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Research article

Apple juice evaluation: Qualitative analysis and microsatellite traceability

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Abstract: Qualitative and DNA analysis can be performed by taking a multidisciplinary approach to evaluate apple juices, the relevant values of which are a function of the origin, processing method and cultivar used. In detail, the aims of this study were to characterize apple juices through physiochemical analysis, sensory analysis and DNA analysis to evaluate the efficiency of simple sequence repeat (SSR) markers for cultivar identification. Six apple juices made with cv Golden Delicious, cv Granny Smith and a mix of these cultivars from an e-commerce platform (Samples A and B), DISAFA (Samples C and D) and a local farm (Piedmont, Italy) (Samples E and F) were considered. Apple juices A, B, E and F (clarified and pasteurized) can be considered as being of high quality, while Samples C and D were unclarified, unpasteurized and made with apples purchased from a local store. Considering the qualitative analysis, it was observed that the cultivar of apple affected the parameters assessed. In the case of total phenolic compounds, the highest values were observed for juices made only with cv Granny Smith, suggesting how this cultivar contributes to maintaining these nutraceutical compounds more than cv Golden Delicious. Regarding DNA analysis, a limited number of markers, i.e., 4 and 3, respectively, for the apple juices originating from e-commerce and a local farm could successfully produce reproducible amplified fragments. These results can be related to the different procedures used in processing apple juices of different origins.

Keywords: origin; DNA; cultivar; polyphenols; panelist

1. Introduction

The apple is one of the most important fruits in the world, with 87,236,221 tonnes produced per year. China (54%) and the European Union (EU) (15%) are the main consumers of fresh apples. Due

to the high production, globally, the apple crop produces ~4 million tons of waste a year, and juice production is one of the most developed industrial sectors able to absorb apple waste [1]. Among all of the fruit juices, apple juice is second in the market in terms of the global demand for flavor [2].

The quality and authenticity of apple juice are elements of primary importance. Actions such as dilution with water, the addition of sugars or organic acids and replacing or mixing the juice with a cultivar of lower value, or of lower quality from an organoleptic point of view, are economic adulteration. They can compromise the image of the product itself, as well as cause damage to the consumer and the market. The authenticity of fruit materials becomes a serious problem when juices and related items are in short supply or are very expensive.

The fruit content, viscosity, color, browning index and natural flavor are some of the parameters used to evaluate the suitability of fruit juice, but the origin of the raw material (e.g., the cultivar, geographical origin and production mode, such as organic) and the type of processing (industrial or artisanal from a short supply chain) can influence the expectation of consumers, also affecting their attitude to purchase and consume. Among the different analytical techniques able to evaluate and detect the different causes of adulteration (e.g., the addition of or dilution with water, the addition of sugar or acidifying agent adulterants, the adulteration of freshly squeezed juice with concentrated fruit juice), DNA-based methods are largely employed, since DNA can also be recovered in enough quality and quantity in heavily processed food matrices [3,4]. Molecular marker-based methods have been found to be particularly effective in the identification of several crops used in the industry [5–8]. For many products of plant origin, it is necessary to know not only the species, but also the cultivar; this is because, in the supply chain industry, the market prices of a product are largely dependent on the variety cultivated [9].

Among the DNA-based markers, simple sequence repeats (SSRs) have proved very effective for the molecular authentication of food [10]; they have a high level of polymorphism, high reproducibility and can be detected in a very small portion of DNA, which, in the case of fragmented DNA, may constitute an important advantage [11]. SSR markers have been successfully applied to trace several crops, including apples, olives, cocoa and hazelnuts used in transformed products [12–15]. Focusing on fruit juices, there are few studies in the literature. Yamamoto et al. [16] tested 15 SSR markers to identify the variety of pear present in processed fruit products, such as juices, preserves and dried fruit; Wu et al. [17] applied DNA barcoding to small berry fruit products, including juices. Hu and Lu [18] developed an analytical method based on loop-mediated isothermal amplification to authenticate pomegranate juice and avoid fraudulent practices such as the addition of cheaper fruit juices.

In this study, we adopted a multidisciplinary approach to enhance the value of processed apple products, such as apple juices of different origins. In detail, the aims of this study were to characterize apple juices of different origin through physiochemical analysis, sensory analysis and DNA analysis to evaluate the efficiency of SSR markers for cultivar identification.

2. Materials and methods

2.1. Apple juice samples

Six apple juices were considered for this work. The descriptions of the samples used in this study (Samples A–F) are reported in Table 1. Apple juices A, B, E and F can be considered as being of high quality. For juices C and D, apples cv Granny Smith and cv Golden Delicious were purchased from a

local store (Turin, Italy) according to the starch iodine pattern index (free of starch and no stain following the ripening chart for each cultivar).

Fruits were cut and squeezed with a juice extractor in the laboratory of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Torino. The resulting juices were then filtered, using a clean muslin cloth, into sterile conical flasks. Images of the different samples are shown in Figure 1.

Table 1. Description of apple juice samples.

Sample	Composition (cv)	Origin	Description
A	Granny Smith	e-commerce	clarified, pasteurized
B	Granny Smith + Golden Delicious	e-commerce	clarified, pasteurized
C	Granny Smith	DISAFA laboratory processing	unclarified, unpasteurized
D	Golden Delicious	DISAFA laboratory processing	unclarified, unpasteurized
E	Granny Smith	local farm, Piedmont (Italy)	clarified, pasteurized
F	Granny Smith + Golden Delicious	local farm, Piedmont (Italy)	clarified, pasteurized

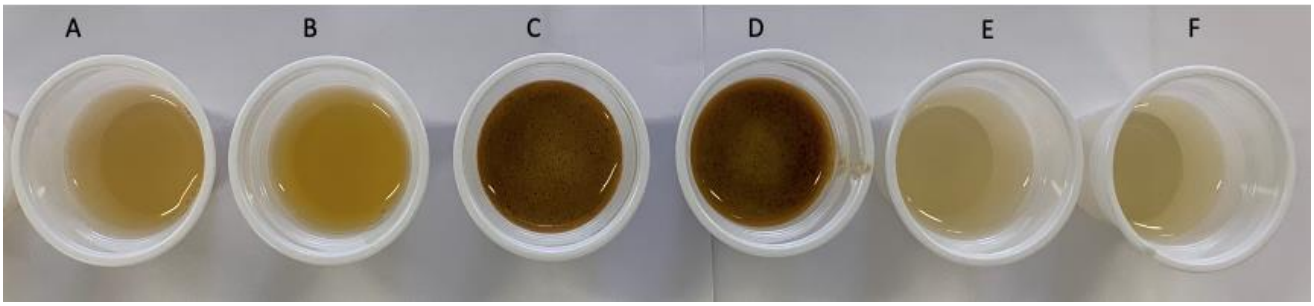

Figure 1. Apple juice samples.

2.2. Physicochemical measurements

The total soluble solids content (TSS) (unit: °Brix) was determined at room temperature (20 ± 1 °C) with an Atago® Pal-1 digital refractometer (Atago Co., Ltd., Tokyo, Japan). Three independent measurements were recorded for each juice sample. The titratable acidity (TA) was measured using an automatic titrator (Titritino 702, Metrohm, Switzerland); it was determined potentiometrically by using 0.1 N NaOH to an end point of pH 8.1. The results are expressed in meq/L. Color was detected with a colorimeter (model: CR-400, Konica Minolta, Langenhagen, Germany). The color parameters were quantified using the CIELAB scale defined by the Commission International de L'Eclairage, in which L* represent lightness/darkness, a* represents redness/greenness and b* represents yellowness/blueness.

The turbidity of the juices was determined by using the method described by Ibrahim et al. [19], which yielded the difference in absorbance at 680 nm of a juice before and after centrifugation (15,000 rpm for 5 min), as expressed as turbidity (%).

The total polyphenolic contents were analyzed by following the protocol of Slinkard and Singleton [20]; 12.5 mL of extraction solvent (500 mL of methanol, 23.8 mL of H_2O, 1.4 mL of HCl) was added to 5 mL of juice; then, 250 µL of the sample was added to 18.5 mL of H_2O, 1.25 mL of Folin–Ciocalteu reagent (Sigma-Aldrich) and 5 mL of 15% Na_2CO_3, followed by incubation for 2 h at room temperature. Absorbance was recorded at 765 nm. The results were calculated as gallic acid equivalents (GAEs) per liter of juice (mg GAE/L). Blank samples were prepared as described for the respective analysis by using the extraction solvent instead of the sample. Spectrophotometric analysis was performed with a UV-Vis spectrophotometer (VWR International).

2.3. Sensory analysis

Sensory analysis was evaluated by considering the visual appearance and taste. Ten untrained panelists (five men and five women, 25–30 years old) from DISAFA received 25 mL of juice for each sample. They provided sample descriptions based on different attributes, including sweetness, acidity, astringency, turbidity, bitterness, color, natural apple flavor and overall judgment [21]. A 9-point scale score was used (ranging from 9, i.e., 'like extremely', to 1, i.e., 'dislike extremely'). Water was used for mouth-rinsing between samples, and the samples were tasted at room temperature (20 ± 1 °C).

2.4. DNA extraction and analysis

The preparation of matrices was carried out by following the protocol that Boccacci et al. [22] applied to musts and wines. For each sample, 30 mL of juice was picked up and placed in Falcon tubes. In each Falcon tube, 0.7 (v/v) of isopropanol was added to promote DNA precipitation. The solutions were incubated at −30 °C for 14 days. Subsequently, the samples were centrifuged at 4 °C and 4000 rpm for 40 min (Centrifuge 5810R, Eppendorf); then, 0.25 g of pellets were taken from each Falcon tube and placed in their respective 2-mL Eppendorf tubes. DNA extraction was performed by following the protocol of Doyle and Doyle [23], which was modified by Han et al. [24] in the following parts: incubation time of 2 h in 2 % cetyltrimethylammonium bromide (CTAB) buffer at 65 °C and incubation time of 48 h in isopropanol at −30 °C to allow DNA precipitation. The final pellet was resuspended in 40 µL of Tris-EDTA buffer.

The concentration and quality of the extracted DNA were checked by using an Ultrospec 2100 Pro® UV-V is spectrophotometer (American Biosciences, Piscataway, New Jersey, USA).

Nine nuclear SSR loci, CH03α04, CH02g09, CH04c07, CH01f02, CH1f07α, CH05e03, CH01d08, CH01h02 and CH04f10 [25], were selected to amplify the extracted DNA. Polymerase Chain Reaction (PCR) amplification was performed in a final volume of 20 µL with the following reagents: 1.7 µL of diluted DNA (10 ng/µL), 0.5 U of Taq-DNA polymerase (Kapa Biosystems, Wilmington, Massachusetts, USA), 2.0 µL of 10X PCR buffer (1.5 mM $MgCl_2$), 200 µM dNTPs, 0.5 µM of each primer and 1 µL of 10% bovine serum albumin (BSA). The PCR conditions included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation (30 s at 94 °C), annealing (45 s at 45 °C) and extension (90 s at 72 °C). The final elongation step was at 72 °C for 30 min.

The fluorochromes 6-FAM, HEX and NED were used to label the forward primers, and the amplification products were analyzed by using a 3130 genetic analyzer sequencer (Applied Biosystems, Foster City, California, USA). GeneMapper software v. 4.0 (Applied Biosystems, Foster City, California, USA) was used to analyze the results, and a GeneScan™ 500 LIZ® size standard ladder was used to estimate the allele sizes.

2.5. Statistical analysis

All statistical analyses were performed by using SPSS Statistics 24 (2017, IBM, Milan, Italy) for a Mac operating system. The data were treated using the analysis of variance method, and the means were separated by using Tukey's test ($P \leq 0.05$).

3. Results and discussion

3.1. Physicochemical measurements

The quality-related parameters for the analyzed samples are reported in Table 2. The quality of the finished juice is heavily dependent on the maturity stage of apples that influences the starchy or green-apple taste. The typical ranges of glucose, fructose and sucrose in apple juice are, respectively, 18–35, 55–80 and 5–30 g/kg. These sugars provide the natural sweetness in juices. Among the six commonly used intense sweeteners permitted in the EU are aspartame, acesulfame K, sucralose and saccharin. They affect the flavor of juice that needs to be labeled as having 'added sugars'. The total content of natural sugars in the studied juices is expressed as the TSS that, as reported in Table 2, varied from 12.8 to 9.2 °Brix. Statistically significant differences can be observed among all of the samples. The quantity and typology of the sugar content in the juice are influenced by the water content and the variety of apple [26], but they are also a function of the geographical location [27]. The cv Golden Delicious in the unclarified and unpasteurized juice (Sample D) showed the highest sweetness, while the lowest value was observed for the clarified and pasteurized juice from the cv Granny Smith cultivar processed by the e-commerce platform (Sample A). The TA of juice, which is correlated with the TSS content, influences juice stability. The samples showed statistically significant differences according to the TSS values. Sweeter samples also showed the lowest TA values, and vice versa. Considering the different origins of the samples, it is possible to affirm that the industrial processing of the e-commerce juices increased the acidity of the cv Granny Smith juices, whose values were, respectively, 114, 105 and 76.0 meq/L for Samples A, E and C.

The color of apple juice is related to phenolic oxidation, which is the main problem for cloudy juice [28]. Among the samples, the cv Granny Smith and cv Golden Delicious juices processed by DISAFA (Samples C and D) were not statistically significantly different in terms of the color parameters. They were the brownest, with b values of 5.19 and 5.08, respectively, which means a color change to red; they also had the highest values of 1.35 and 1.59, respectively, which means a color change to yellow from green. As can be observed in Figure 1, the other juices were colorimetrically similar to each other as a function of their origin; the turbidity values highlight the same.

Table 2. Physicochemical parameters of apple juices and sensory score.

Parameter	Samples					
	A	B	C	D	E	F
TSS (°Brix)	9.2 ± 0.2 e*	11.4 ± 0.0 c	11.4 ± 0.1 c	12.8 a ± 0.1	11.7 ± 0.1 b	10 ± 0.0 d
TA (meq/L)	114 ± 4.3 a	65.9 ± 1.6 d	76 ± 0.1 c	46.3 ± 0.4 e	105 ± 1.5 b	46 ± 0.5 e
L	21.1 ± 1.3 c	23.2 ± 2.5 c	32.9 ± 3.4 a	29.6 ± 1.3 b	24.2 ± 0.6 bc	22.9 ± 1.3 c
a	−0.27 ± 0.1 b	−0.4 ± 0.1 b	1.35 ± 0.6 a	1.59 ± 0.8 a	−0.1 ± 0.4 b	−0.25 ± 0.1 b
b	0.9 ± 0.1 b	0.87 ± 0.1 b	5.19 ± 1.4 a	5.09 ± 1.5 a	0.2 ± 0.7 c	−0.05 ± 0.3 c
Turbidity (%)	0.0005	0.002	0.266	0.283	0.105	0.013
Polyphenols (GAE/1)	27.6 ± 2.1 bcd	17.8 ± 0.4 cd	33.7 ± 4.7 ab	12.9 ± 0.4 d	37.4 ± 6.6 a	27.4 ± 1.4 bc

*Note: Values followed with different letters in a row are significantly different for Tukey's test ($P \leq 0.05$).

The content of phenols is very high in apples, but different studies have reported that these compounds are present at a lower concentration in juices compared to the original matrix. The different processing steps influence their qualitative and quantitative composition, as previously reported [29,30]. Most of the phenolic compounds (58%) [25], such as proanthocyanidins, are known to be maintained in the waste or solid pomace part during juice processing [29,31]. The total phenolic compounds in the samples show statistically significant differences. The highest values were observed for juices made only from cv Granny Smith, suggesting how this cultivar contributes to maintain these nutraceutical compounds more than cv Golden Delicious.

3.2. Sensory analysis

The results of the sensory evaluation performed by panelists are reported in Figure 2. The sensory panel responses show differences among the samples in terms of all attributes considered. Samples B, D and E were the sweetest juices; they were also less turbid and astringent. These juices are based on cv Golden Delicious, which also had the highest °Brix. They were scored best for the overall judgment, to which the natural apple flavor attribute also contributed. These results are preliminary, and they give an indication of a possible consumer judgment, because a specific consumer test should be performed.

3.3. DNA analysis

DNA-based technologies can unequivocally identify a species or variety, and they have proved to be useful tools for a number of food authentication approaches; nevertheless, the number of applications in the assessment of fruit juice authenticity is rather limited [32]. In the present study, we evaluated the results of DNA extraction and SSR analysis for apple juice traceability.

Genomic DNA was extracted from six different samples of fruit juices, which were derived from three different sources (Table 1). DNA extraction from fruit juice is rather difficult; in particular, it is hard to obtain high-quality DNA. A very important step is the preparation of the sample before extraction.

For the sample preparation, the protocol of Boccacci et al. [22] was used, which involves the treatment of the matrix with isopropanol, incubation at −30 ℃ for 14 days and subsequent extraction of DNA from the pellets obtained by centrifugation of the same matrix. It should be noted that the

formation of pellets depended on the type of juice processing and, in particular, on juice turbidity. In fact, for clarified and heat-treated juices such as the samples of industrial origin (both e-commerce and local farm) (Samples A, B, E and F), it was difficult to observe pellet formation.

For DNA extraction, the CTAB-based extraction method [23] was used, but some modifications were necessary to improve the quality of the extracted DNA, such as increasing the incubation time of the samples with the CTAB buffer to 2 h at 65 ℃, as well as increasing the incubation time of the sample after the addition of isopropanol and sodium acetate to 48 h at −30 ℃, to promote DNA recovery.

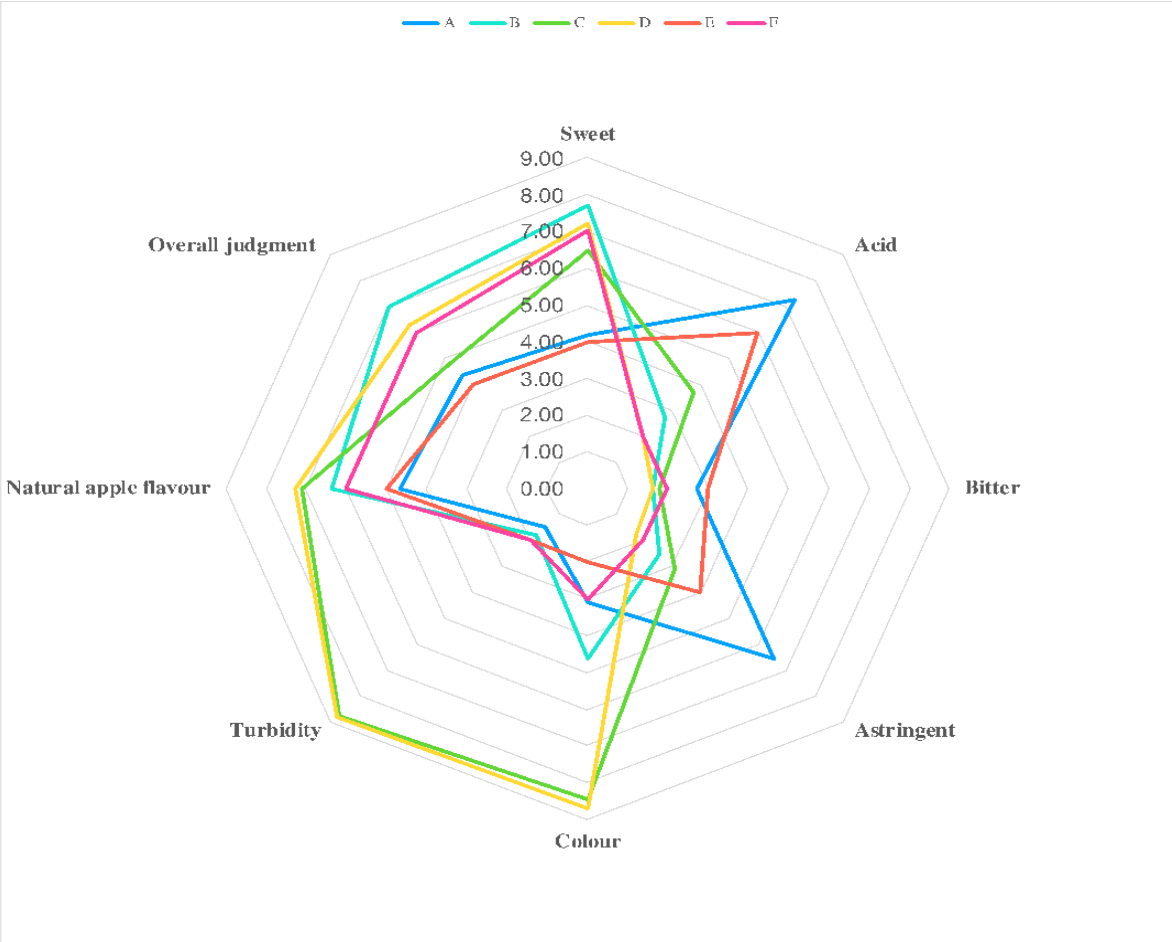

Figure 2. Results of sensory analysis of apple juice samples.

The amount and quality of genomic DNA were evaluated both by agarose gel electrophoresis (Figure 3) and by spectrophotometric analysis; the values of DNA concentration and the 260/230 nm and 260/280 nm ratios are reported in Table 3.

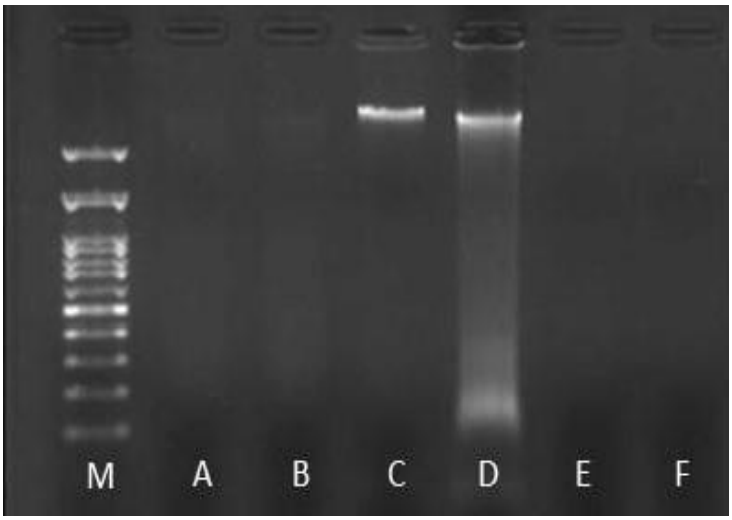

Figure 3. Agarose gel electrophoresis results for the DNA extracted from apple juices by using the method of Doyle and Doyle [23]. Juice from the e-commerce platform (A, B), DISAFA (C, D) and a local farm (E, F); 100 bp DNA Ladder H3 RTU marker (M).

Table 3. Concentration and quality (valued by 260/280 and 260/230 ratios) of DNA extracted from apple juices.

Sample	Composition (cv)	Origin	DNA concentration (ng/μL)	260/280	260/230
A	Granny Smith	e-commerce	82	1.15	0.90
B	Granny Smith + Golden Delicious	e-commerce	95	1.27	0.75
C	Granny Smith	DISAFA laboratory processing	180	1.42	1.61
D	Golden Delicious	DISAFA laboratory processing	210	1.65	1.54
E	Granny Smith	Piedmont (Italy) local farm	59	1.16	0.67
F	Granny Smith + Golden Delicious	Piedmont (Italy) local farm	41	0.88	0.82

PCR amplification of the DNA extracted from all juice samples was performed at nine nuclear SSR loci: CH03α04, CH1f07α, CH04c07, CH01d08, CH02g09, CH04f10, CH01f02, CH01h02 and CH05e03 [33] (Table 4). In order to optimize the amplification of the extracted DNA, BSA was used to overcome the presence of inhibitors, which could hinder the amplification; according to Schrader et al. [34], "the addition of BSA to the PCR reaction mixture is effective against some PCR inhibitors that can interact with nucleic acids, hinder primer pairing or inhibit/alter/degrade DNA polymerase".

The data were compared to the cv Golden Delicious and cv Granny Smith SSR reference profiles, which were obtained by using the DNA extracted from leaves and included in the database developed at DISAFA [35] (data unpublished) in order to check their compliance.

Table 4. Primers used, annealing temperature, allele size range (bp) [33], and cv Golden Delicious and cv Granny Smith leaf SSR reference profiles.

Locus	Ta (°C)	Allele size range (bp)	cv Golden Delicious profile (bp)	cv Granny Smith profile (bp)
CH03α04	55	92–124	119–119	115–117
CH02g09	55	98–138	117–136	117–136
CH04c07	55	98–135	95–112	107–112
CH01f02	55	174–206	170–180	182–206
CH1f07α	55	174–206	176–196	178–200
CH05e03	55	158–190	178–184	168–180
CH01d08	55	238–290	249–271	253–253
CH01h02	55	236–256	246–248	188–242
CH04f10	55	144–254	188–188	242–242

The genetic profiling and subsequent final identification of the cultivar were possible only for the juices produced by DISAFA (Samples C and D), while it was only partially possible or completely impossible for the other juices. In Samples C and D, in fact, all nine primer pairs tested could successfully produce reproducible SSR profiles, and the amplified fragments were the same size as those from the cv Granny Smith and cv Golden Delicious leaf samples.

Regarding the juices of e-commerce and local farm origin, only four SSR markers (CH03α04, CH1f07α, CH04c07 and CH01d08) and three SSR markers (CH04c07, CH01h02 and CH05e03), respectively, could successfully produce reproducible amplified fragments. The other SSR markers did not produce amplification. Nevertheless, by comparing the SSR genotypes at the amplified loci with those included in the database developed at DISAFA, the cv Granny Smith and cv Golden Delicious alleles were identified.

Regarding the juices of local farm origin, in Sample F, only one SSR marker (CH04f10) produced reproducible amplified fragments, while, in Sample E, none of the nine SSR markers produced amplification. In this case, the SSR markers could not be utilized for cultivar identification.

The results obtained depend on the production process. DNA is much more resistant to industrial transformation than other biological components, but physical fragmentation and chemical treatment can affect the yield, integrity and quality of DNA [36]. The production process of the DISAFA juices (Samples C and D) did not include filtration, clarification and heat treatment; the juice was obtained by performing simple pulp pressing, facilitating DNA extraction. In contrast, the production processes of the local juices from the Piedmont area (Samples E and F) and those from e-commerce (Samples A and B) included filtration, clarification and pasteurization, which hindered DNA extraction. Processing of the juices of local farm origin included filtration through a screen, which separates the juice from solid residues and pasteurization at 82 °C for 20 s; the production process of the e-commerce juices (Samples A and B) included filtration, clarification and pasteurization, but no additional details of the production process are available.

Although the production process, for most industrial companies, constitutes an industrial secret, there are several phases of the industrial transformation of apple juices that involve temperatures above 90–95 °C [37], such as thermal inactivation with a high-temperature short-time system of pectinolytic enzymes at temperatures of 95–105 °C in order to maintain the natural opalescence of the juice, or hot filling of glass or tinplate containers at temperatures not lower than 90–95 °C during the product

packaging phase [38]. It is considered that heat treatments result in the breaking down of DNA into small fragments [39] and DNA degradation [40]. In fact, when considering heat treatments, almost all compounds and DNA in apple juice are susceptible to thermal degradation [16], which hinders the amplification of the extracted DNA and the subsequent varietal identification.

4. Conclusion

The development of a multidisciplinary approach to trace apple cultivars along the food chain, such as in juice, could be an important tool to protect apples with appellations of origin, apples belonging to ancient varieties or those whose production is regionally relevant, and to protect consumers and producers from fraudulent practices (e.g., misleading product labeling, the blending of multiple apple varieties into juices sold as monovarietal, substitution of the declared cultivar). This study evidences how the results of physicochemical analysis are largely dependent on the cultivar of apples, while those of DNA analysis are mostly related to apple juice processing. In particular, the high temperature of the processing method can compromise the results obtained, so DNA analysis is very sensitive for apple juice. The simple approach used in this study in terms of the methodology applied could be interesting to share more detailed information about the juice product and supply chain traceability to the consumer via label packaging.

Conflict of interest

The authors declare no conflict of interest.

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