nitrogen	temp (°C)	darkness	daylight
				supplement			storage condition		
A	control	38	27	0.6	/	/	20	+	/
B	pectin supplementation	38	27	1.2	/	/	20	+	/
C	sugar supplementation	43	33	0.6	/	/	20	+	/
D	storage at 4 °C	38	27	0.6	/	/	4	+	/
E	storage under N_2	38	27	0.6	/	+	20	+	/
F	natural colorant supplementation	38	27	0.6	0.09	/	20	+	/
G	storage in daylight	38	27	0.6	/	/	20	/	+

[a] +, condition used; /, condition not used.

Table 2. Total Phenolic Contents in the Strawberry Spreads during Storage[a]

spread variety	total phenolics (mg gallic acid equiv/100 g spread)				
	0 weeks	6 weeks	12 weeks	16 weeks	19 weeks
A	303.2 ± 42.7	336.2 ± 64.0	347.2 ± 52.5	380.7 ± 53.6	356.6 ± 33.2
B	319.6 ± 64.1	318.7 ± 86.7	336.4 ± 94.4	348.4 ± 24.4	336.3 ± 12.7
C	322.1 ± 36.6	297.6 ± 32.5	338.9 ± 68.1	349.1 ± 36.3	354.9 ± 62.5
D	340.1 ± 72.8	313.8 ± 30.3	309.5 ± 90.6	318.9 ± 54.8	336.3 ± 31.8
E	331.4 ± 50.2	319.6 ± 81.5	361.7 ± 47.9	372.0 ± 60.0	371.5 ± 8.8
F	330.9 ± 73.6	308.1 ± 29.8	337.3 ± 89.4	379.1 ± 42.9	368.2 ± 5.3
G	337.6 ± 35.0	333.9 ± 89.9	339.1 ± 15.9	368.0 ± 43.8	393.1 ± 7.9

[a]Data are expressed as means ± standard deviation ($n = 6$). There were no significant differences between the data within columns or rows. See Table 1 for spread varieties.

The mobile phase components were 3% formic acid (A) and acetonitrile (B). The anthocyanins were separated at 40 °C with the following gradient: 0−2 min, 7−9% B; 2−4 min, 9−11% B; 4−12 min, 11−12% B; 12−13 min, 12% B; 13−25 min, 12−13% B; 25−40 min, 13−100% B. The flow rate of the mobile phase was 0.250 mL/min from 0 to 4 min, 0.225 mL/min from 4 to 13 min, and 0.200 mL/min from 13 to 40 min. The injection volume was 10 μL.

The quantitation of individual anthocyanins was calculated relative to pelargonidin 3-glucoside (the external standard) and determined from the calibration curve of 10 points with spiked spread samples that covered the range from 1 to 80 mg/L; this was linear and had a correlation coefficient of 0.994. The limit of detection and the limit of quantitation were determined in our previous study.[16]

LC−MS Determination of Phenolic Acids, Flavonols, Flavanols and Flavones. The mass spectrometer operated in negative ion mode with the following parameters: capillary voltage, 3.0 kV; cone voltage, 25 V; and extractor voltage, 5 V. The source temperature was 100 °C, the desolvation temperature was 350 °C, the cone gas flow was 50 L/h, and the desolvation gas flow was 400 L/h. The mobile phase components were 1% formic acid (A) and acetonitrile (B). The mobile-phase gradient used was: 0−5 min, 10% B; 5−50 min, 10−60% B; 50−52 min, 60−80% B; 52−60 min, 80% B; 60−70 min, 80−10% B; 70−80 min, 10% B. The injection volume was 20 μL, and the column temperature was 25 °C. The flow rate of the mobile phase was 0.200 mL/min.

The phenolic acids, flavanols, flavonols and flavones were identified on the basis of their retention times, MS spectra, and molecular-ion identification. Quantitation of these compounds was calculated relative to the corresponding external standards from the linear calibration curves of 10 points with spiked spread samples that covered the range from 0.1 to 40 mg/L; these were linear and had correlation coefficients up to 0.9903. These compounds were quantitated according to their corresponding standards: phenolic acids: protocatechuic acid, vanillic acid, salicylic acid and gallic acid; flavonols: quercetin and kaempferol; flavanol catechin and flavone luteolin. The limit of detection and the limit of quantitation were determined in our previous publication.[16]

Statistical Analysis. The experimental data were evaluated statistically using the SAS/STAT program.[17] The basic statistical parameters were calculated by the MEANS procedure. The data were tested for normal distributions and analyzed using the general linear model procedure. The statistical model included the main effects of spread variety and storage time as well as the interactions between variety and storage time. The means for the experimental groups were obtained using the Duncan procedure, and they were compared at the 5% probability level.

RESULTS AND DISCUSSION

Total Phenolics. The total phenolics content of the strawberry spreads were determined by the Folin−Ciocalteu method. The total phenolics obtained were expressed as mg gallic acid equiv/100 g spread (Table 2). Their values in the spreads at time 0 of storage ranged from 303.2 to 340.1 mg gallic acid equiv/100 g, whereas generally, total phenolics content ranged from 297.6 to 393.1 mg gallic acid equiv/100 g. By statistical analyses, the differences between the values were not

Table 3. Mass Spectrometric Data and Retention Times of the Individual Anthocyanins

anthocyanin	M_w[a] (g/mol)	MS/MS (m/z)	retention time (min)
cyanidin 3-glucoside	449	287	11.8
pelargonidin 3-glucoside	433	271	14.0
pelargonidin 3-rutinoside	579	271	16.2
pelargonidin 3-(6″-malonyl glucoside)	519	271	29.9
cyanidin 3-(6″-malonyl glucoside)	535	287	36.4
cyanidin 3-(6″-succinyl glucoside)	550	287	37.0

[a]M_w, molecular mass.
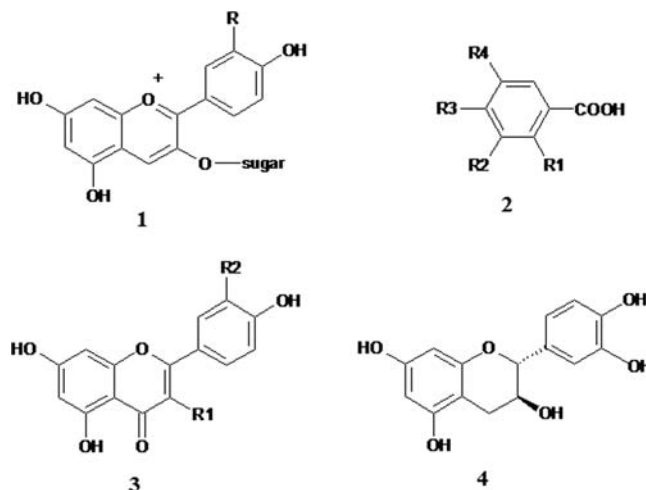
Figure 1. Structural formulas of pelargonidin (R = H) and cyanidin (R = OH) glycosides (**1**); protocatechuic (R1 = R4 = H, R2 = R3 = OH), vanillic (R1 = R4 = H, R2 = OCH₃, R3 = OH), salicylic (R1 = OH, R2 = R3 = R4 = H) and gallic acid (R1 = H, R2 = R3 = R4 = OH) (**2**); quercetin (R1 = R2 = OH), kaempferol (R1 = OH, R2 = H) and luteolin (R1 = H, R2 = OH) (**3**); catechin (**4**).

significant ($P = 0.2921$ to 0.9344). Thus, the total phenolics content was not affected by spread varieties and storage periods. Generally speaking, there were no important differences due to spread variety and storage period. Indeed, according to a previous study,[18] no changes in the total phenolics in strawberry and cherry jams have been observed, even over five months of storage.

Anthocyanins. The individual anthocyanins of the strawberry spreads were determined by LC−MS/MS. The identification was performed by MS according to the m/z values of the anthocyanin positive ions and based on the mass spectra, molecular-ion, anthocyanin retention times, and comparisons with the literature.[19,20]

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Table 4. Effects of Spread Varieties and Storage Times on Anthocyanins Content in the Strawberry Spreads, As Determined by LC−MS/MS[a] (Duncan Test, α = 0.05)

| anthocyanin	storage time (weeks)	anthocyanin concentration in the different spread varieties (mg pelargonidin 3-glucoside equiv/100 g)						
		A	B	C	D	E	F	G
pelargonidin 3- glucoside	0	37.1 ± 10.7Xx	36.3 ± 13.2Xx	27.6 ± 7.3Xx	35.9 ± 6.7Xx	38.7 ± 2.4Xx	41.7 ± 12.6Xx	29.6 ± 14.5Xx
	6	16.9 ± 8.0Yxyz	12.8 ± 9.3Yz	15.5 ± 6.2Yyz	29.8 ± 7.9Xx	18.3 ± 10.4Yxyz	26.6 ± 18.5Xxy	12.0 ± 6.9Yz
	12	4.7 ± 1.9Yy	4.6 ± 2.1Yy	6.6 ± 3.7Zy	29.3 ± 5.7Xx	7.9 ± 6.9Zy	7.7 ± 2.4Yy	4.2 ± 1.2Yy
	16	5.8 ± 5.6Zy	4.2 ± 3.3Yy	5.0 ± 2.5Zy	23.8 ± 7.4Xx	5.1 ± 4.1Zy	4.3 ± 1.5Yy	3.7 ± 1.3Yy
	19	4.0 ± 0.5Zy	2.5 ± 0.5Yy	4.1 ± 1.2Zy	33.4 ± 5.7Xx	4.2 ± 0.4Zy	7.8 ± 3.5Yy	3.6 ± 1.2Yy
cyanidin 3- glucoside	0	1.5 ± 1.0Xxy	1.0 ± 0.9Xxy	0.7 ± 0.4Xy	1.7 ± 0.8Xx	1.7 ± 0.7Xxy	1.5 ± 1.2Xxy	1.0 ± 1.0Xxy
	6	1.0 ± 0.2XYx	0.9 ± 0.1Xx	0.5 ± 0.2XYx	1.4 ± 0.7Xx	1.1 ± 1.2Xx	1.3 ± 1.2Xx	0.4 ± 0.3Xx
	12	0.2 ± 0.0Yy	0.3 ± 0.1Xxy	0.3 ± 0.00XYxy	1.9 ± 1.3Xx	0.3 ± 0.2Yxy	0.3 ± 0.2Yxy	0.1 ± 0.1Xy
	16	nd	0.1 ± 0.1YXy	nd	1.1 ± 0.6Xx	0.2 ± 0.1Yy	0.1 ± 0.1Yy	0.2 ± 0.1Xx
	19	0.1 ± 0.1Yy	nd	0.1 ± 0.1Yy	2.2 ± 0.7Xx	nd	nd	nd
pelargonidin 3-(6″-malonyl glucoside)	0	5.5 ± 2.3Xx	4.4 ± 1.1Xx	3.1 ± 2.3Xx	5.2 ± 1.4Xx	5.4 ± 0.6Xx	5.3 ± 1.6Xx	4.1 ± 1.9Xx
	6	1.5 ± 0.5Yx	1.4 ± 0.6Xx	1.2 ± 0.6XYx	2.7 ± 1.8Yx	2.8 ± 3.3XYx	2.9 ± 2.7XYx	1.2 ± 0.6Yx
	12	0.3 ± 0.1Yx	nd	0.5 ± 0.00XYx	5.0 ± 1.0XYx	0.6 ± 0.4Yx	0.4 ± 0.2Yx	0.2 ± 0.1Yx
	16	0.7 ± 0.8Yx	3.5 ± 5.4Xx	0.1 ± 0.1Yx	2.7 ± 1.1Yx	0.5 ± 0.2Yx	0.4 ± 0.1Yx	0.1 ± 0.1Yx
	19	0.2 ± 0.0Yy	nd	nd	4.0 ± 0.4XYx	nd	nd	nd
cyanidin 3-(6″- malonyl- glucoside)	0	8.2 ± 5.3Xx	10.1 ± 6.2Xx	7.9 ± 4.9Xx	9.2 ± 5.4Xx	7.4 ± 4.5Xx	9.5 ± 5.5Xx	8.5 ± 5.6Xx
	6	7.1 ± 5.2Xx	6.8 ± 5.8XYx	4.7 ± 4.5Xx	9.6 ± 6.4Xx	6.3 ± 4.3Xx	6.7 ± 5.3XYx	4.7 ± 3.7XYx
	12	5.4 ± 2.9Xx	4.1 ± 2.5XYx	3.9 ± 2.2Xx	7.5 ± 5.0Xx	4.5 ± 3.5Xx	3.7 ± 1.3XYx	3.9 ± 3.2XYx
	16	2.8 ± 2.0Xy	4.6 ± 4.3XYy	3.9 ± 3.4Xxy	9.5 ± 8.1Xx	3.6 ± 2.9Xy	3.3 ± 2.3XYy	3.0 ± 1.8XYy
	19	2.1 ± 0.7Xy	1.7 ± 0.4Yy	1.6 ± 0.3Xy	4.9 ± 1.5Xx	1.9 ± 0.4Xy	3.1 ± 0.8Yy	1.5 ± 0.2Yy
cyanidin 3-(6″- succinyl- glucoside)	0	2.4 ± 1.2Xx	1.9 ± 1.0Xx	1.4 ± 0.7Xx	3.2 ± 1.6Xx	3.6 ± 1.2Xx	1.4 ± 1.6Xx	2.5 ± 1.6Xx
	6	3.5 ± 3.1Xx	2.9 ± 2.0Xx	1.5 ± 1.1Xx	2.9 ± 0.7Xx	3.5 ± 2.9Xx	2.7 ± 0.6Xx	2.9 ± 1.9Xx
	12	4.0 ± 2.0Xx	3.5 ± 3.0Xx	2.0 ± 1.3Xx	3.9 ± 2.7Xx	1.8 ± 1.1Xx	3.1 ± 1.1Xx	2.1 ± 1.4Xx
	16	2.0 ± 1.4Xx	2.3 ± 0.5Xx	1.9 ± 1.6Xx	3.4 ± 2.3Xx	1.9 ± 1.4Xx	3.2 ± 1.2Xx	2.5 ± 2.5Xx
	19	3.6 ± 1.1Xx	3.2 ± 1.9Xx	1.7 ± 0.6Xx	3.7 ± 1.5Xx	2.1 ± 0.8Xx	3.1 ± 1.9Xx	1.8 ± 0.2Xx
pelargonidin 3-rutinoside	0	1.0 ± 0.6Xx	1.0 ± 0.8Xx	0.6 ± 0.5Xx	1.3 ± 0.1Xx	1.2 ± 0.6Xx	1.8 ± 0.5Xx	0.8 ± 0.7Xx
	6	0.8 ± 0.3Xx	0.8 ± 0.4Xx	0.5 ± 0.3Xx	1.2 ± 0.2Xx	1.4 ± 1.8Xx	1.2 ± 0.6XYx	0.6 ± 0.0Xx
	12	nd	0.2 ± 0.0Xx	nd	1.4 ± 1.1Xx	0.6 ± 0.5Xx	0.3 ± 0.2YZx	0.2 ± 0.1Xx
	16	0.4 ± 0.3Xy	0.3 ± 0.2Xy	0.2 ± 0.1Xy	1.0 ± 0.3Xx	0.4 ± 0.3Xy	0.2 ± 0.0Zy	nd
	19	nd	nd	nd	1.4 ± 0.3Xx	nd	nd	nd
totals	0	55.8 ± 14.6Xx	54.7 ± 15.3Xx	41.2 ± 13.1Xx	56.4 ± 6.1Xx	58.0 ± 4.2Xx	60.9 ± 12.0Xx	46.4 ± 16.9Xx
	6	30.9 ± 6.7Yxy	25.7 ± 11.8Yy	23.8 ± 8.9Yy	47.9 ± 13.5Xx	33.3 ± 7.5Yxy	41.4 ± 20.0Xx	21.9 ± 9.3Yy
	12	14.5 ± 1.9Zy	12.8 ± 4.4Yy	13.3 ± 4.0YZy	45.3 ± 15.3Xx	15.7 ± 6.9Zy	15.8 ± 4.2Yy	10.8 ± 3.8Yy
	16	11.8 ± 5.6Zy	15.2 ± 5.5Yy	11.2 ± 2.7YZy	41.4 ± 8.6Xx	11.7 ± 2.6Zy	11.4 ± 3.3Yy	9.6 ± 2.5Yy
	19	10.1 ± 2.3Zyz	7.4 ± 2.8Yyz	7.5 ± 0.4Zz	49.6 ± 1.3Xx	8.2 ± 0.8Zyz	14.1 ± 6.9Yy	7.0 ± 1.6Yz

[a]Data are expressed as means ± standard deviation (n = 6). Contents of anthocyanins (mg/100 g) in strawberry spreads were quantitated as equivalents of pelargonidin 3-glucoside. Statistically significant differences are marked with upper case letters (X, Y, Z) within each column (for each anthocyanin), and with lower case letters (x, y, z) within the same row. See Table 1 for spread varieties. nd; not detected.

Fragmentation ions m/z 271 were identified as a derivative of pelargonidin, with m/z 287 as a derivative of cyanidin. The chromatographic retention times of individual anthocyanins are given in Table 3, and are in agreement with previous findings.[19,20] The quantitation is expressed as mg pelargonidin 3-glucoside equivalents per 100 g spread and is given in Table 4.

In all of the strawberry spreads, we analyzed the levels of six individual anthocyanin glycosides belonging to cyanidin and pelargonidin anthocyanidins. Structural formulas of individual anthocyanins (1) are presented in Figure 1 while the LC−MS/MS chromatograms are presented in Figure 2. The predominant anthocyanin in the freshly prepared strawberry spreads (at time 0 of storage) was pelargonidin 3-glucoside (67%), with the others at lower relative levels: cyanidin and pelargonidin 3-(6″-malonyl-glucosides) (16 and 9%, respectively), cyanidin 3-(6″-succinyl-glucoside) (4%), cyanidin 3-glucoside (2%), and pelargonidin 3-rutinoside (2%). A similar anthocyanin profile has been reported for fresh strawberries.[1,20] During the storage,

nearly all of the individual anthocyanins in the spreads decreased, with almost the same trend, and with pelargonidin 3-(6″-malonyl-glucoside) and pelargonidin 3-rutinoside becoming undetectable; only cyanidin 3-(6″-succinyl glucoside) remained stable (P = 0.1111−0.8689). Thus, for the first time, we have observed here that an individual anthocyanin can remain unchanged during storage.

The sum of the anthocyanins in the freshly prepared spreads (at time 0 of storage) ranged from 41.2 to 60.9 mg pelargonidin 3-glucoside equiv/100 g (Table 4). During the storage, the sum of the anthocyanins was greatly reduced, which is not surprising as the anthocyanins are known to be very unstable compounds.[11] Our data show that the anthocyanin concentrations were halved in only six weeks. Considering that the shelf life of strawberry spreads lasts from six months to one year, this rapid anthocyanin decay will lead to deterioration in the nutritional and consequently sensorial quality of the spreads. According to our unpublished data, we have also observed significant sensorial
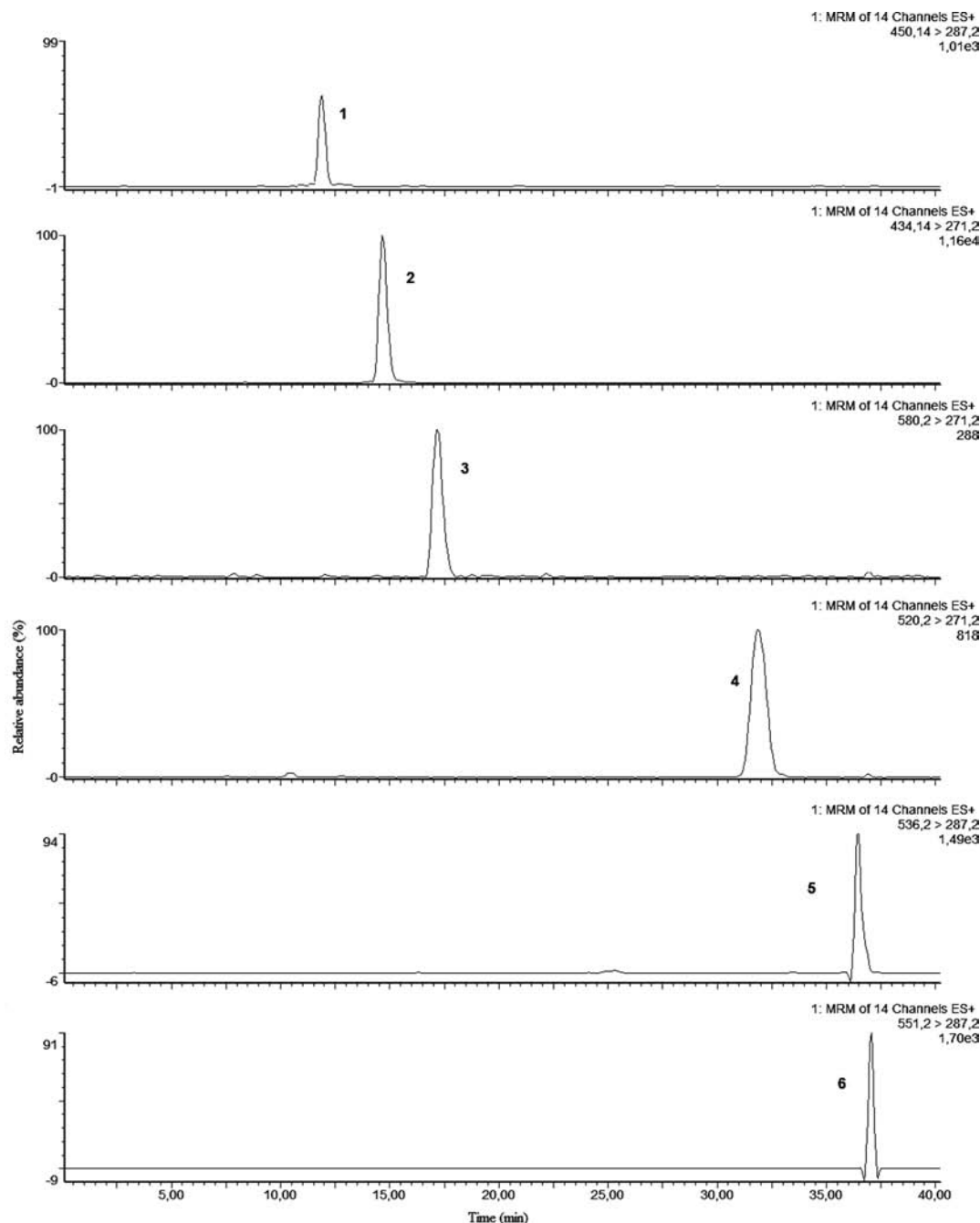
Figure 2. LC−MS/MS chromatograms of individual anthocyanins: cyanidin 3-glucoside (**1**), pelargonidin 3-glucoside (**2**), pelargonidin 3-rutinoside (**3**), pelargonidin 3-(6″-malonyl glucoside) (**4**), cyanidin 3-(6″-malonyl glucoside) (**5**), and cyanidin 3-(6″-succinyl glucoside) (**6**).

quality loss of spreads during storage, with the color intensity significantly decreased as an important effect of anthocyanin decay. However, it has already been reported that during storage differences in the stabilities between individual anthocyanins are not observed (i.e., cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside). Their loss proportions were similar even after one year of storage in a freezer.[15] From our results, after 19 weeks of storage, the anthocyanins significantly declined by 77−87% for the spread varieties of A (control), B (pectin supplementation), C (sugar supplementation), E (storage in N_2), G (storage in daylight) and F (natural colorant supplementation). Only a 12% decrease was seen for variety D (storage at 4 °C), and the statistical analysis showed that this decay was not significant ($P = 0.1622$).

However, the supplementation of pectin in the strawberry spreads did not have any protective effects on anthocyanin stability during the storage of the spreads, although it was doubled to 1.2% (variety B), in comparison to the control of 0.6% (variety A). Contrary to this, a recently published study showed that addition of pectin markedly enhances anthocyanin stability in viscous model solutions,[21] presumably because pectin can strongly lower water activity.[22] It has also been reported that during storage, the anthocyanin stability in spreads and jams depends on the pectin type[23] and also its source. The anthocyanin stability was better in a pectic model system that contained citrus pectin, compared to that of apple and sugar beet pectins.[21]

Sugars are known to decrease anthocyanin stability.[9] Our data also show that the addition of a greater quantity of sugar (sucrose)
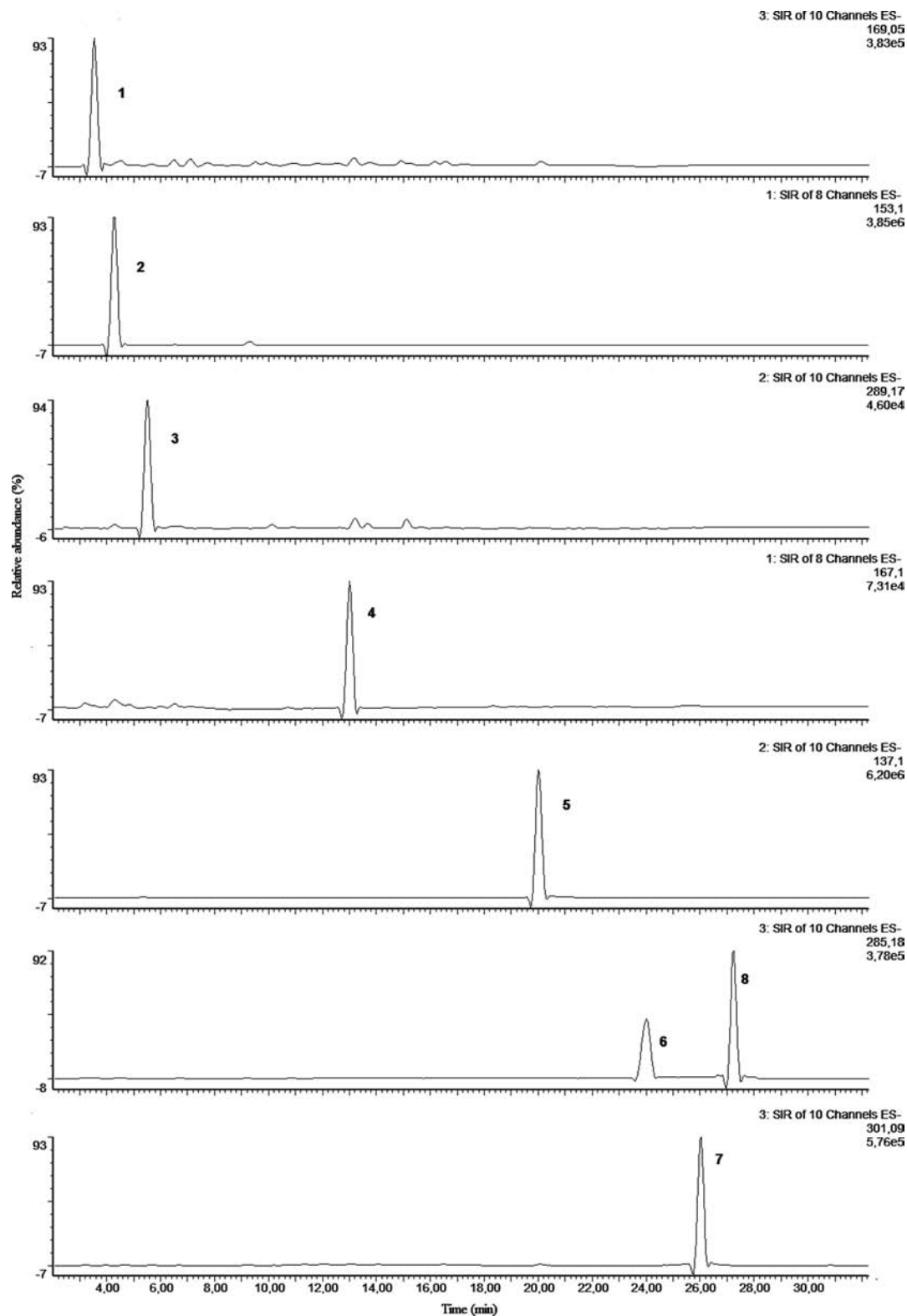
Figure 3. LC−MS chromatograms of gallic acid (**1**), protocatechuic acid (**2**), catechin (**3**), vanillic acid (**4**), salicylic acid (**5**), luteolin (**6**), quercetin (**7**), and kaempferol (**8**).

does not prevent anthocyanin loss during the storage of the spreads, even the content of soluble solids significantly increased from 38 to 43 °Brix. This observation is in agreement with a previous study[24] that showed no protective effects on anthocyanins when the sugar concentration was higher than 20%. In our study, both of the concentrations compared, as 43% (variety C) and 38%

(variety A), were higher than the 20% reported previously. For the addition of other sugar types, including sucrose, glucose and fructose, no different effects on anthocyanin stability were seen previously.[25] However, some previous studies have shown better pigment stability with higher concentrations of sugar during jam storage, presumably due to lower water activities.[22]

The anthocyanin stability depends on the temperature of the storage of the spread. High temperatures have been shown to cause large anthocyanin losses.[9] In the same way, lowering the temperature in the present study (i.e., variety D, for storage at 4 °C) resulted in the best anthocyanin retention, and also for color stability (our unpublished data). As reported by others,[26] the color and antioxidant properties of strawberry jams stored at 4 °C are significantly better than those stored at 20 °C. Therefore, storage in the fridge appears to be suitable to help to conserve the anthocyanins in the spread. Probably, the low temperature slows the degradation processes of the anthocyanin molecules.

The presence of oxygen can have detrimental effects on anthocyanins because they undergo degradation through direct oxidation or through the enzymatic reactions of polyphenol oxidase.[9,11] In the present study, the enzymatic degradation of the anthocyanins will not be possible due to the high temperatures during the spread processing, which will have inactivated all of the enzymes. From our data, it can also be seen that after storage of the spread in the presence of nitrogen, the anthocyanin decrease was significant ($P < 0.0001$). Also, after 19 weeks of storage, there were no major differences detected in the anthocyanin contents between the spreads treated with nitrogen (variety E) and without (variety A). Therefore, an absence of the oxygen does not prevent anthocyanin degradation, and so use of an inert atmosphere does not appear to be an appropriate way for storing strawberry spreads.

The anthocyanin decline for variety F (supplementation with 0.09% natural colorant) was similar to the control (variety A). Thus, the addition of the natural colorant, which was a concentrate of blackcurrant and hibiscus that contained anthocyanins, did not influence their decay during storage. Conversely, some studies have reported positive effects on anthocyanins with the addition of natural colorants. It has been reported that addition of anthocyanins increases their own concentrations, as this appears to promote higher anthocyanin stability, and hence color stability, which is known as the concentration effect.[9] With the addition of anthocyanins (e.g., black carrot and elderberry juice concentrate), canned strawberries maintained a suitable color after weeks of storage at 16 to 18 °C.[27] Anthocyanins from berries have also been suggested as appropriate colorants for food rich in fats (e.g., yogurt).[14]

Anthocyanins have been reported to be relatively sensitive to natural light, because this accelerates their degradation.[9] From our data, the anthocyanin loss in the spreads stored in the daylight (variety G) was significant. However, after 19 weeks of storage, no differences were seen between the anthocyanin in the spreads stored in the daylight (variety G) and those stored in the dark (variety A). This is in good agreement with other reports that have shown similar decay of anthocyanins in strawberry jams and spreads stored in the dark and under illumination.[23] Thus, it can be assumed that storage in the dark or in daylight does not promote any differences in the anthocyanin levels in strawberry spreads.

Phenolic Acids, Flavonols, Flavanol and Flavones. Using LC−MS analytical techniques, the phenolic acids (protecatechuic, vanillic, salicylic, gallic acid), flavonols (quercetin, kaempferol), flavanol (catechin) and flavones (luteolin) were determined. Their structural formulas (**2**, **3** and **4**) are presented in Figure 1 while LC−MS chromatograms are presented in Figure 3. The identification was performed by MS according to the m/z of their negative ions, and based on the mass spectra, molecular-ion, retention times, and comparison with the literature.[16,28,29]

Table 5. Retention Times of the Individual Phenolic Acids and Flavonols and of Flavanol and Flavone

compound group	compound	$M_w{}^a$ (g/mol)	retention time (min)
phenolic acids	protocatechuic acid	154	4.7
	vanillic acid	168	13.0
	salicylic acid	138	20.7
	gallic acid	170	3.0
flavonols	quercetin	302	26.3
	kaempferol	286	27.8
flavanol	catechin	290	5.7
flavone	luteolin	286	24.2

$^a M_w$, molecular mass.

These findings agree with our data (chromatographic retentions and molecular weight), as demonstrated in Table 5. The contents were expressed as mg equivalents of the corresponding standard compounds per 100 g of the spreads (Table 6).

Phenolic acids, flavonols, flavanols and flavones are known to be relatively unstable. Under processing and storage they can readily undergo numerous reactions and modifications.[30] From our data, the content of phenolic acids, flavonols, flavanols and flavones in the freshly prepared strawberry spreads (at time 0 of storage) were apparently lower than the anthocyanins, although their occurrence and changes during the storage period were not negligible. Some of them decreased, and some increased or remained stable. After 19 weeks of storage, there was an obvious decline in luteolin (99%), vanillic acid (79%) and kaempferol (73%) in all of the spread varieties, except in variety D (storage at 4 °C), where the decline was relatively low (77, 54, 51%, respectively). An increase in gallic acid (2-fold to 6-fold) and salicylic acid (1-fold to 2-fold) was also noted in almost all of the spread varieties, although these two phenolic acids were stable in the spreads stored at 4 °C (variety D), as shown by the statistical analysis. The contents of protocatechuic acid and quercetin did not change during the storage period ($P = 0.0729 − 0.7101$).

To date, there have been very few publications on this topic. Previous investigations into the influence of processing and storage on the phenolics content in foods have been reviewed recently.[31] Thus, storage has been seen to modify the content of phenolic acids, with both losses and increases in these compounds. In our unpublished data, the phenolic acids rose significantly during industrial and homemade processing of bilberry spread. Changes in the storage temperature were the major factor that contributes to the maintenance of constant levels of the phenolic acids, which is also evident from our data here. The opposite varying trends of individual phenolic acids have already been noted by others.[32] They observed that over six months of storage at different temperature, the content of caffeic acid in orange juice significantly decreased, while the occurrence of p-coumaric and ferulic acids increased. The increase could result from the degradation of anthocynins[9,10] and/or the release of free acids from their bound forms.[32,33] Also, different effects of storage time and temperature were shown on different fruit for flavanol levels;[31] in general, higher temperatures decrease their content. However, the influence of food storage on the flavonol content of foods was summarized as being ambiguous: increases, decreases and no changes have been reported. Similar conclusions are also seen from our data. However, from our observations we can conclude that the storage at 4 °C (variety D) shows the lowest changes in the contents of the phenolic acids and flavonols that were determined in the present study.

Table 6. Effects of Spread Variety and Storage Time on Contents of Individual Phenolic Acids and Flavonols and of Flavanol and Flavone in the Strawberry Spreads, As Determined by LC–MS[a] (Duncan Test, α = 0.05)

compounds	storage time (weeks)	concentrations in the different spread varieties (mg equiv/100 g)						
		A	B	C	D	E	F	G
phenolic acids								
protocatechuic acid	0	0.07 ± 0.04Xx	0.08 ± 0.03Xx	0.06 ± 0.04Xx	0.08 ± 0.03Xx	0.10 ± 0.04Xx	0.11 ± 0.02Xx	0.06 ± 0.02Xx
	6	0.05 ± 0.04Xx	0.05 ± 0.03Xx	0.04 ± 0.01Xx	0.07 ± 0.03Xx	0.05 ± 0.03Xx	0.08 ± 0.02Xx	0.05 ± 0.02Xx
	12	0.03 ± 0.01Xz	0.04 ± 0.01Xyz	0.04 ± 0.01Xyz	0.05 ± 0.01Xy	0.04 ± 0.01Xyz	0.08 ± 0.01Xx	0.04 ± 0.01Xyz
	16	0.04 ± 0.01Xx	0.04 ± 0.01Xx	0.04 ± 0.02Xx	0.06 ± 0.03Xx	0.05 ± 0.03Xx	0.08 ± 0.03Xx	0.04 ± 0.01Xx
	19	0.07 ± 0.01Xx	0.02 ± 0.00Xx	0.05 ± 0.02Xx	0.06 ± 0.03Xx	0.04 ± 0.02Xx	0.09 ± 0.03Xx	0.04 ± 0.01Xx
vanillic acid	0	0.53 ± 0.30Xx	0.59 ± 0.41Xx	0.64 ± 0.36Xx	0.90 ± 0.53Xx	0.73 ± 0.39Xx	0.87 ± 0.47Xx	0.54 ± 0.03Xx
	6	0.17 ± 0.16Yy	0.20 ± 0.11Yy	0.17 ± 0.08Yy	0.58 ± 0.04Xx	0.17 ± 0.05Yy	0.58 ± 0.18XYx	0.12 ± 0.05Yy
	12	0.12 ± 0.09Yy	0.11 ± 0.02Yy	0.11 ± 0.03Yy	0.57 ± 0.18Xx	0.10 ± 0.02Yy	0.49 ± 0.22XYx	0.27 ± 0.04Yy
	16	0.09 ± 0.02Yy	0.10 ± 0.03Yy	0.09 ± 0.02Yy	0.36 ± 0.21Xx	0.10 ± 0.05Yy	0.24 ± 0.09Yx	0.11 ± 0.06Yy
	19	0.13 ± 0.05Yx	0.11 ± 0.04Yx	0.13 ± 0.06Yx	0.41 ± 0.02Xx	0.14 ± 0.05Yx	0.25 ± 0.08Xx	0.09 ± 0.00Yx
salicylic acid	0	0.21 ± 0.03Yy	0.28 ± 0.10Xx	0.30 ± 0.07Xxy	0.24 ± 0.06Xy	0.28 ± 0.09Xxy	0.22 ± 0.12Xy	0.23 ± 0.03Yy
	6	0.23 ± 0.07Yx	0.24 ± 0.11Xx	0.26 ± 0.06Xx	0.28 ± 0.05Xx	0.31 ± 0.07Xx	0.27 ± 0.08Xx	0.27 ± 0.05Yx
	12	0.28 ± 0.07XYyz	0.27 ± 0.07Xyz	0.36 ± 0.08Xxy	0.23 ± 0.03Xz	0.38 ± 0.09Xx	0.35 ± 0.06Xxy	0.27 ± 0.04Yyz
	16	0.36 ± 0.11Xx	0.36 ± 0.10Xx	0.35 ± 0.06Xx	0.24 ± 0.03Xx	0.35 ± 0.14Xx	0.34 ± 0.10Xx	0.38 ± 0.09Xx
	19	0.30 ± 0.06Xx	0.36 ± 0.12Xx	0.43 ± 0.16Xx	0.21 ± 0.04Xx	0.43 ± 0.03Xx	0.41 ± 0.01Xx	0.45 ± 0.02Xx
gallic acid	0	0.39 ± 0.36Yx	0.85 ± 0.63Yx	0.46 ± 0.24Yx	0.36 ± 0.14Xx	0.40 ± 0.23Wx	0.38 ± 0.32Xx	0.46 ± 0.06Yx
	6	0.55 ± 0.24Yx	0.89 ± 0.74XYx	0.37 ± 0.23Yx	0.39 ± 0.16Xx	0.77 ± 0.27ZWx	0.48 ± 0.18Xx	0.80 ± 0.09XYx
	12	1.23 ± 0.83XYx	1.35 ± 0.78XYx	0.85 ± 0.57Yx	0.40 ± 0.14Xx	1.23 ± 0.63YZx	1.10 ± 0.09Xx	1.74 ± 0.27Xx
	16	2.49 ± 1.81Xx	1.68 ± 0.99Xx	0.96 ± 0.09Yx	0.49 ± 0.21Xx	1.51 ± 0.39XYx	1.25 ± 0.36Xx	1.95 ± 0.17Xx
	19	1.06 ± 0.70XYx	1.97 ± 0.34Xx	2.66 ± 0.52Xx	0.52 ± 0.18Xx	2.15 ± 0.63Xx	1.88 ± 0.31Xx	1.76 ± 0.56Xx
flavonols								
quercetin	0	0.09 ± 0.03Xx	0.15 ± 0.06Xx	0.14 ± 0.05Xx	0.13 ± 0.05Xx	0.27 ± 0.34Xx	0.12 ± 0.07Xx	0.09 ± 0.03Xx
	6	0.05 ± 0.03Xx	0.06 ± 0.05Xx	0.07 ± 0.04Xx	0.09 ± 0.02Xx	0.06 ± 0.04Xx	0.76 ± 1.57Xx	0.08 ± 0.01Xx
	12	0.05 ± 0.02Xx	0.04 ± 0.01Xx	0.06 ± 0.06Xx	0.09 ± 0.02Xx	0.05 ± 0.02Xx	0.06 ± 0.01Xx	0.09 ± 0.03Xx
	16	0.06 ± 0.02Xx	0.06 ± 0.03Xx	0.06 ± 0.05Xx	0.07 ± 0.02Xx	0.08 ± 0.04Xx	0.08 ± 0.01Xx	0.09 ± 0.04Xx
	19	0.07 ± 0.03Xx	0.05 ± 0.04Xx	0.09 ± 0.01Xx	0.08 ± 0.02Xx	0.09 ± 0.04Xx	0.07 ± 0.04Xx	0.06 ± 0.01Xx
kaempferol	0	0.65 ± 0.06Xx	0.73 ± 0.06Xx	0.57 ± 0.06Xx	0.71 ± 0.17Xx	0.73 ± 0.32Xx	0.80 ± 0.06Xx	0.59 ± 0.23Xx
	6	0.42 ± 0.26Yx	0.30 ± 0.10Yx	0.37 ± 0.20Yx	0.50 ± 0.11Yx	0.42 ± 0.24Xx	0.30 ± 0.14Xx	0.25 ± 0.04Yx
	12	0.22 ± 0.11Yx	0.25 ± 0.14Yx	0.28 ± 0.10Yx	0.43 ± 0.12Yx	0.28 ± 0.09Xx	0.21 ± 0.07Xx	0.21 ± 0.08Yx
	16	0.20 ± 0.06Yx	0.23 ± 0.11Yx	0.16 ± 0.10Yx	0.43 ± 0.08Yx	0.26 ± 0.15Xx	0.27 ± 0.05Xx	0.31 ± 0.11XYx
	19	0.19 ± 0.03Yx	0.15 ± 0.04Yx	0.17 ± 0.13Yx	0.35 ± 0.05Yx	0.19 ± 0.04Xx	0.21 ± 0.02Xx	0.17 ± 0.07Yx
flavanol								
catechin	0	3.75 ± 1.25Xx	5.60 ± 2.11Xx	4.60 ± 1.43Xx	3.54 ± 1.38Xx	3.23 ± 1.56Xx	0.28 ± 0.18Xx	4.15 ± 1.67Xx
	6	1.88 ± 0.98Xx	2.44 ± 0.41Xx	3.92 ± 1.21Xx	3.43 ± 3.02Xx	3.25 ± 1.07Xx	4.21 ± 2.77Xx	1.99 ± 0.19Xx
	12	1.97 ± 0.56Xx	1.38 ± 0.58Xx	2.80 ± 0.42Xx	2.93 ± 0.21Xx	2.10 ± 0.99Xx	3.69 ± 0.57Xx	1.67 ± 0.21Xx
	16	6.49 ± 1.38Xx	5.68 ± 0.21Xx	2.50 ± 1.38Xx	1.83 ± 0.73Xx	5.83 ± 8.31Xx	1.69 ± 0.31Xx	2.67 ± 0.77Xx
	19	3.07 ± 0.46Xx	3.99 ± 0.48Xx	1.14 ± 0.43Xx	0.21 ± 0.02Xx	0.09 ± 0.01Xx	5.42 ± 1.40Xx	1.85 ± 0.31Xx
flavone								
luteolin	0	2.96 ± 0.59Xx	3.03 ± 0.57Xx	2.12 ± 0.55Xxyz	2.35 ± 1.2Xxy	2.43 ± 1.02Xxy	1.14 ± 0.25Xz	1.79 ± 0.80Xyz
	6	0.16 ± 0.10Yy	0.21 ± 0.18Yy	0.31 ± 0.03Yy	1.30 ± 0.57XYx	0.02 ± 0.06Yy	0.25 ± 0.06Yy	0.13 ± 0.02Yy
	12	0.08 ± 0.05Yy	0.08 ± 0.07Yy	0.04 ± 0.02Yy	0.94 ± 0.19Yx	0.05 ± 0.04Yy	0.09 ± 0.06Yy	0.04 ± 0.02Yy
	16	0.02 ± 0.01Yy	0.03 ± 0.01Yy	0.07 ± 0.07Yy	0.55 ± 0.27Yx	0.02 ± 0.01Yy	0.04 ± 0.01Yy	0.02 ± 0.00Yy
	19	0.05 ± 0.02Yy	0.03 ± 0.00Yy	0.04 ± 0.03Yy	0.55 ± 0.27Yx	0.02 ± 0.01Yy	0.02 ± 0.01Yy	0.02 ± 0.00Yy

[a]Contents of individual phenolic acids, flavonols, flavanol and flavone (mg/100 g) in strawberry spreads were quantitated as equivalents of the respective phenolic acid, flavonole, flavanol or flavone. Data are expressed as means ± standard deviation (n = 6). Statistically significant differences are marked with upper case letters (X, Y, Z, W) within each column (for each compound) and with lower case letters (x, y, z) within the same row. See table for spread varieties.

The fate of phenolics such as phenolic acids, flavonols, flavanols and flavones during the storage of strawberry spreads is still relatively poorly understood. The modifications of phenolics and novel compounds formed have been more thoroughly studied during winemaking and wine aging.[31] Therefore, this topic still represents a challenge for further studies in the future.

To the best of our knowledge, this is the first report of a comprehensive and systematic evaluation of phenolic compounds in a fruit spread that has been processed and stored as a range of different varieties. We analyzed the total phenolics and individual phenolics in strawberry spreads that were processed according to different industrial procedures and were then stored for several weeks under various storage conditions. In all of the varieties of the strawberry spreads we observed that, in general, all of the phenolics monitored underwent modifications during the storage period. The best phenolic retention was observed in the spreads stored at 4 °C. Also, for the first time, an anthocyanin (cyanidin 3-(6″-succinyl-glucoside)) is shown to remain at stable

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levels through 19 weeks of storage. However, to clarify all of the phenolic reactions, modifications and mechanisms, further studies are needed for each compound individually. From our data, we would speculate that the possible options for anthocyanin reactions in fruit spreads during storage are generally degradation and polymerization. The products of these reactions are phenolic acids and procyanidins, respectively.[11] In the present study, these compounds also react with the Folin–Ciocalteu reagent, and therefore the contents of the total phenolics were not changed during the storage period. To achieve strawberry spreads that remain as a high phenolic source and to preserve their nutritional quality for acceptable periods of time, the industry should consider our suggestion that these products are stored at low temperatures (4 °C). Thus, it is very important to maintain phenolics unchanged as far as possible, or at least to minimize their decreases and modifications in processed food during manufacture and storage (use of a cold chain), to keep their nutritional and organoleptic attributes, and therefore to contribute to improved human health.

■ AUTHOR INFORMATION

Corresponding Author
*Tel: +386-1-320-37-43. Fax: +386-1-256-62-96. E-mail: tomaz.polak@bf.uni-lj.si.

Notes
The authors declare no competing financial interest.

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