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Changes in Antioxidant and Metabolite Profiles during Production of Tomato Paste

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Tomato products and especially concentrated tomato paste are important sources of antioxidants in the Mediterranean diet. Tomato fruit contain well-known antioxidants such as vitamin C, carotenoids, flavonoids, and hydroxycinnamic acids. The industrial processing of this fruit into tomato paste involves several treatments that potentially affect the final profile of antioxidants and other metabolites in the commercial product. Here we have used both biochemical and metabolomic techniques to assess the effect of each separate step in the industrial production chain starting from fresh fruit to the final tomato paste. Material was collected from five independent tomato paste production events spread over two successive years. Samples comprised the intact ripe fruits and semifinished products after fruit-breaking, separation of the pulp from skin and seeds, evaporation, and finally after canning and pasteurization. The effect of each processing step was determined by different types of analysis. First, the total antioxidant capacity and total phenolic content were determined by commonly used spectrophotometric methods. Second, individual antioxidants in the extracts were identified and compared using an HPLC with online antioxidant detection. Third, in each sample the levels of the major individual antioxidants present, i.e., vitamin C, phenolic compounds (such as rutin and chlorogenic acid), tocopherols, and carotenoids, were quantified. Fourth, an untargeted metabolomic approach using LC-QTOF-MS was used to identify those production steps that have the largest impact on the overall metabolic profile in the final paste as compared to the original fruits. This multifaceted approach has revealed that each processing step induces specific alterations in the metabolic profile, as determined by the different analysis procedures, and that in particular the fruit-breaking step and the removal of seed and skin significantly affect the levels of antioxidants and many other metabolites present in commercial tomato paste.

KEYWORDS: Antioxidants; flavonoids; carotenoids; metabolomics; tomato processing; tomato paste; vitamin C

INTRODUCTION

Tomato fruit is widely consumed either fresh or after processing into various (cooked) products. The consumption of tomatoes has been proposed to reduce the risk of several chronic diseases such as cardiovascular diseases and certain types of cancer and especially prostate cancer (*1, 2*). In addition, tomato consumption leads to decreased serum lipid levels and low-density lipoprotein oxidation (*3*). These health protective effects have been widely attributed to the presence of key antioxidants such as lipid-soluble lycopene and β-carotene, as well as water-soluble vitamin C, and compounds of intermediate hydrophobicity such as quercetin glycosides, naringenin chalcone, and chlorogenic acid. All of these are known to contribute significantly to the antioxidant activity of tomato fruit (*2, 4*).

Besides fresh fruit, tomato paste or the more concentrated tomato puree is a significant component in the human diet. For example, in Turkey, tomato paste is included in the majority of homemade dishes. About 115000 tons of tomato paste was consumed in Turkey in 2006 (*5*). From this perspective, it is important to understand the effect of industrial scale tomato paste making on health-associated compounds. Generally, the industrial paste-making process involves several steps, as outlined in **Figure 1**. Briefly, fruits are chopped in a "breaker" unit, after which the pulp is shortly heated and separated from seed and skin and some moisture is removed by evaporation. Finally, the product is canned and pasteurized. This production

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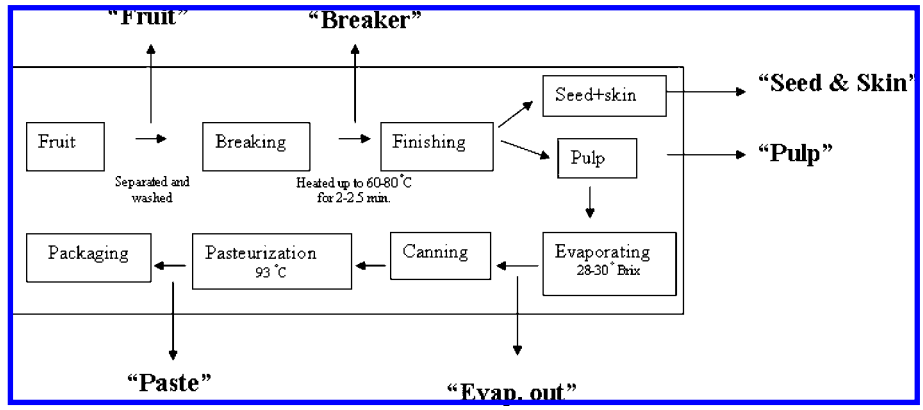

Figure 1. Tomato paste production scheme (arrows outward indicate the sampling steps of the study). The different steps are explained in the Results section.

process involves a number of heating steps, which may be expected to have an effect on heat-labile and oxidizable compounds. A number of studies, mostly performed on a laboratory scale, indicate that the content of carotenoids and vitamin C in tomato products may be negatively affected by various thermal treatments such as boiling, frying, drying, and microwaving (*4, 6*). Also, it has been reported that some carotenoid is lost during production of tomato paste in a factory (*6*). In contrast, little is known about the effects of these treatments on (poly)phenolic antioxidants including flavonoids (*7*). With regard to the percentage of fruit lycopene ending up in the final paste, the available literature data are inconsistent with values ranging from a ca. 20% decrease to a ca. 33% increase (*6, 8*). Clearly, these variable findings indicate that results obtained from laboratory experiments are hard to translate to the actual effects taking place during factory scale paste making. Each processing step, to a more or lesser extent, will likely influence the composition of metabolites, including health-related antioxidants, and thus the quality of the final tomato paste. There is need for a better understanding of the physiological and biochemical processes that can take place during the entire route from the field harvest of fresh fruit up to the pasteurized and canned tomato paste ready for retail. Understanding these processes will result in the development of tools and metabolic markers to monitor the quality of the paste. This, in turn, will allow us to optimize further and control industrial scale tomato processing and improve the nutritional value of tomato paste.

The science of metabolomics is being established to help us gain a broader insight into the biochemical composition of living organisms and how this changes in time and after external perturbations. With the recent developments in plant metabolomic techniques (*9*), it is now possible to detect several hundred metabolites simultanously and to compare samples reliably for differences and similarities in a semiautomated and, essentially, untargeted manner. In tomato, for example, such comprehensive metabolomic approaches have been used to compare the metabolic profiles of fruit peel and flesh tissues (*10*), to define metabolic alterations in fruits induced by mutations such as the spontanously occurring light-hyperresponsive $Hp2^{dg}$ mutation (*11*), and to investigate metabolic changes taking place upon fruit development and ripening (*12, 13*).

To our knowledge, comprehensive biochemical studies on the effect of the individual steps in factory scale tomato processing have not yet been reported. The aim of the present study was, therefore, to investigate industrial processing of fresh fruits into tomato paste both by analyzing the fate of specific antioxidants, using dedicated analyses, and by taking a broader overview, using an approach based on liquid chromatography–mass spectrometry (LC-MS) for nontargeted metabolomics (*10, 14*). Five independent tomato paste production events, spread over two successive years, were followed at each step in the paste production process starting with fresh fruit material. The results reveal both the reproducibility of industrial processing and the relative impact of each step on the antioxidant and overall metabolite composition of the paste.

MATERIALS AND METHODS

Tomato Material. Fresh tomato samples, material from the different processing steps, and the final tomato paste products were collected at an established tomato paste factory (Tamek Co.) in Bursa, Turkey. The majority of tomato material used by this factory belongs to the Shasta variety, obtained from surrounding villages in Karacabey. At six processing steps, samples of >100 g were collected. This was repeated five times, so five independent processing events using different, independent tomato batches (i.e., five biological replicates of each step) were tested: two in 2005 and three in 2006 (seasonal replicates), each sampling time separated by about 4 weeks. All samples were snap-frozen in liquid nitrogen, transported in frozen state to The Netherlands in dry ice, and subsequently ground to a fine powder using a precooled grinder. All samples were individually freeze-dried to compensate for the differences in water content and stored at −80 °C until analysis. Samples were analyzed in triplicate (technical repetitions). The standard deviation of the technical replicates was always lower than 5% of the mean value.

Moisture Content Analysis. Moisture content of the samples were analyzed according to Association Official of Analytical Chemists (AOAC) method 925.10. Approximately 2 g of each sample was taken and exactly weighed before and after air-drying at 130 °C in an oven.

Preparation of Extracts. For the estimation of the total phenolic content and total antioxidant activities using spectrophotometric assays, 2 mL of 75% methanol was added to a 0.2 ± 0.01 g freeze-dried sample and sonicated for 15 min. After centrifugation at 2500 rpm for 10 min, the supernatant was collected, another 2 mL of 75% methanol was added to the pellet, and the extraction procedure was repeated. Both supernatants were combined and adjusted to a final volume of 5 mL. This solution was used as the hydrophilic extract. Then, lipophilic compounds were extracted from the pellet following the procedure for carotenoid extraction described below and using 5 mL of 2-propanol to finally dissolve the metabolites. This solution was used as the lipophilic extract.

For quantitative analyses of specific antioxidants by HPLC, different extraction methods were applied. For (poly)phenolic antioxidants, a 25 (±0.05) mg freeze-dried sample was extracted with 2.0 mL of 75% methanol in ultrapure water following the procedure described before (*11*). Using this procedure, the recovery of phenolic compounds is reported to be >90% (*11*). Hydrolysis of flavonoids was performed as described (*15*) by weighing a 30 ± 0.5 mg freeze-dried sample and

adding 0.57 mL of MQ water, 1 mL of methanol containing 0.5% (w/v) TBHQ, and 0.4 mL of 6 M HCl, followed by 1 h hydrolysis at 90 °C. For lipophilic antioxidants (carotenoids and tocopherols), five different extraction methods (*11, 16*) were first tested for their efficiency in extracting lycopene from both "fruit" and "paste" samples. The method giving the highest recovery in both types of samples (*11*) was selected for analyses in our further experiments. Briefly, 25 (±0.05) mg dry weight was extracted three times using 4.5 mL of MeOH−$CHCl_3$ (2.5:2.0 v/v) and 2.5 mL of Tris buffer (pH 7.5). The $CHCl_3$ fractions were pooled, dried under nitrogen gas, and finally taken up in ethyl acetate before HPLC analyses. For vitamin C, 25 (±0.05) mg dry weight was weighed and extracted in 0.475 mL of 5% metaphosphoric acid in ice-cold water (*17*).

For metabolite profiling using LC-MS, 25 mg dry weight of freeze-dried material was extracted using 2.0 mL of 0.1% formic acid (v/v) in 75% aqueous−methanol, as described (*10*).

Spectrophotometric Assays. The total phenolic content was estimated using Folin−Ciocalteu reagent as described before (*18*), using 100 μL of lipophilic extract, 900 μL of pure water, and 5 mL of reagent. For the preparation of a standard curve, 0.10–0.50 mg/mL gallic acid was used, and data were expressed in milligrams of gallic acid equivalents (GAE) per 100 g dry weight.

Total antioxidant levels were estimated by four different *in vitro* tests. All assays were performed on both the hydrophilic and the lipophilic extracts (see above). In all assays, trolox was used as a reference compound, and results were expressed in terms of micromoles of trolox equivalent antioxidant capacity (TEAC) per 100 g dry weight.

The ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] method used was as according to that in ref *19* with some slight modifications. For the hydrophilic extracts the ABTS stock solution was diluted in 50 mM potassium phosphate buffer, pH 8.0, instead of 5 mM PBS. Then, 100 μL of sample extract or standard was mixed with 1 mL of ABTS-working solution (final pH of the reaction mixture was about 7.4), and after exactly 40 s the remaining ABTS radicals were measured at 734 nm.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method (*20*) was performed by mixing 100 μL of both types of sample extract with 2 mL of 0.1 mM DPPH in methanol (not buffered). After incubation at room temperature for 30 min in the dark, the absorbance of the mixture was measured at 517 nm.

The FRAP (ferric reducing antioxidant power) method (*21*) was performed using 100 μL of both types of sample extract or trolox, which was pipetted into 1.5 mL reaction tubes, followed by the addition of 900 μL of FRAP reagent (pH 3.6). This mixture was then quickly vortexed for 20 s. Absorbance was measured at 593 nm against a reagent blank exactly 4 min after the addition of sample to the FRAP reagent.

The CUPRAC (copper reducing antioxidant capacity) method was performed according to that previously described (*22–24*). The pH of the reagent was 7.0 and the reaction time 1 h.

HPLC Analyses of Antioxidant Compounds. Chlorogenic acid, flavonoids, carotenoids, tocopherols, and vitamin C were all analyzed by HPLC as recently described for fresh tomato fruit (*11*), using calibration curves of reference compounds for quantification. Briefly, (poly)phenolic compounds were separated on a Luna C18 column (Phenomenex) on a Waters HPLC system (W600) using a gradient of 0.1% formic acid and acetonitrile. Carotenoids and tocopherols were separated on a YMC-Pack C30 column with a gradient of methanol and *tert*-butyl ether. Vitamin C was analyzed using a YMC-Pro C18 column and a gradient of acetonitrile in phosphoric acid buffer at pH 4.4. Eluting compounds were detected by PDA (Waters 996 detector) or fluorescence (Waters 2475 detector).

Online HPLC Antioxidant Detection. An HPLC-PDA system coupled to postcolumn antioxidant detection, in which ABTS cation radicals are mixed online with the separated compounds, was used to determine which antioxidant species are present in the aqueous−methanol tomato extracts (*25*). The online ABTS antioxidant reaction lasted 30 s at 40 °C and pH 7.4.

LC-QTOF-MS-Based Metabolomics. The 75% aqueous−methanol extracts were subjected to a nontargeted LC-MS-based metabolomic approach (*10, 14*), using an Alliance HPLC system, a PDA detector, and a high-resolution quadrupole time-of-flight (QTOF) MS (Waters). Electrospray ionization in negative mode was used to ionize compounds separated by the LC column. Mass signals were extracted and aligned across all samples in an unbiased manner using the dedicated Metalign software (www.metalign.nl), and a data matrix of intensities of all mass signals × samples was created. Mass signals were identified by comparing their retention times and accurate masses to the tomato fruit metabolite database (MoTo DB) available at http://appliedbioinformatics.wur.nl/moto/ (*13*), using an mass accuracy window of 5 ppm. Annotations thus obtained were further checked by manually comparing the PDA spectra and mass fragments (if present) with those published in the MoTo DB.

Statistical Analysis. All analyses were performed using the five biological replicates. Data were subjected to statistical analysis using SPSS software (version 11.5 for Windows XP, SPSS Inc.) for the analysis of variance (ANOVA). Duncan's new multiple range test was used to analyze differences between treatments. For multivariate analysis, the LC-MS data were read into GeneMaths software (Applied Maths) after 2 log transformation of mass signal intensities. Mass signals (variables) were normalized by dividing by the mean of each variable. To test LC-MS data for significant differences in relative mass signal intensities between processing steps, Student's *t*-test was used.

RESULTS

Sampling. The scheme of factory scale tomato paste production is shown in **Figure 1**. The first sample ("fruit") was taken from tomatoes as they arrived at the factory. The second sample ("breaker") was taken after the tomatoes had been sorted, washed in a water bath, transported to the breaker unit by means of water tunnels, chopped with knives in the breaker for a few seconds, and finally collected in tanks ready to be sent to the finisher unit. The third and fourth samples were taken after the material had been heated to 60–80 °C for 2–2.5 min in thefinisher unit, after which the seed and skin parts (sample "seed and skin") were removed from the remaining pulp by means of filters (sample "pulp"). The amount of seed and skin removed at this step represented about 3.5% of the original whole fruit wet weight. The fifth sample ("evap out") was taken after the pulp had been treated in a three-effect evaporator unit, where water was removed, by heating up to 80 °C, until a Brix value of 28–30° was reached. The sixth sample ("paste") was collected after the final paste was canned and pasteurized (5–10 min at 93 °C). For each sample, five biological replications (from starting fruit material and all processing steps) were collected at five different time points, over two seasons. The moisture content was analyzed for each sample (**Table 1**). The dry matter content from fruit to paste increased from 5% to 30%.

Spectrophotometric Assays. The total phenolic content (**Table 1**), estimated using the Folin−Ciocalteu method, did not change significantly ($p > 0.05$) during the entire process toward the final paste.

Total antioxidant capacity was measured using four different methods on both the hydrophilic and the lipophilic extracts. The different analyses show different trends (**Table 1**). On the basis of the ABTS method, the total antioxidant activity was not significantly altered in paste as compared to fruit for both hydrophilic and lipophilic extracts. In contrast, according to the DPPH and FRAP methods, both lipophilic and hydrophilic antioxidant activities decreased significantly during processing. As judged by the CUPRAC method, hydrophilic antioxidants do not change significantly, while hydrophobic antioxidants strongly decrease. The seed and skin fraction generally had high levels of antioxidants, compared to the final paste.

Online Antioxidant Analysis. The relative contribution of individual compounds to the total antioxidant activity of the extracts, and the potential chemical basis of any changes observed, was assessed (cf. ref *25*). This was done by an HPLC

Table 1. Water Content, Total Phenolic Level, and Antioxidant Activity in Samples Taken from Various Tomato Processing Steps[a]

analyses		fruit	breaker	finisher pulp	seed and skin	evap out	paste
moisture content (%)		94.6 ± 0.9 a	94.2 ± 0.7 a	94.5 ± 1.4 a	78.7 ± 3.1 b	71.6 ± 5.1 c	71.9 ± 2.7 c
total phenolics (mg of GAE/100 g)		576.5 ± 76.2 a	592.0 ± 21.8 a	565.2 ± 47.5 a	543.5 ± 83.8 a	596.3 ± 54.0 a	608.5 ± 52.5 a
ABTS (μmol of TEAC/100 g)	H[b]	4385.7 ± 514.3 a	4409.4 ± 82.7 a	4056.3 ± 338.3 a	4235.1 ± 710.9 a	4155.6 ± 355.1 a	3988.0 ± 385.0 a
	L[c]	139.1 ± 22.1 a	143.8 ± 11.7 a	140.1 ± 9.4 a	103.7 ± 54.1 b	123.8 ± 4.4 ab	118.5 ± 10.4 ab
	T[d]	4524.8 ± 529.4 a	4553.2 ± 86.7 a	4196.4 ± 341.8 a	4338.8 ± 753.2 a	4279.4 ± 353.2 a	4106.5 ± 391.6 a
CUPRAC (μmol of TEAC/100 g)	H	3819.4 ± 452.1 ab	3744.1 ± 286.3 ab	3347.0 ± 309.1 b	4098.6 ± 801.6 ab	3985.2 ± 803.8 ab	4311.3 ± 807.9 a
	L	595.2 ± 167.2 a	544.3 ± 102.1 a	324.5 ± 45.4 b	492.2 ± 24.9 a	282.1 ± 173.7 bc	162.6 ± 80.6 c
	T	4415.6 ± 588.0 b	4288.4 ± 219.7 b	3671.5 ± 270.2 b	4590.8 ± 769.6 a	4267.3 ± 820.9 b	4473.9 ± 877.1 b
FRAP (μmol of TEAC/100 g)	H	1709.2 ± 139.4 a	1402.1 ± 54.0 b	1234.8 ± 260.7 bc	1131.1 ± 232.1 c	1178.7 ± 104.6 bc	1054.1 ± 90.3 c
	L	357.6 ± 125.6 a	298.7 ± 95.2 ab	143.2 ± 44.1 c	240.7 ± 15.8 b	101.7 ± 5.2 c	79.9 ± 29.0 c
	T	2066.8 ± 132.6 a	1700.8 ± 90.7 b	1378.0 ± 221.5 c	1371.8 ± 218.8 c	1280.4 ± 100.7 c	1134.0 ± 73.3 c
DPPH (μmol of TEAC/100 g)	H	1211.4 ± 217.5 a	980.4 ± 83.4 b	872.3 ± 241.1 bc	879.4 ± 184.6 bc	849.4 ± 47.5 bc	706.7 ± 33.5 c
	L	109.3 ± 21.4 b	108.1 ± 17.4 b	103.6 ± 10.9 bc	132.6 ± 10.2 a	84.0 ± 5.9 cd	78.1 ± 20.7 d
	T	1320.7 ± 217.9 a	1088.5 ± 74.7 b	975.9 ± 231.6 bc	1012.0 ± 189.9 b	933.4 ± 48.5 bc	784.8 ± 45.3 c

[a] The data presented in this table consist of average values ± standard deviation of five independent processing events. All contents are expressed per 100 g dry weight. Different letters in the rows represent statistically significant differences ($p < 0.05$). [b] H = hydrophilic extract. [c] L = lipophilic extract. [d] T = total.

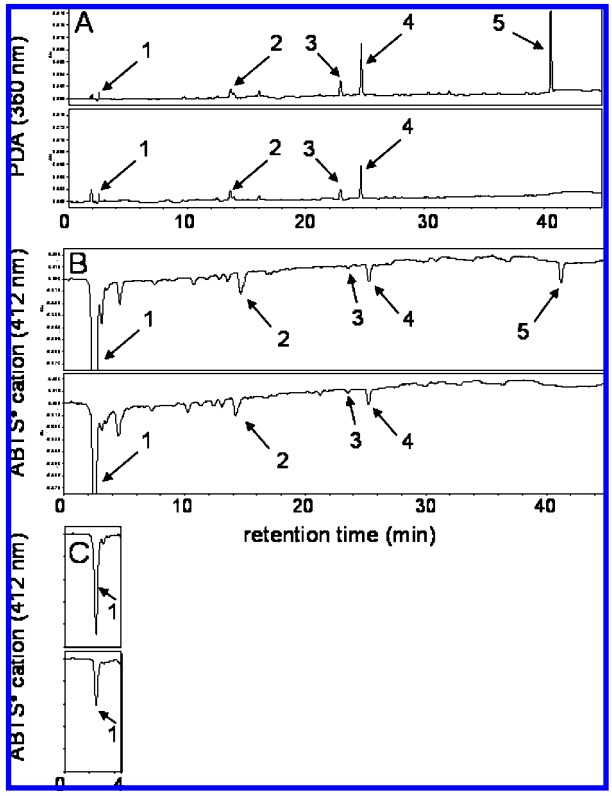

Figure 2. Profiling antioxidants in aqueous−methanol extracts using HPLC-PDA with online antioxidant detection. (**A**) Typical chromatograms (PDA, recorded at 360 nm) of fruit (upper panel) and paste (lower panel). (**B**) Antioxidants in fruit (upper panel) and paste (lower panel) as determined from reaction with ABTS• cation radicals. (**C**) Same as (**B**), comparing the antioxidant activity in the first 4 min of the chromatogram. *Y*-axes in upper and lower panels are directly comparable. Numbers refer to the main antioxidants identified: 1, ascorbic acid (vitamin C); 2, chlorogenic acid; 3, rutin apioside; 4, rutin; 5, naringenin chalcone.

analysis with a specific online antioxidant detection system based on postcolumn reaction with ABTS cation radicals. As shown in **Figure 2** (top panels), in fruit samples the first part of the chromatogram (until 5 min) comprised approximately 85% of the total antioxidant activity (based on total peak area). This region contains polar antioxidants including vitamin C (main contribution) and glutathione. Between 5 and 35 min after sample injection, phenolic compounds such as chlorogenic acid, rutin, and rutin apioside eluted which were responsible for about 12.5% of the antioxidant activity in the extracts. Naringenin chalcone, eluting at a retention time of 42 min, showed an activity equivalent to about 2.5% of the total antioxidant activity. In the paste samples (**Figure 2**, bottom panels), the antioxidant activity of the peak eluting within the first 5 min was 79% of the total, while that of the compounds eluting between 5 and 35 min was 21%. Since naringenin chalcone was completely lost upon tomato paste production, and the content of its isomeric form naringenin was relatively low, no other compound with significant antioxidant activity was observed in the paste after 35 min of chromatography.

Individual Antioxidants. The changes in specific antioxidant compounds were monitored during the paste production process. To this end, hydrophilic and hydrophobic extracts were analyzed by HPLC coupled to PDA and fluorescence detection, and known antioxidant compounds were quantified using commercially available standards (**Table 2**). Although there was considerable variation between batches in the antioxidant content, the trends reflecting the effect of processing were similar in all batches, as shown for lycopene in **Figure 3A**,**B** and for rutin in **Figure 3C**.

In all lipophilic extracts all-*trans*-lycopene, β-carotene, and lutein were identified as the main carotenoids. The content of these three carotenoids gradually decreased from fruit to paste by 32%, 36%, and 75%, respectively. Within the five tomato pasting events, the decrease in all-*trans*-lycopene varied between 19% and 42% (**Figure 3B**). Other carotenoid species detected, such as all-*trans*-phytoene and *cis*-lycopene, could not be quantified due to lack of standards. However, no significant changes in peak areas were observed for these compounds between fruit and paste (data not shown). With regard to the tocopherols (**Table 2**), both α- and β-forms did not change significantly, while their corresponding biosynthetic precursors decreased during processing.

Naringenin chalcone, naringenin, rutin, and rutin apioside were the most abundant flavonoids in the samples (**Table 2**). Notably, the abundance of all these flavonoids significantly increased more than 2-fold as a result of the breaking step. As shown for rutin (**Figure 3C**), this increase upon breaking was highly reproducible between tomato batches. To assess whether

Table 2. Carotenoids, Flavonoids, and Vitamin C Content at Various Steps of Industrial Tomato Paste Production[a]

compound (mg/100 g dry weight)	fruit	breaker	finisher pulp	seed and skin	evap out	paste
all-*trans*-lycopene	146.0 ± 39.5 a	130.8 ± 28.9 ab	114.1 ± 31.8 abc	61.9 ± 14.5 d	89.2 ± 10.0 cd	98.9 ± 25.5 bc
β-carotene	4.7 ± 1.4 a	4.8 ± 1.0 a	4.2 ± 0.9 a	4.2 ± 0.6 a	2.8 ± 0.4 b	3.0 ± 0.5 b
lutein	2.0 ± 0.4 a	2.1 ± 0.4 a	1.9 ± 0.4 a	1.4 ± 0.2 b	0.9 ± 0.2 c	0.5 ± 0.1 c
rutin	19.8 ± 11.8 c	43.7 ± 16.7 b	12.5 ± 8.2 c	167.3 ± 22.2 a	16.5 ± 5.4 c	16.9 ± 6.1 c
rutin apioside	4.8 ± 1.9 c	10.6 ± 2.3 b	4.7 ± 1.8 c	35.1 ± 7.0 a	5.1 ± 1.2 c	4.9 ± 1.3 c
naringenin	0.0 ± 0.0 d	1.1 ± 0.2 c	2.5 ± 0.6 b	3.6 ± 1.2 a	2.0 ± 0.8 b	2.2 ± 0.9 b
naringenin chalcone	19.2 ± 8.7 bc	43.8 ± 8.1 b	2.8 ± 2.1 c	166.2 ± 50.0 a	0.0 ± 0.0 c	0.0 ± 0.0 c
chlorogenic acid	21.0 ± 9.4 a	15.1 ± 7.2 a	18.4 ± 14.0 a	13.3 ± 4.2 a	16.2 ± 7.6 a	16.7 ± 8.0 a
vitamin C	245.7.1 ± 89.7 a	174.3 ± 34.9 ab	124.8 ± 51.6 b	10.0 ± 11.8 c	184.9 ± 6.1 ab	122.0 ± 28.7 b
α-tocopherol	31.62 ± 8.84 a	32.99 ± 15.01 a	24.39 ± 12.67 a	31.36 ± 12.41 a	23.95 ± 5.98 a	38.67 ± 2.29 a
β-tocopherol	1.08 ± 1.49 ab	0.0 ± 0.0 b	2.07 ± 0.60 a	1.38 ± 1.33 ab	1.48 ± 0.40 ab	2.46 ± 0.17 a
δ-tocopherol	0.35 ± 0.19 b	0.28 ± 0.11 bc	0.18 ± 0.09 bcd	0.71 ± 0.17 a	0.07 ± 0.04 d	0.11 ± 0.05 cd
γ-tocopherol	8.85 ± 5.38 b	7.75 ± 2.24 b	2.17 ± 1.12 c	23.18 ± 6.32 a	1.20 ± 0.31 c	1.45 ± 0.25 c

[a] Data represent average quantities ± standard deviation (determined by HPLC-PDA or HPLC-fluorescence detection) of five independent processing events. Different letters in the rows represent statistically significant differences ($p < 0.05$).

this was due to an increased extractability or a partial deglycosylation or deesterification of more complexed flavonoid species, an acid hydrolysis of the samples followed by HPLC-PDA analyses of the resulting flavonoid aglycons was performed (data not shown; method according to ref *15*). After hydrolysis a similar ratio between fruit and breaker flavonoids was observed as in nonhydrolyzed extracts, suggesting that the increase in these compounds by the breaker step was the result of *de novo* synthesis, rather than release from more complex forms. In the later stages of the paste-making process, the increase in flavonoids during the breaking step was reversed upon removal of the seed and skin fraction, resulting in levels in the paste that were similar to those in the original fruit. In contrast to the flavonoids, chlorogenic acid, the main hydroxycinnamic acid compound in tomato, was not increased in the breaker samples, relative to the fruit samples.

Another change in flavonoids that occurred during processing was the conversion of naringenin chalcone into its isomeric flavanone form, naringenin (*26*). While the flavanone was undetectable in any of the fruit samples, small amounts were already detected in the breaker samples and more in samples from the subsequent processing steps. In contrast, the chalcone form was highest after breaking and was mainly lost on separation of the seed and skin fraction. At the end of the total paste-making procedure, the chalcone was undetectable in paste samples while the flavanone naringenin represented 11% of the amount of total naringenin/naringenin chalcone present in fruit samples.

As a result of processing, about half of the vitamin C present in the original fruit was lost, resulting in about 120 mg/100 g dry weight in the final paste. The main processing step causing a significant loss in vitamin C was the pulping step, which included a heat treatment (2.5–3.0 min at 60–80 °C). The next heat treatment, during the evaporation stage (up to 80 °C until the desired Brix value was reached), did not further affect the vitamin C content, while the subsequent pasteurization step (5–10 min at 93 °C) toward the final paste reduced it by 34% (compared to the evap out sample). In contrast to the flavonoids, no increase in vitamin C was observed as a result of the breaking step, and a relatively low content was detected in the seed and skin fraction as compared to the pulp and final paste.

Untargeted Metabolomic Analysis. In order to establish the variation between the five replicate processing events and to determine which of the processing steps mostly affects the overall metabolite composition of the final paste, all tomato samples were extracted in aqueous−methanol and analyzed using an untargeted LC-QTOF-MS-based metabolomic approach. The mass profiles thus obtained (**Figure 4**) were processed and aligned across all samples using Metalign software. After filtering out mass signals that were <10 times the local noise in all 30 samples, the resulting data matrix contained intensity values (calculated as peak heights) for 3177 mass signals aligned across all samples. The mass intensity data were 2 log transformed and then subjected to multivariate analysis using GeneMaths software.

To visualize the effect of each industrial tomato processing step, principal component analysis (PCA) of the data set was performed after range scaling by subtracting the mean value of the log-transformed values per variable and sample. As shown in the score plot (**Figure 5**), the five biological replicates of samples clustered together according to each processing step. This result indicates that the metabolite profiles were mainly influenced by the various processing steps, rather than by variation between individual tomato fruit batches, season, or processing event. The first principal component (*X*-axis) explained 36% of the total variation in the data set. This component points to the most dominant step in the paste production process and clearly corresponded to the separation of seed and skin from the rest of the tomato material, with the largest difference in metabolite profiles between seed and skin and paste. The second component (*Y*-axis) explained 20% of the variation and corresponded to the stepwise processing from fruit to paste. The largest overall variation between the biological replicates at each processing step, calculated over all 3177 mass signals detected, was observed within the pulp samples: 44%, as compared to about 32% in the other samples. Nearly 43% (1356 signals) of the total number of mass signals differed significantly (Student's *t*-test, $p < 0.05$) between fruit and paste samples. Likewise, about 60% of the signals were statistically different between fruit and seed and skin and about 25% between fruit and breaker.

The compounds that are affected by the various processing steps were deduced from the differential mass signals by manually retrieving the corresponding accurate masses from the original chromatograms and comparing the observed accurate masses and retention times with those recently published in the MoTo DB of tomato fruit metabolites (*10*). Among the compounds that were significantly higher in fruit versus paste (**Table 3**) were a range of glycosylated alkaloids (lycoperoside and esculeoside isomers), hydroxycinnamates (mono-, di-, and tricaffeoyl derivatives), flavonoids (specifically naringenin chalcone and two of its glycosides), and the saponin tomatoside A. In contrast, the alkaloid lycoperoside H and the flavonoid

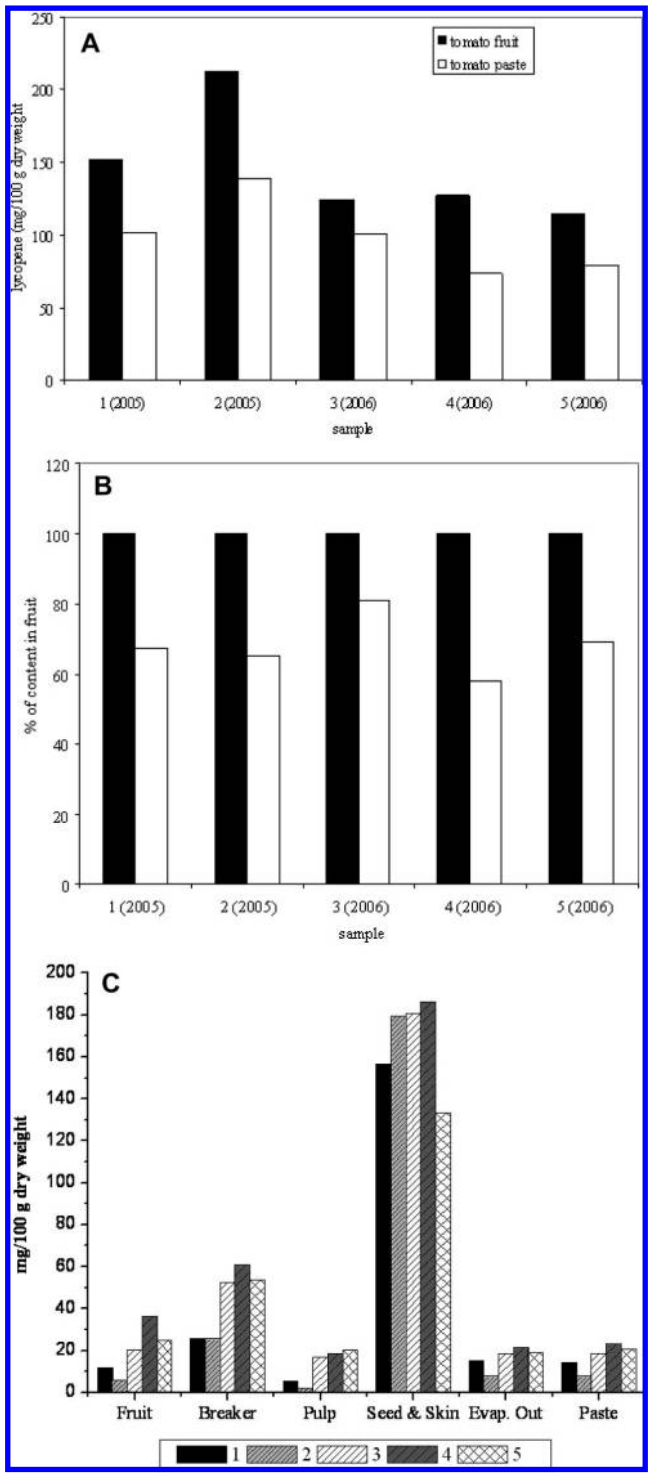

Figure 3. Antioxidants in different samples. (**A**) Variation in lycopene content of tomato fruit (filled bars) and paste (open bars) among five independent replicates harvested over a 2 year period. (**B**) As in (**A**) but normalized for lycopene content of fruit. (**C**) Rutin content in different processing stages among five independent replicates harvested over a 2 year period. All analyses were performed in triplicate.

naringenin were respectively 2-fold and 6-fold higher in paste compared to original fruit. Clearly, most compounds that were lower in paste were lost from the production chain upon removal of the seed and skin fraction (**Table 4**). This fraction therefore contained relatively high levels of all flavonoids, as well as several alkaloids. On the other hand, the compounds that were relatively low in the seed and skin fraction were several hydroxycinnamates (chlorogenic acid and three caffeic acid hexosides), citric acid, a glycoside of pantothenic acid (=vitamin B_5), and UDP-glucose. Among the compounds that were significantly different between fruit and breaker samples were, again, several flavonoids and glycoalkaloids (**Table 5**). The relative levels of these compounds increased after the breaking treatment as compared to the intact fruits.

DISCUSSION

In the literature there are several reports on the effects of processing on tomato fruit compounds, such as lycopene, β-carotene, and vitamin C, or on the total antioxidant potential of the processed tomato materials (*4, 6–8*). However, most of these studies involved laboratory scale experiments. In contrast, the samples used in the present study were collected from a real life factory situation. Since mimicking the process in the laboratory may not provide the same end products as the industrial scale process, it seems relevant to follow the real commercial process in order to evaluate properly the effects of processing on the product actually reaching the consumer. In this study we focused on samples taken from a Turkish factory producing tomato paste and analyzed a number of different well-defined processing steps. In order to estimate batch-to-batch variations, for instance resulting from differences in original fruit source or season, each step in the process was independently sampled five times from different fruit batches processed during two successive years.

Antioxidants Are Differentially Affected by Paste Making. The fate of antioxidants during the paste-making process was initially broadly monitored by determining the total antioxidant capacity. Two different extraction methods and a range of spectrophotometric assays were employed, as it is impossible to extract and analyze all antioxidants present in tomato or its products using a single method. The principle of the antioxidant assay, the radical that is generated, the end-point detection, and the allocated reaction time vary considerably from one test to another, and each method has its advantages and disadvantages (*27–29*). We therefore applied different tests to get the broadest overview of the antioxidant capacity and used both hydrophilic and hydrophobic tomato extracts. Notwithstanding the variation in antioxidant capacity values between the different tests applied (**Table 1**), the hydrophilic extracts always contained the majority of antioxidants in all samples analyzed. The general effect observed during tomato processing from fresh fruit toward paste was a modest decrease, both in hydrophilic and lipophilic antioxidant capacities. In the analysis of lipophilic extracts (**Table 1**), the CUPRAC values were highest, indicating that this is a sensitive assay in organic solvents (*23, 24*). The CUPRAC values broadly followed the trend of lycopene (**Table 2**) during the processing. However, generally speaking, the various total antioxidant assays were inconsistent. In our view, such variable results obtained by different assays indicate that total antioxidant capacity data are difficult to interpret without additional investigations toward the individual compounds. Using HPLC with an online antioxidant detection system, we were able to identify vitamin C as the key antioxidant in the hydrophilic extracts, both in the original fruit and in the final tomato paste (**Figure 2**). Next to vitamin C, phenolic antioxidants including rutin, rutin apioside, chlorogenic acid, and naringenin chalcone (only in original fruit) were also identified as major antioxidants in these hydrophilic extracts. In the hydrophobic (chloroform) extracts, all-*trans*-lycopene was by far the most abundant compound detected (**Table 2**). Lycopene is a good antioxidant, with *in vitro* activities higher than those of vitamin C and vitamin E (*30*).

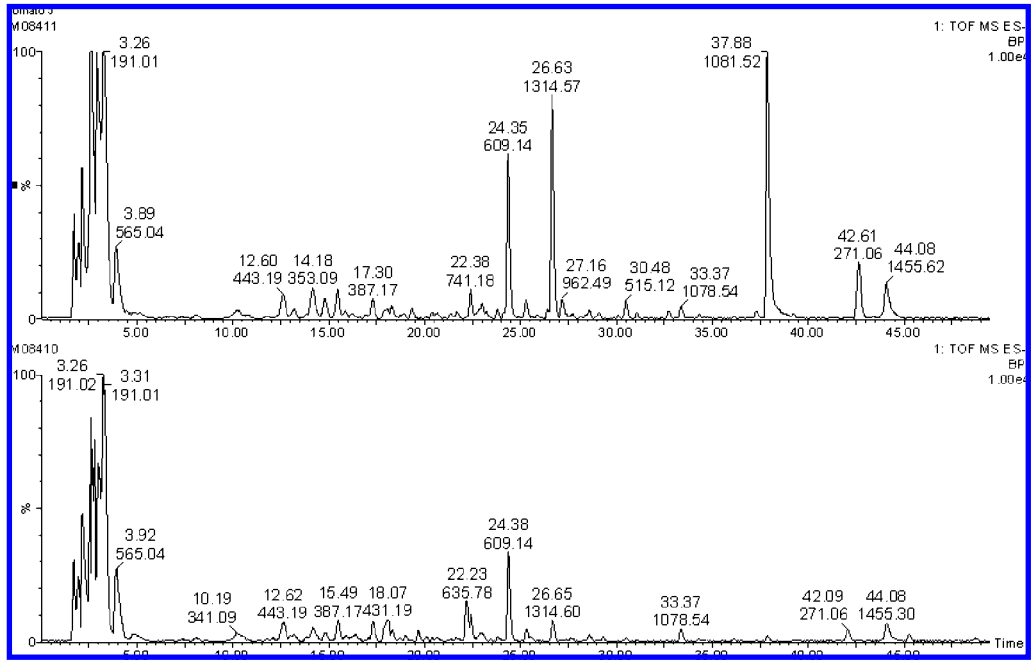

Figure 4. Representative LC-MS chromatograms of aqueous−methanol extracts from fruit (upper panel) and paste samples (lower panel). Numbers at chromatographic peaks indicate retention time (in minutes: above) and accurate *m*/*z* (ESI negative mode: below). Chromatograms are on the same scale.

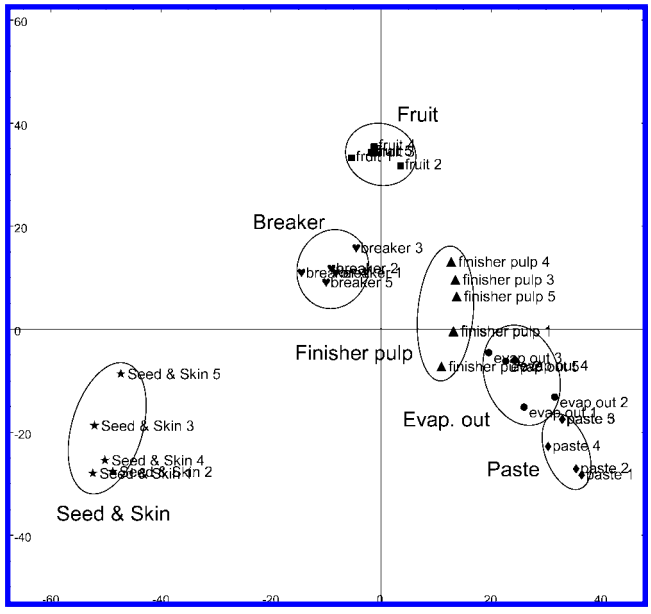

Figure 5. Principal component analysis of untargeted LC-MS based metabolomic data. LC-MS chromatograms of all samples were processed and aligned in an unbiased manner using Metalign software, and mass signal intensities were subjected to multivariate analyses after 2 log transformation and normalization. Independent replicate samples per processing step have been numbered 1−5.

Clearly, paste making has a negative effect on a number of dominant antioxidants in tomato. A gradual and significant decrease (final loss of 50%) in vitamin C was observed in paste compared to the original fruit (**Table 2** and **Figure 2C**). A reduction in vitamin C by heat applications and processing has been reported previously (*4, 8, 18, 31*). In addition to vitamin C, several carotenoids, including lycopene, were significantly affected by the paste-making process (**Table 2**). For lycopene, many researchers have reported significant losses as a result of heat treatments (*4, 6*). In contrast, increased lycopene contents through several thermal treatments and by homogenization in juice or paste production have also been reported (*8*). These increases in lycopene are supposed to result from an increased extractability during fruit processing. Our results, obtained with samples from a tomato paste factory, indicate a gradual and significant decrease in carotenoids (final loss of 32–75%, depending on carotenoid species) upon industrial processing from fruit to tomato paste. It is possible that this result was caused by differential extractability in carotenoids between processing fractions. However, the efficiency of carotenoid extraction by the method used in the present study was the best among five commonly used methods tested, and no residual color remained in the material after extraction. Therefore, we assume that the observed carotenoid losses most likely resulted from oxidation reactions taking place during factory scale tomato processing. Notably, the lipid-soluble antioxidant α-tocopherol (vitamin E) was not affected by the industrial processing, while its biosynthetic precursor γ-tocopherol was significantly lower in paste than in fruit. The concentration of γ-tocopherol was relatively high in the seed and skin fraction, which explains its lower content in the final paste, while that of α-tocopherol was about similar over all fractions. The same trend was observed for the other tocopherol forms, i.e., β-tocopherol and its biosynthetic precursor δ-tocopherol.

The data of total phenolic content (**Table 1**), using the Folin−Ciocalteu assay, indicated that there were no significant changes in these compounds during processing. According to this assay, all fractions contained more or less the same amount of phenolic compounds. In contrast, both the dedicated quantitative HPLC analyses (**Table 2**) and the untargeted metabolic profiling approach (**Tables 3**−**5**) point to significant differences in flavonoids at specific processing steps, with relatively high levels in the seed and skin fraction. It should be noticed that this Folin−Ciocalteu assay is not specific for phenolic compounds as other reducing compounds present in crude extracts may interfere (*32*). We therefore conclude that the Folin−Ciocalteu assay is not suitable for monitoring changes in phenolic compounds during tomato fruit processing.

Table 3. Identified Metabolites Detected by LC-QTOF-MS That Were Significantly Different (Student's *t* Test, $p < 0.05$, $n = 5$) between Original Fruit and Final Paste[a]

RT (min)[b]	metabolite	fruit:paste ratio
42.10	naringenin	0.16
26.57	lycoperoside H	0.54
7.29	pantothenic acid−hexose	1.36
13.17	caffeic acid−hexose	1.46
14.81	chlorogenic acid	1.79
23.85	lycoperoside G or F or esculoside A	2.26
31.06	quercetin−hexose−deoxyhexose−pentose−*p*-coumaric acid	2.36
39.27	tricaffeoylquinic acid	2.87
20.45	esculeoside B	3.04
30.52	dicaffeoylquinic acid	3.14
33.39	lycoperoside A, B, or C	3.39
33.42	naringenin chalcone−hexose	6.70
32.75	naringenin chalcone−hexose	8.64
25.87	lycoperoside G or F or esculoside A	14.53
26.64	lycoperoside G or F or esculoside A	16.86
42.67	naringenin chalcone	22.82
37.90	tomatoside A	62.33

[a] Indicated for each compound is the calculated ratio of the signal intensities of the compound in both samples and the level of significance (*p*-value) of the difference between fruit and paste samples. [b] RT: chromatographic retention (in minutes).

Table 4. Identified Metabolites Detected by LC-QTOF-MS That Were Significantly Different (Student's *t* Test, $p < 0.05$, $n = 5$) between Fruit and Seed and Skin Samples[a]

RT (min)	metabolite	fruit:seed and skin ratio
33.39	a-tomatine	0.07
42.10	naringenin	0.09
22.47	rutin apioside	0.12
33.39	lycoperoside A, B, or C	0.14
42.67	naringenin chalcone	0.16
24.40	rutin	0.19
15.51	quercetin−dihexose−deoxyhexose	0.19
27.41	kaempferol rutinoside	0.19
15.82	naringenin−dihexose	0.23
31.06	quercetin−hexose−deoxyhexose−pentose−*p*-coumaric acid	0.26
37.90	tomatoside A	0.26
39.27	tricaffeoylquinic acid	0.31
32.75	naringenin chalcone−hexose	0.34
26.57	lycoperoside H	0.39
13.85	coumaric acid−hexose	0.39
3.26	citric acid	1.92
13.17	caffeic acid−hexose	2.29
14.81	chlorogenic acid	2.50
11.73	caffeic acid−hexose	2.63
4.83	phenylalanine	2.68
15.90	benzyl alcohol−hexose−pentose	3.15
7.29	pantothenic acid−hexose	3.72
10.19	caffeic acid−hexose	4.51
3.92	UDP-glucose	21.53

[a] Indicated for each compound is the retention time (RT) and the calculated ratio of the signal intensities of the compound in both samples and the level of significance (*p*-value) of the difference between fruit and seed and skin samples.

Metabolic Profiling Using LC-MS. An untargeted LC-MS analysis gave a more comprehensive view of the biochemical consequences of paste making, beyond just carotenoids and vitamin C. In fact, more than 40% of a total of 3177 metabolite-related mass signals were significantly changed during the entire process from fruit to paste. It appears that two steps are specifically important in changing the metabolic profiles during the paste making (**Figure 5**; **Tables 3**−**5**).

First, the removal of seed and skin in the finisher step caused a major change in the overall metabolic profile. This change includes a strong reduction in the level of a number of flavonoids and alkaloids, relative to the original fruit samples (**Table 4**). For the flavonoids for which standards were commercially available, these results were confirmed in the targeted HPLC-PDA analysis (**Table 2**). These flavonoids and alkaloids mostly end up in the seed and skin fraction, which is therefore relatively rich in these compounds. Both the flavonoid and the alkaloid compounds identified have been reported to occur mainly in the epidermal tissue of tomato fruits (*10, 13, 33*). It is therefore likely that the loss of flavonoids and alkaloids during the pasting process resulted from incomplete extraction of these compounds from the epidermal tissue. An exception in this respect is tomatoside A, which is a seed-specific compound (*13*), and is therefore relatively abundant in the seed and skin fraction. The removal of seed and skin material thus seems to have a strong effect, but it should be noted that it consists of only about 3.5% of the total dry weight of the complete fruit. Therefore, the quantitative effects are less dramatic. For instance, the amount of rutin ending up in seed and skin represented about 30% of the amount present in fruit and 14% of that in breaker samples. Likewise, from the total amount of γ-tocopherol present in the

Table 5. Identified Metabolites Detected by LC-QTOF-MS That Were Significantly Different (Student's *t* Test, $p < 0.05$, $n = 5$) between Original Fruit and Breaker Samples[a]

RT (min)[b]	metabolite	fruit:breaker ratio
42.10	naringenin	0.23
33.39	lycoperoside A, B, or C	0.35
24.40	rutin	0.41
42.67	naringenin chalcone	0.47
22.47	rutin apioside	0.49
15.82	naringenin−dihexose	0.56
27.41	kaempferol rutinoside	0.59
26.57	lycoperoside H	0.66
15.51	quercetin−dihexose−deoxyhexose	0.67
20.45	esculeoside B	0.79
7.29	pantothenic acid−hexose	1.34
3.92	UDP-glucose	1.80
12.90	ferulic acid−hexose	1.98

[a] Indicated for each compound is the retention time (RT) and the calculated ratio of the signal intensities of the compound in both samples and the level of significance (*p*-value) of the difference between fruit and breaker samples. [b] RT: chromatographic retention (in minutes).

original fruit, about 10%, was lost by removing the seed and skin fraction.

A second important step is the transition from fruit to breaker (**Tables 2** and **5**). After this step, most flavonoids and some alkaloids were reproducibly increased by a factor 2−3. During this transition, the fruits are washed, transported, and chopped. It is yet unclear which of these activities contribute to the observed enrichment in flavonoids and alkaloids. One explanation could be that the breaking of the fruit triggers a wound response in the fruit tissue. Increases in flavonoids have been frequently reported upon cutting (wounding) of plant tissues (e.g., refs *34* and *35*). Moreover, it is known that wounding of tomato fruit induces production of enzymes such as ascorbate free radical reductase, which are involved in the regeneration of antioxidant compounds (*36*).

The increase of flavonoid-type antioxidants by the breaking step was not accompanied by an increase in the total antioxidant activity but rather by a slight reduction according to most assays (**Table 1**). This apparent discrepancy can be well explained by the fact that vitamin C is by far the most abundant antioxidant (**Figure 2**, **Table 2**), and this specific antioxidant decreases during the same processing step. Notably, we also did not observe a significant change in hydroxycinnamates (phenylpropanoids) such as chlorogenic acid during the breaker step, although hydroxycinnamates and flavonoids share the same precursor (phenylalanine) in their biosynthesis.

In conclusion, on the basis of both dedicated antioxidant analyses and broader metabolomic techniques, we have observed that the most critical events in tomato processing were the breaking step, which causes a significant increase in a range of flavonoids and alkaloids, and the pulping step, after which the seed and skin are removed. Recovery of flavonoids and other compounds from the seed and skin fraction might be considered to serve as a source of functional ingredients for the food industry (*37*) and as a high quality plant protein source (*38*). One could also aim to produce tomato paste that is comparable, with respect to metabolic composition, to the original fruit. This would primarily require a specific improvement in the pulping step, in order to obtain a more efficient extraction from seed and skin tissues. Alternatively, compounds present in the seed and skin fraction may be separately extracted and then returned to the paste. In addition, as the breaker step results in increased amounts of flavonoids, further research into the biochemical processes underlying this aspect and adjustment of the breaker step toward optimized flavonoid levels may help to enhance the levels of these health-related compounds in the final paste.

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