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Changes in the proteomics and metabolomics profiles of *Cormus Domestica* (L.) fruits during the ripening process

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Abstract

Background *Cormus domestica* (L.) is a monophyletic wild fruit tree belonging to the Rosaceae family, with well-documented use in the Mediterranean region. Traditionally, these fruits are harvested and stored for at least 2 weeks before consumption. During this period, the fruit reaches its well-known and peculiar organoleptic and texture characteristics. However, the spread of more profitable fruit tree species, resulted in its progressive erosion. In this work we performed proteomic and metabolomic fruit analyses at three times after harvesting, to characterise postharvest physiological and molecular changes, it related to nutritional and organoleptic properties at consumption.

Results Proteomics and metabolomics analysis were performed on fruits harvested at different time points: freshly harvested fruit (T0), fruit two weeks after harvest (T1) and fruit four weeks after harvest (T2). Proteomic analysis (Shotgun Proteomic in LC-MS/MS) resulted in 643 proteins identified. Most of the differentially abundant proteins between the three phases observed were involved in the softening process, carbohydrate metabolism and stress responses. Enzymes, such as xyloglucan endotransglucosylase/hydrolase, pectin acetylesterase, beta-galactosidase and pectinesterase, accumulated during fruit ripening and could explain the pulp breakdown observed in *C. domestica*. At the same time, enzymes abundant in the early stages (T0), such as sucrose synthase and malic enzyme, explain the accumulation of sugars and the lowering of acidity during the process. The metabolites extraction from *C. domestica* fruits enabled the identification of 606 statistically significant differentially abundant metabolites. Some compounds such as piptamine and resorcinol, well-known for their antimicrobial and antifungal properties, and several bioactive compounds such as endocannabinoids, usually described in the leaves, accumulate in *C. domestica* fruit during the post-harvest process.

Conclusions The metabolomic and proteomic profiling of the *C. domestica* fruit during the postharvest process, evaluated in the study, provides a considerable contribution to filling the existing information gap, enabling the

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molecular and phytochemical characterisation of this erosion-endangered fruit. Data show biochemical changes that transform the harvested fruit into palatable consumable product.

Keywords *Cormus Domestica L.*, Proteomics, Metabolomics, Ethnobotanical heritage, Post-harvest

Background

The valorisation of niche plant species linked to territorial cultural heritage strictly depends on the characterisation of their properties to avoid the risk of cultural erosion. In fact, these plant species represent essential genetic resources for the maintenance of biodiversity and ecosystem services they provide. However, entropic activities and economically more remunerative intensive monocultural agricultural production systems have led to the progressive erosion of these resources [1, 2].

The taxonomy of *Sorbus* historically has been controversial, but molecular phylogenetic analyses have indicated that Sorbus *sensu lato* is polyphyletic, but Sorbus *sensu stricto* and the other five segregated genera, Aria, Chamaemespilus, Cormus, Micromeles and Torminalis are monophyletic [3]. The Service Tree, *Cormus domestica (=Sorbus domestica L.)* (Rosaceae), is a rare and uncommon species of wild fruit tree with a fragmented low-density population found mainly in southern and central Europe. This fruit tree has been known and cultivated since Roman times due to its good resilience and ability to grow on several soil types, even those poor in nutrients. It needs good sun exposure and a mild temperate climate but tolerates water stress, so it is well adapted to the Mediterranean climate. Considered as a forest fruit tree, it provides several ecosystem services for biodiversity maintenance and is also used as an ornamental tree in urban areas [4]. Unfortunately, the natural spread of this species has progressively eroded, and cultivation for fruit production is now even more limited [5]. *C. domestica* fruits can be distinguished according to whether they are pear- or apple-shaped. These fruits with distinctive flavours can be used for various culinary and medicinal purposes [6]. Ripe *C. domestica* fruits suitable for consumption are brown or yellowish coloured and ripen in September-October. The average weight of a *C. domestica* fruit is 25 g and its diameter can reach a maximum of 4.5 cm [7–9].

The Rosaceae family contains several fruits of great economic value, such as apple, pear, cherry, peach and many others, and for these fruits, it is possible to find various multi-omics analyses (metabolomics, transcriptomics and proteomics) which characterise them from a physiological, nutritional and organoleptic point of view during the ripening and post-harvest phases [10–14]. The physiological process of ripening involves drastic changes like alteration in colour, alteration in composition and quantity of sugars, organic and volatile acids that modify flavour and aroma, and alteration to the cell walls that make the fruit softer [15, 16]. Although the ripening process generally makes fruits more palatable, the *C. domestica* fruit flesh remains astringent and tough when harvested. In fact, the fruits are only consumed several weeks after harvest in a state of incipient decay, when the flesh finally reaches a very soft texture and the flavour becomes sweet and pleasant [17]. At this stage, the fruit can be eaten as it is or used to make jam, juice, fruit wine, or cider [18]. Although the *C. domestica* fruits are rich in nutrients and suitable for processing, they are currently undervalued or even unknown by consumers and will need to be enhanced to compete with the wide range of fruits on the market [8, 19]. To date, there are few manuscripts in the scientific literature analysing the phytochemical changes of this peculiar fruit during the post-harvest phases. Multi-omics data collection and integration to follow a physiological process is an approach that provides a high degree of insight, especially when applied to species that have so far been little studied and need to be enhanced [10, 20–22]. Therefore, given the physiological process that precedes the consumption of this fruit, and the lack of information regarding its proteome and metabolome, the aim of this work is to fill these gaps to characterise and valorise this ancient fruit.

Results

Cormus Domestica (L.) post-harvest proteomic profiles

As shown in Fig. 1A and B, proteomic analysis allowed us to identify 643 protein species (Supplementary File1), of which 12% were present exclusively at the time of harvest (T0), 14% shared exclusively between T0 and T1, and most (71.6%) were common to the three maturation stages observed (T0, T1, T2). Interestingly, the extraction yield, and the number of identified proteins decreased at later stages, from T0 to T2 (Table 1).

Statistical analysis, performed by means of Dunn test ($p<0.05$), allowed us to filter the original data set to 160 differentially abundant proteins between the three maturation stages, i.e. between the freshly harvested C. domestica fruit (T0), after 2 weeks (T1) and 4 weeks (T2) of post-harvest ripening (Table 1, Supplementary File 2). To obtain an overview of the functional classes they belong, proteins were sorted according to GO classes for Molecular Function (Supplementary Fig. 1), Biological Process (Supplementary Fig. 2) and Cellular Component (Supplementary Fig. 3) via Mercator4 V2.0 (https://mapman.gabipd.org/app/mercator) [23] and are depicted in Fig. 1B. Most proteins were involved in carbohydrates, amino acids, protein and lipid metabolism, cell

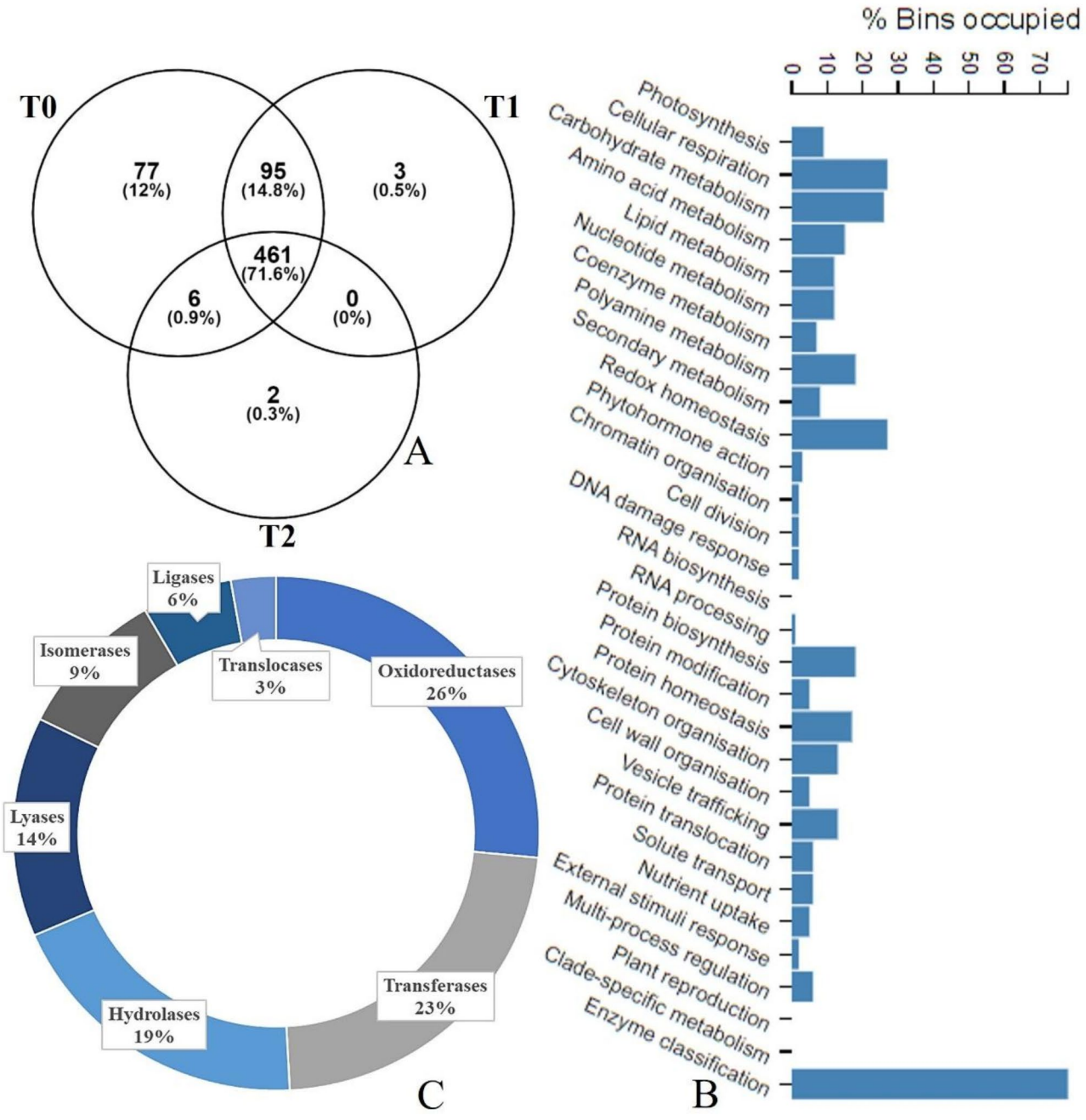


Fig. 1 A: Venn diagram showing the number of identified proteins shared at the three different ripening stages analysed (T0 freshly harvested fruit, T1 fruit at the usual stage of consumption, 2 weeks after harvest, T2 fruit 4 weeks after harvest). B: Percentages of proteins identified at the three stages T0, T1, T2, identified as belonging to functional classes according to Mercator (https://mapman.gabipd.org/app/mercator). C: Follow-up on proteins identified as enzymes divided into enzyme classes in percentage abundance

wall and cytoskeleton reorganisation. Several proteins corresponding to enzymes involved in cell wall modification and fruit softening process, such as xyloglucan endotransglucosylase/hydrolase (A0A498IEK2), expansin (A0A498IEG1), pectin acetylesterase (A0A498IJ39, A0A498IYP7, A0A5N5HPZ0), beta-galactosidase (A0A498KCW4, A0A5N5GFK6) and pectinesterase (A0A0B5W3U1, A0A0M3TB64, A0A5N5FU34) were most abundant in T1 stage. The enzyme malate dehydrogenase (A0A498I601, A0A498I8M1, A0A540KIX2) was consistently abundant in the three observed stages, whereas fructose-bisphosphate aldolase (A0A5N5HXN8,

Table 1 Average protein extraction yield (µg/g) obtained from the three biological replicates for each ripening stage. Number of proteins identified for each post-harvest stage observed (T0: freshly harvested fruit, T1: fruit 2 weeks after harvest, T2: fruit 4 weeks after harvest). Number of differentially abundant proteins (Dunn test $p < 0.05$) between the different post-harvest stages observed (supplementary File1-2). The terms up/down stand for more/less protein abundance in the first sample compared to the second one

	T0	T1	T2	Statistically significantly different		
Extraction Yield µg/g sample	804	836	700	**T0 vs. T1**	84	80 up	4 down
Identified Proteins	638	559	469	**T0 vs. T2**	128	116 up	12 down
Ripening stage-exclusive protein	77	3	2	**T1 vs. T2**	29	22 up	7 down

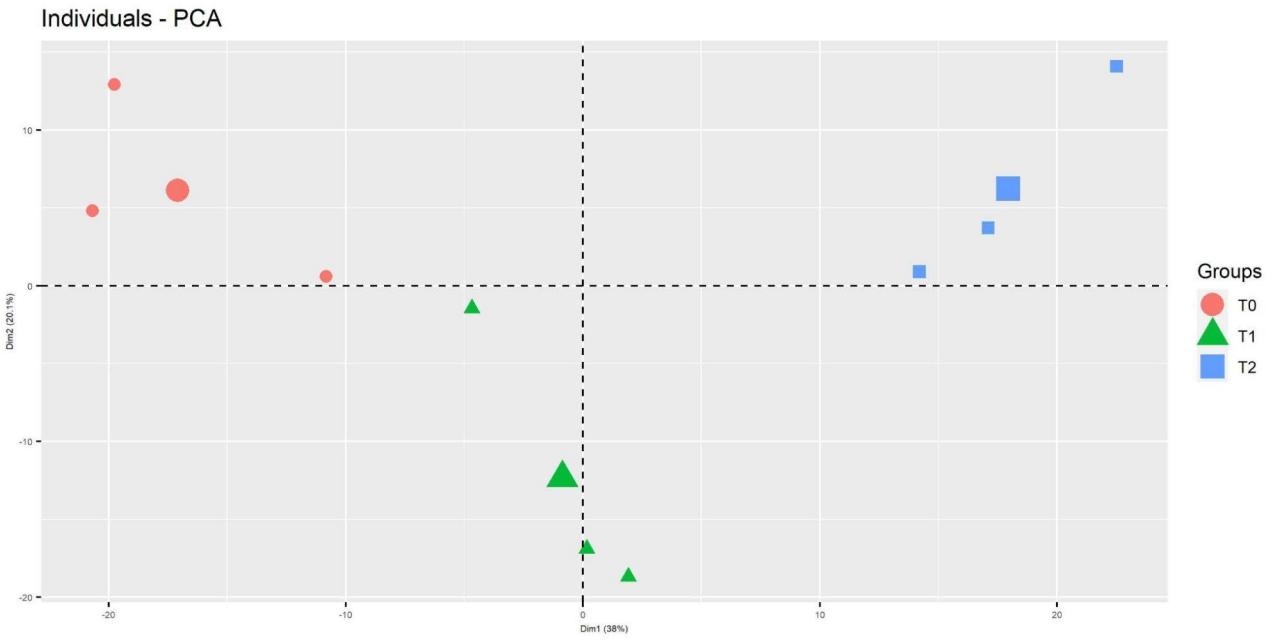
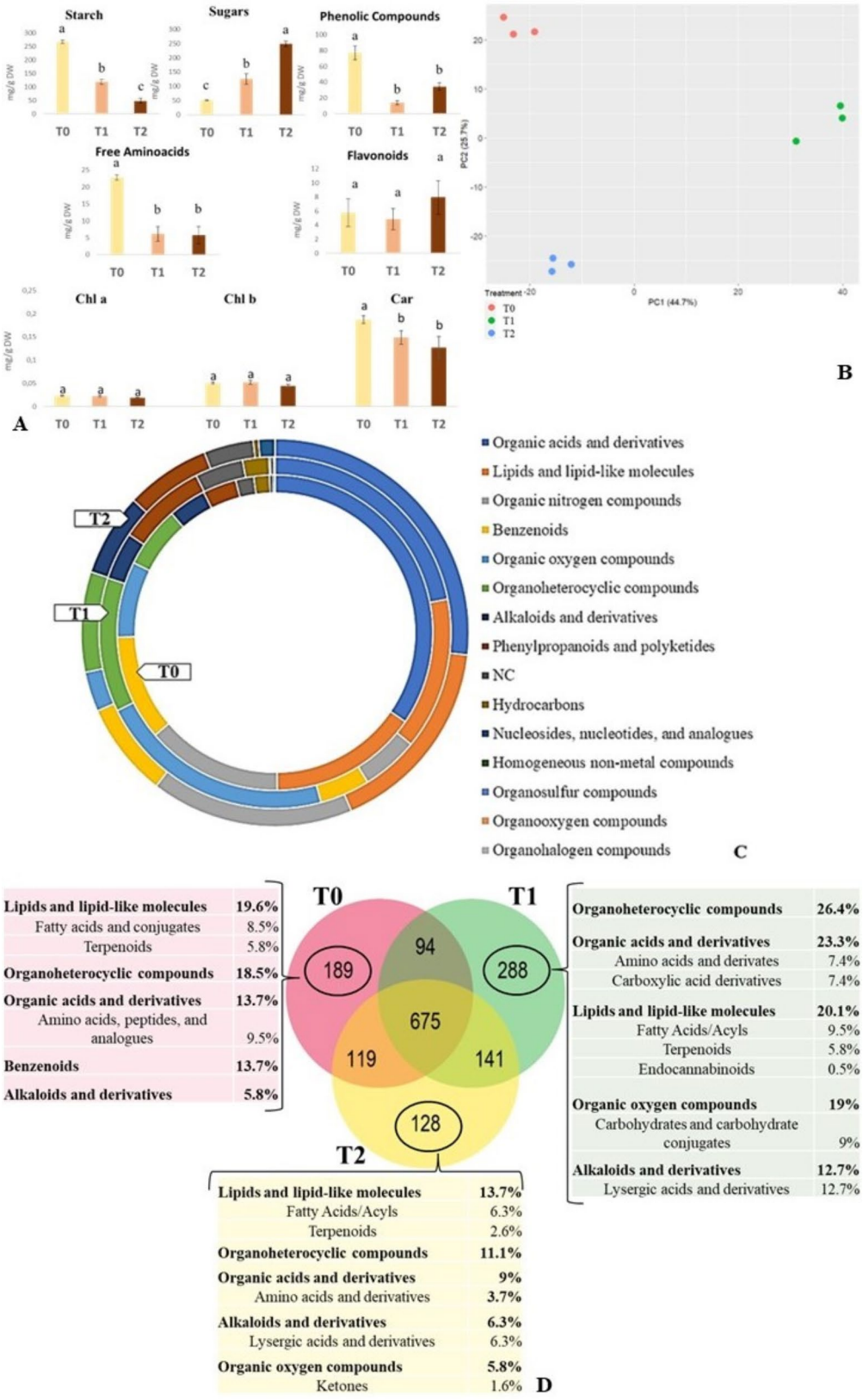


Fig. 2 Two-dimensional principal component analysis (PCA) score scatter plot showing clusters of samples (three replicates for each ripening stage under analysis) based on their similarity. Mean point is also plotted as the biggest icon for each group

A0A540KUY8) was more abundant ($p < 0.05$) in T2 and showed the opposite trend to glucose-1-phosphate adenylyltransferase (A0A498HC29) and glucose-6-phosphate 1-dehydrogenase (A0A5N5GTK5). Regarding the decrease in acidity, several malate dehydrogenases (A0A498I601, A0A498I8M1, A0A540KIX2) were identified with a constant or significantly increasing trend during the ripening process. In addition, citrate synthase (A0A165FX72) was more abundant in the early stages of post-harvest process T1 > T0, being non-detected in T2 (Supplementary File1). Furthermore, proteins with enzymatic activity represented the most abundant class, comprising 181 accessions. The intense enzymatic activity, evident when observing the ripening process of *C. domestica*, involved all the enzyme macroclasses (Fig. 1C) with the following abundances: 26% oxidoreductases, 22% transferases, 19% hydrolases, 13% lyases, 9% isomers, 5% ligases, and 2% translocases. In addition to several enzymes involved in scavenging reactive oxygen species (ROS), proteomic analysis revealed the abundance of several Heat Shock Proteins. Also, some peculiar proteins related to the physiological stress induced by the ripening process in the fruit have been identified. It was the case of abscisic stress ripening protein (A0A498JY74), that accumulated in T2, remorin (A0A498JK49) that showed a peak in abundance in T1, and spermidine synthase (A0A5N5HGV6) that did not change in abundance among the three stages.

The PCA analysis (Fig. 2) clearly showed the degree of homogeneity of the biological replicates and the good separation of the samples at their maturation stages. It also showed that the main multivariate difference was observed between T0 and T2, in agreement with the univariate test (Dunn test). The top fifty proteins that contribute most to the variability among stages bases on cos2, (Supplementary Fig. 4) were identified, showing how certain proteins of significant relevance to the physiological process of ripening were characteristic variables across different phases. For example, beta-galactosidase (A0A498KCW4) and citrate synthase (A0A498HN15) were characteristic of T0, pectinesterase (A0A5N5FU34) of T1, and lipoxygenase (S4ULE0) and mandelonitrile lyase (A0A498KFV8) of T2.



Lipids and lipid-like molecules	19.6%
Fatty acids and conjugates	8.5%
Terpenoids	5.8%
Organoheterocyclic compounds	18.5%
Organic acids and derivatives	13.7%
Amino acids, peptides, and analogues	9.5%
Benzenoids	13.7%
Alkaloids and derivatives	5.8%

Organoheterocyclic compounds	26.4%
Organic acids and derivatives	23.3%
Amino acids and derivates	7.4%
Carboxylic acid derivatives	7.4%
Lipids and lipid-like molecules	20.1%
Fatty Acids/Acyls	9.5%
Terpenoids	5.8%
Endocannabinoids	0.5%
Organic oxygen compounds	19%
Carbohydrates and carbohydrate conjugates	9%
Alkaloids and derivatives	12.7%
Lysergic acids and derivatives	12.7%

Lipids and lipid-like molecules	13.7%
Fatty Acids/Acyls	6.3%
Terpenoids	2.6%
Organoheterocyclic compounds	11.1%
Organic acids and derivatives	9%
Amino acids and derivatives	3.7%
Alkaloids and derivatives	6.3%
Lysergic acids and derivatives	6.3%
Organic oxygen compounds	5.8%
Ketones	1.6%

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 A: Concentrations of Starch, Sugars, Phenolic Compounds, Free Amino Acids, Flavonoids and Photosynthetic Pigments (Chlorophyll a, b and Carotenoids) at the different ripening stages (T0 - freshly harvested fruit, T1 - fruit after 2 weeks of post-harvest ripening at the usual stage of consumption, T2 - fruit after 4 weeks of post-harvest ripening); different letter indicate significant difference among ripening stages (ANOVA with Tukey post-hoc comparison, $p < 0.05$). B: PCA analysis of the identified metabolites shows a good replicates clustering between the tree stages T0, T1 and T2. C: Metabolites macro-clustering with relative percentage abundances in *C. domestica* fruits during the post-harvest ripening process. D: Venn diagram showing unique or shared metabolites in multiple stages with focus on metabolites present exclusively in one of the three stages (T0, T1, T2) and most abundant classes in percentage (Supplementary File 4)

Cormus Domestica (L.) metabolomic profiles at post-harvest ripening

Initial analyses carried out on the fruit samples under analysis (T0, T1 and T2) showed a marked reduction in starch content from an average of 267 mg/g in the freshly harvested fruit (T0) to 48 mg/g in the fruit at the end of the ripening process (T2), and an increase in sugar levels from an average of 50 mg/g to 248 mg/g during ripening. In contrast, no significant change in chlorophyll and carotenoid content was observed (Supplementary File 3, Fig. 3).

Metabolomic analysis resulted in 4377 features resolved in positive ionisation mode, of which 2334 had putative annotation (Supplementary File 4). A statistical analysis was performed on the 1752 variables thus selected (consistency 0.25), obtaining 606 statistically significant differentially abundant metabolites. All the metabolites were classified using ClassyFire (http://classyfire.wishartlab.com/). As shown in Fig. 3C, the metabolic profile of the three ripening stages underwent consistent alterations, and the profile of the intermediate stage (T1), had more Alkaloids and Organoheterocyclic compounds and less Benzenoids than the under and over-ripened stages (Supplementary File 4). Figure 3D shows a Venn diagram where it can be seen that of the metabolites identified, 675 were common to the three ripening stages (T0, T1, T2), 94 shared between T0 and T1, 141 between T1 and T2 and 119 between T2 and T1. It is also possible to observe a focus on the metabolites unique to only one of the three post-harvest phases, and which classes were the most represented. T1 has a higher percentage of metabolites belonging to the Organoheterocyclic compounds and Organic acids class than the T0 and T2 phases. As far as molecules of a lipidic nature are concerned, including terpenoids, although these were unique compounds peculiar to each class, the trend in class abundance remained almost constant at T0 and T1 and then decreased at T2 (Supplementary File 4).

A principal component analysis (PCA) showed a good clustering of the replicates and separation of the stages (Fig. 3B). Table 2 shows the main metabolites characterising the T1 stage. The main metabolites at T0 and T2 stages, in which the fruit is not routinely consumed, are presented in Supplementary Tables 1, and prominent among them were Chlorogenic acid, 4-Coumarylalcohol, Caffeic acid, Citric acid and Pipecolic acid. These were filtered maintaining a variable consistency of 0.25. Furthermore, a PCA with the 50 top-score variables (based on cos2) is shown in Supplementary Fig. 5.

As the results showed, the class of organoheterocyclic compounds was the most represented in the T1 stage. To this superclass belonged certain metabolites present exclusively at one of the three post-harvests ripening stages under analysis. For example, freshly harvested (T0) fruit had compounds such as patulin and nicotinamide that were not found in the other stages. The usual eating stage, the richest in these metabolites, had exclusive compounds such as furanone, aspilactonol C, pantolactone, kinetin and carbazoles. The last observed stage (T2) had unique compounds such as piperidine and agnestin B. In the case of metabolites belonging to the class of carbohydrates and carbohydrate conjugates, an increased abundance was also observed at the T1 stage. In fact, while for some compounds, such as glucose and maltose, an accumulation trend was observed during the process, others had a Gaussian trend, or were present exclusively at the consumption stage (T1) such as apiitol or biflorin. The accumulation of these sugars was consistent with respect to starch splitting, as confirmed by the results shown above. Among the metabolites belonging to the class of lipids and lipid-like molecules, the most abundant in our samples were oleamide and hexadecanamide. To the same class of metabolites belonged several terpenoids found in all post-harvest ripening phases analysed (T2) such as maslinic acid and p-cymene. In addition, several compounds that contribute to the fruit's aromatic notes, such as citral, limonene, pulegone and camphor, although present at all three ripening stages analysed, reached a peak in abundance at the T1 stage, in which the aromatic bouquet was enriched with compounds such as borneol and capsidiol. An interesting result was the presence of endocannabinoids (2-Arachidonoyl glycerol) most abundant at the fruit harvest stage (T0). The class of lysergic acids and derivatives belonged several compounds that exhibited different abundance trends. Among these, the metabolites most representative of the T0 stage were chlorogenic acid and caffeic acid, whose values then decreased with ripening. The T1 stage was characterized by the abundance of citric acid, pipecolic acid, sorbic acid, and adipic acid and the exclusive presence of compounds such as almecillin and mirubactin. In contrast, some of the metabolites present in all stages of ripening of the *C. domestica* fruit, but most abundant in T2, were acetoacetic acid, asperterreusine A, salicylic acid

Table 2 List of compounds most characteristic of the usual stage of consumption of the fruit of *Cormus Domestica* (L.)

Compound	Class/Subclass
5-hydroxy-4-methoxy-5,6-dihydro-2 H-pyran-2-one	Pyranones and derivatives
Oleamide	Fatty Amides
2-Hexadecylpyridine	Pyridines and derivatives
D-(+)-Proline	Amino acids, peptides, and analogues
(4-Methylphenyl)-2-thienylmethanone	Carbonyl compounds
(R)-4-Dehydropantoate	Fatty Acyls
4-(Trifluoromethoxy)phenylacetic acid	Phenol ethers
acetoacetic acid	Lysergic acids and derivatives
2-Hydroxy-2_4-pentadienoate	Fatty Acyls
2-(1-Piperidinylmethyl)cyclododecanone	Piperidines
Piptamine	Phenylmethylamines
Trihexyl 2-(butyryloxy)-1,2,3-propanetricarboxylate	Tetracarboxylic acids and derivatives
Bis-D-fructose2′_1:2_1′-dianhydride	Carbohydrates and carbohydrate conjugates
2-ACETYL-3,5-DIMETHYLBENZO(B) THIOPHENE	Benzothiophenes
13(S)-HOTrE	Fatty Acyls
1-Dodecyl-2-pyrrolidinone	Fatty Acyls
Resorcinol	Benzenediols
Rickiol C	Macrolides and analogues
Dimethylpyruvic acid	Short-chain keto acids and derivatives
D-(+)-Glucose	Carbohydrates and carbohydrate conjugates
(R)-Malate	Hydroxy acids and derivatives
(2R_3S)-2_3-Dimethylmalate	Hydroxy acids and derivatives
Hexadecanamide	Fatty amides
Bis(2,2-dihydroxyethyl) hydrogen phosphate	Non-metal phosphates
3-Hydroxyaminophenol	N-phenylhydroxylamines
Sulcatol	Fatty alcohols
Myristamine oxide	Aminoxides
2,4-Xylidine	Xylenes
(3aS,4R,5 S,7aR)-4,5-dihydroxy-7-methyl-3a,4,5,7a-tetrahydrobenzo[1,3]dioxol-2-one	Carbonic acid diesters
Gephyronic acid (hemiketal)	Terpene glycosides

and palmitic acid. Another class of particular interest in the delineation of the metabolomic profile of the fruit is that of the phenolics and polyketides, to which belonged several well-known phytocompounds such as coumarin, fustin, and taxifolin, whose concentration decreased appreciably in the observed phases (T0>T1>T2), and others with peaks of abundance at T1 such as Quercetin-3β-D-glucoside and trifolin.

Discussion

Although much information is available for other fruits belonging to the Rosaceae family regarding physiological and biochemical changes along the ripening process [13], little is known about postharvest changes in *Cormus domestica* (L) fruit. This small, ancient fruit, widespread in the Mediterranean, and closely connected to traditional and rural culture, is unfortunately at serious risk of cultural erosion due to the wider spread of intensive cultivation of fruits with wider economic value such as the apple or pear [24]. The ripening process is a genetically programmed, irreversible and highly regulated process involving changes in the fruit structure, organoleptic properties and chemical composition. Generally, the combination of physiological changes makes the fruit more palatable for consumption. The main alterations of a ripening fruit involve its colour, texture, flavour and aroma. The colour change is mainly due to the chloroplasts turning into chromoplasts and the accumulation of neo-synthesised pigments. The pulp softening is due to the alteration of the cell walls, the sweetening to the accumulation of sugars and the reduction of sour components, and the production of various metabolites contribute to the attainment of aroma and flavour [15, 25–27]. Traditionally, *C. domestica* fruits are harvested fully developed, and eaten after a ripening period in a cool, dry and airy room in the dark for about a month until they reach a soft consistency. Only then will the fruit have lost its acrid, woody flavour and become very sweet and juicy. At this stage it is consumed as it is or used for the production of jams, jellies or spirits [5, 28]. In this work, we collected proteomics and metabolomics data at different stages of post-harvest ripening in order to delineate the major physiological changes in the fruit of *C. domestica*, for which few data were available in the literature. The pronounced colour change, usually observed during the fruit ripening [29] was not observed in *C. domestica* fruit, which, as shown by analyses of photosynthetic pigment content and in the sample photos shown, did not accumulate carotenoids during ripening, but rather took on a brownish colour mainly due to the natural oxidative processes involving the fruit in postharvest ripening [30]. The phenomenon of pulp browning was due, as evidenced by metabolomic analysis, to the accumulation of chlorogenic acid and its subsequent oxidation by polyphenol oxidase (PPO). This enzyme, which was found to be constant by proteomics analysis in the post-harvest ripening stages, catalyses the oxidation of phenols to quinones and the reduction of oxygen to water, with concomitant protein complexation and the formation of melanin pigments that give this ripe fruit its

characteristic colour [31]. In addition to colour change, ripening includes an essential softening process due to hydrolytic processes resulting in the breakdown of cell wall polymers (cellulose, hemicelluloses and pectins) by wall enzymes that are essential for softening the fruit and improving its chewability [16, 32]. One of the most abundant functional classes revealed by our proteomic analysis is that involved in cell wall changes. In fact, the results highlighted that the abundance of enzymes such as b-galactosidase and pectinesterase acting on the pectic component of the cell wall, which is mainly responsible for the hardness of unripe fruits. Specifically, the role of β-galactosidase (β-Gal) in the apple ripening process has been studied and its involvement in modulating the firmness and the pectin content has been confirmed and appears to be induced by ethylene [33, 34]. These enzymes, like others that act on hemicelluloses (XET) and non-enzymatic proteins such as expansin, accumulated abundantly in the fruit of *C. domestica* already in the early stages of post-harvest ripening. The enzymatic activity of the cell wall in the fruit of *C. domestica* made it particularly soft in consistency, but this, together with the accumulation of sugars and oxidative processes, significantly reduced its shelf life [16]. Reactive oxygen species (ROS) are inevitably produced by metabolic pathways activated during the fruit ripening process. The ROS accumulation causes oxidative damage by reacting with nucleic acids, proteins and lipids. Simultaneously with their production and accumulation in plant tissue, plants activate the recovery system and produce enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), glutathione reductase (GR), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and antioxidant compounds such as ascorbate, glutathione, flavonoids, and tocopherols, which were found to accumulate in the *C. domestica* fruits under analysis and as in other fruits belonging to the Rosaceae family [13, 35]. The accumulation of antioxidant enzymes and soluble antioxidant compounds such as flavonoids has also been observed in our samples. In particular, the accumulation of quercetin-3β-D-glucoside, taxifolin, fustin and coumarin increased the nutraceutical power of this ancient fruit [36, 37], which has a general anti-inflammatory effect and can modulate the lipid peroxidation involved in several diseases such as atherogenesis, thrombosis, and carcinogenesis, especially at the habitual stage of fruit consumption to decay then 4 weeks after harvest. Ripening is a physiological process, highly regulated with pathways that are still not clearly defined and understood, and which interconnects at several levels with abiotic stress response pathways [25, 38]. The post-harvest ripening process induced in *C. domestica* fruit the accumulation of Abscisic stress ripening protein (ASR), a protein induced by ABA, stress and ripening. Several studies have shown that ASR gene expression varies between species, organs and conditions, and appears to be involved in fruit ripening, although its mechanism of action is not yet well understood [39, 40]. Similarly, Remorin, a protein involved in the regulation of several physiological processes, showed a peak in abundance two weeks after *C. domestica* fruit harvest, and its contribution in ripening process regulation is known from the literature [41]. Other proteins involved in stress and energy metabolism such as Small Heat Shock Proteins (sHSPs) were found to be abundant in the ripening of *C. domestica* fruit. These chaperones have been found in several proteomics studies related to abiotic and developmental stress response that have been characterised in ripening fruits [34, 42]. During ripening, a complex regulatory mechanism shifts metabolism from starch synthesis to starch degradation and the accumulation of soluble sugars, mainly sucrose. This conversion is responsible not only for fruit sweetness, but also for providing energy for the metabolic processes taking place during ripening [43, 44]. As expected and evidenced by both metabolomic and proteomic analyses, also in the post-harvest ripening process of the *C. domestica* fruit followed in this experiment, a marked conversion of starch to soluble sugars was observed and the levels of the enzyme sucrose synthase remained high until the end of the ripening process. The main soluble sugars in fruits such as apples or pears are sucrose, fructose and glucose, which were also particularly abundant in the *C. domestica* stage of consumption; as were some sugar alcohols such as sorbitol [11]. Furthermore, metabolic analysis confirmed the gradual but significant accumulation of maltose and glucose as ripening progressed. In addition to sweetness, another important parameter that determines the organoleptic characteristics of the fruit is acidity, mainly due to the presence of organic acids accumulated in the vacuole [45]. In apples and pears, the main ones are malic acid and citric acid, and the lowering of the degree of acidity is mainly due to the investment of these organic acids in the respiration process and the production of secondary metabolites [32]. Citrate synthase and Malic enzyme accumulated in the *C. domestica* fruit during ripening, causing a reduction in malate and contributing to the decline in fruit acidity. Several peculiar metabolites were found to be particularly abundant in our samples, especially in the abiutal consumption stage. Among these piptamine and resorcinol, are well-knownfor their antimicrobial and antifungal properties [16]. In addition to firmness and sweetness, another important parameter characterising a ripe fruit is its olfactory characteristics. These depend on hundreds of volatile compounds that are produced differentially during the various stages of ripening and are responsible

for aroma production. Volatile compounds vary widely in different fruits and are generally aldehydes, alcohols, ketones, lactones, esters and terpenoids [46]. Because aroma is closely associated with phytochemicals that make fruit healthy, the health benefits for the consumer are not limited to the satisfying experience for the senses (taste and smell). Metabolomic analysis has allowed us to identify several compounds particularly abundant in the preferential eating stage of the *C. domestica* fruit that together provided the fruit with a peculiar aroma. Citral and limonene, in addition to inducing the jasmonic acid pathway to endow fruit with resistance to biotic stresses, confered fresh and citrus notes [47]. The abundance of camphor, pulegone and borneol added intense minty notes, while benzophenone soft, fresh notes with touches of fruit and flowers. Typically these compounds not only impart the final aroma to the fruit but also have antimicrobial and anti-inflammatory properties [15, 48, 49].

Conclusions

Cormus domestica is an ancient fruit tree, widespread in the Mediterranean area, closely linked to traditional culture and at serious risk of cultural erosion. This work analysed the post-harvest ripening process of the *Cormus domestica* fruit through a multi-omics approach, integrating the proteomic and metabolomic approaches to characterise both the biological process and the peculiarities of this ancient fruit and fills an information gap. The multiomics approach used revealed the involvement of several enzymes in C. domestica fruit that participate in cell wall softening such as xyloglucan endotransglucosylase/hydrolase, expansin, pectin acetylesterase, beta-galactosidase and pectinesterase. In response to reactive oxygen species (ROS) generated during ripening, *C. domestica* activates a robust antioxidant defense system. Enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), and peroxidase (POX) work alongside non-enzymatic antioxidants like flavonoids (quercetin-3β-D-glucoside, taxifolin, fustin, and coumarin) to mitigate oxidative damage. These antioxidants not only protect the fruit but also enhance its nutraceutical value. However, especially in the final stage, close to the fruit's decay, a marked browning of the pulp appears to be associated with the accumulation of chlorogenic acid and its subsequent oxidation by polyphenol oxidase. While the particularly sweet taste of this ancient fruit is mainly due to the rapid conversion of starch into simple sugars and the reduction of the acid component due to the accumulation of citrate synthase and malic enzyme during ripening and the reduction of malate. The aroma, appreciated and recognised by consumers, seems to depend on a broad spectrum of compounds such as citral, limonene, camphor, pulegone and borneol, which in addition have antimicrobial and anti-inflammatory properties. The latter were of particular interest because, in addition to characterising the aromatic bouquet of the fruit under analysis, they also lay the foundations to establish the nutraceutical properties already known and inherited from in rural popular culture.

Methods

Plant material

The fruits of *Cormus domestica* (L.) were collected in Campania, Italy in September 2021 from three different trees located in the same area with slightly alkaline, sandy and well drained soils (Fig. 4). The voucher specimens (PAL-22824) were deposited at the *Herbarium Mediterraneum Panormitanum* of the botanical garden of the University of Palermo. Samples were identified by Professor Carmine Guarino using the dichotomous key found in Flora d'Italia [50] and performed, after the species identification, nomenclatural correction of synonyms through the digital resources of the Kew Botanical Garden (http://www.plantsoftheworldonline.org/). The climate in this area is typically Mediterranean, with average minimum winter temperatures of 8 °C and 24 °C in summer and average annual rainfall of around 900 mm [51]. The harvested fruits (stage T0) were transported to the laboratory where the traditional post-harvest ripening procedure for these fruits was applied, which involves staying in a ventilated, cool and dry place in the dark for a period of about 2 weeks (stage T1). Ripening continued for a further 2 weeks until the fruit had completely turned brown (stage T2).

Proteomics analysis

Protein extraction and digestion

Three fruits for each stage considered (T0-T1-T2) were washed in deionised water, freeze-dried, and pulverised in liquid nitrogen to extract proteins separately from three independent biological replicates. For each sample 0.2 gram of pulverised material was used for total protein extraction with a modified protocol of the classical TCA/Acetone-Phenol extraction [52]. After precipitation in 5 volumes of ammonium acetate in methanol and three washes with cold acetone (3 ml), the pellet was dried under vacuum and solubilised in 100 µl of a solution containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% (w/v) Triton X-100, and 100 mM DTT) and centrifuged to remove insoluble material (12,000 g 3min 4 °C). Using the Bradford method and bovine serum albumin as a standard, the protein concentration obtained was then estimated [53]. The protein extract (80 µg) was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN cell (8.6×6.7 cm; Bio-Rad) using 12% polyacrylamide gel and voltage of

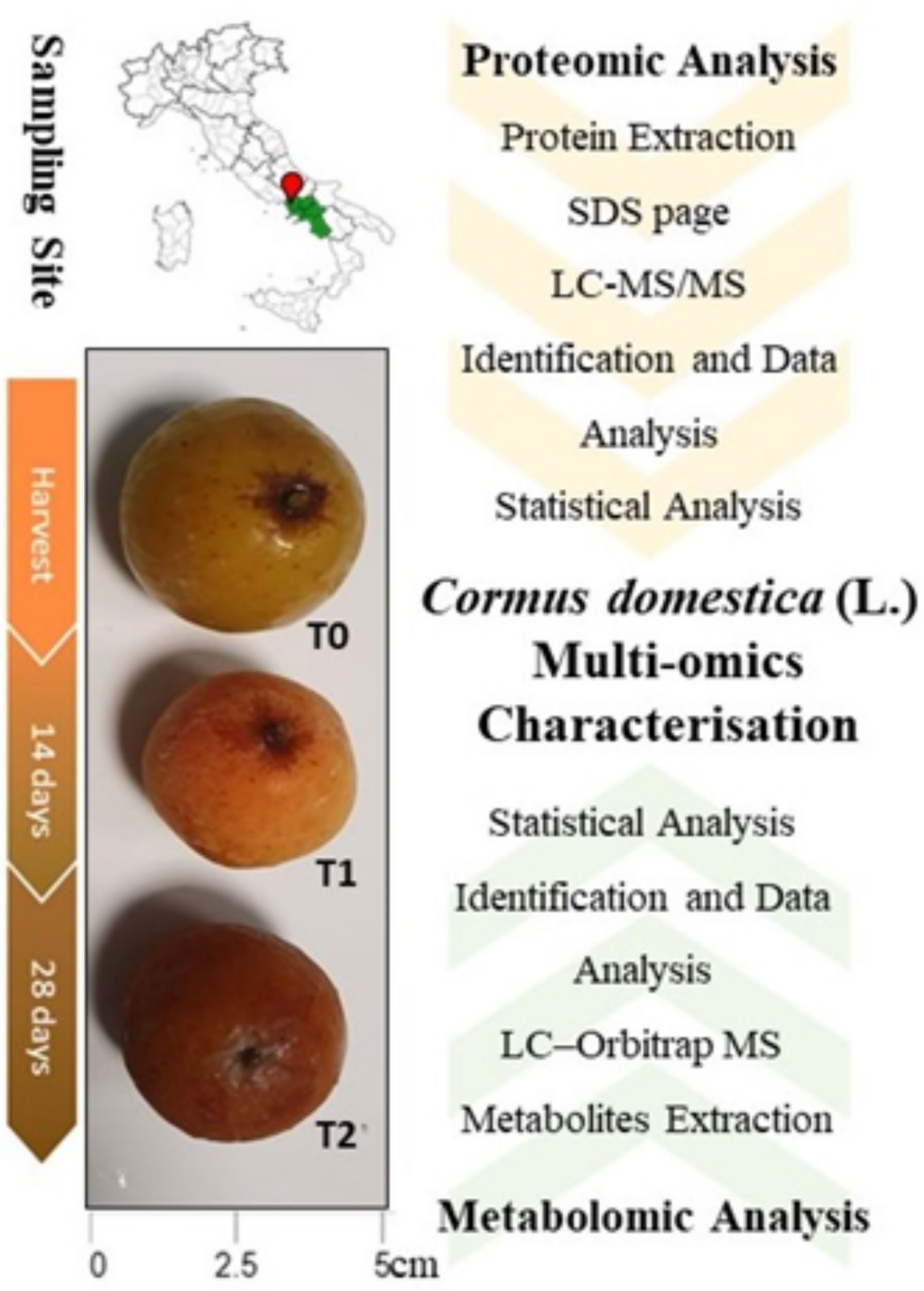


Fig. 4 Plant material sampling area, *C.domestica* fruit in the three post-harvest stages taken for analysis, and summary of the proteomic and metabolomic workflow applied in the work

80 V. The electrophoresis was stopped when the bromophenol blue had advanced 0.5 cm into the resolving gel. The gel was then stained with Coomassie Brilliant Blue R-250 and bleached overnight in 30% methanol and 10% acetic acid in water. The unique band obtained from each sample was cut out with a scalpel into portions of size<1 mm^3 and transferred individually into 1.5 ml tubes for digestion with 12.5 ng µL -1 sequential grade trypsin from Promega (Madison, WI, USA), following the detailed parameters described by Castillejo et al. [54].The gel bands were destained by incubating them twice for 30 min in a solution containing 200 mM ammonium bicarbonate in 40% acetonitrile at 37 °C. Afterward, they underwent three cycles of dehydration/rehydration using pure acetonitrile and 25 mM ammonium bicarbonate in 40% acetonitrile, respectively, followed by air-drying at room temperature for 10 min. Subsequently, 20 µL of trypsin, at a concentration of 12.5 ng µL−1 in 25 mM ammonium bicarbonate, was added to the dried gel pieces, and digestion proceeded at 37 °C for 12 h. The digestion process was terminated, and peptides were extracted from the gel plugs by adding 10 µL of 1% (v/v) trifluoroacetic acid (TFA) and incubating for 15 min. The eluent produced after the digestion is purified using C18, this process of desalting peptides involves elution with 70% acetonitrile.

Shotgun proteomic analysis

Samples were acidified with 0.5% Trifluoroacetic acid. Desalting and concentration step was performed with ZipTip C18 (Millipore) and the digested samples were finally Speedvac dried (200 ng per sample). LC-TIMS-MS/MS was carried out using a nanoElute nanoflow ultrahigh-pressure LC system (Bruker Daltonics, Bremen, Germany) coupled to a timsTOF Pro 2 mass spectrometer, equipped with a CaptiveSpray nanoelectrospray ion source (Bruker Daltonics). 200 ng of peptide digest was loaded onto a Bruker FIFTEEN C18 capillary column (15 cm length, 75 µm ID, 1.9 µm particle size, 120 Å pore size; Bruker Daltonics). Peptides were separated at 30 °C using a 20 min gradient at a flow rate of 300 nL/min (mobile phase A (MPA): 0.1% FA; mobile phase B (MPB): 0.1% FA in acetonitrile). A step gradient from 0 to 35% MPB was applied over 13 min, followed by a 35 to 90% MPB step of 13 to 15 min, and finished with a 90% MPB wash for an additional 5 min for a further time. The total run time was of 20 min per analysis, timsTOF Pro 2 was run in DIA-PASEF mode. Mass spectra for MS and MS/MS scans were recorded between 100 and 1700 m/z. Ion mobility resolution was set to 0.85–1.30 V s/cm2 over a ramp time of 100 ms. Data-dependent acquisition was performed using 4 PASEF MS/MS scans per cycle with a duty cycle close to 100%. A polygonal filter was applied on the *m/z* space and ion mobility, to exclude low *m/z*, mainly single-charged ions from the selection of PASEF precursors. An active exclusion time of 0.4 min was applied to precursors that reached 20,000 intensity units. The collision energy was increased stepwise as a function of the ion mobility ramp, from 27 to 45 eV.

Protein identification and quantitation

The parameters applied were set as follows: precursor mass tolerance 10 ppm, fragment ion mass tolerance 0.1 Da, charge state>+2, false discovery rate (FDR) 1%. Proteins were classified according to the law of parsimony. The degree of confidence in protein identification was obtained by setting at least 2 matching peptides, a minimum score of 2 and a sequence coverage mostly above 9.5% [55]. Raw data was analyzed in Spectronaut (see 15.1.210713.50606 – Rubin) (Biognosys). The reference library is acquired from UniProt (uniprot-maleae_29-2022.11.08-12.51.25.04–157351 entries). The directDIA algorithm was used for raw data analysis and library generation was created with the Pulsar search algorithm using the database itself. All settings were set to default (BGS factory settings). To account for post-translational modifications and chemical labelling, the following adjustments were used: carbamidomethylation of cysteine residues was set as fixed modification, methionine oxidation and acetylation (N-terminal protein) were set as variable modification.

Metabolomics analysis

Metabolites extraction

For metabolite extraction, three replicates were taken for each ripening stage considered (T0-T1-T2). Fruits were washed in deionised water, freeze-dried and pulverised in liquid nitrogen. Metabolites were then extracted from 30 mg of sample using 600 uL of cold ethanol: water (4:1) extraction solution while stirring for 10 s. The mixture was sonicated (40 kHZ for 10 min) and centrifuged (20,000 g, 4 °C, 4 min), the supernatant thus obtained was transferred to a new tube and the extraction was repeated on the pellet with a further 600 uL of ethanol: water (4:1).

Total sugar, phenolic compounds, free amino acids, flavonoids and photosynthetic pigments content analysis

Extracts obtained from the samples (in three biological replicates) were used to assess the content of total sugars, phenolic compounds, free amino acids, flavonoids and photosynthetic pigments, according to classic protocols [56]. The 3,5-dinitrosalicylic acid (DNS), Folin-Ciocalteu and ninhydrin methods were used to quantify the total sugar, phenolics and amino acid content, respectively; and glucose, chlorogenic acid and glycine as reference standards. Absorbance was measured at 570 nm for sugars and amino acids and at 765 nm for phenols. The photosynthetic pigments, (chlorophylls and carotenoids)

were evaluated by diluting the extract 1:2 in 80% ethanol and reading the absorbances at 663, 647, 537 and 470 nm. All readings were taken with a Thermo Scientific Evolution 201 UV-Visible spectrophotometer.

Metabolite identification and quantification using LC–Orbitrap MS analysis

The extract obtained from each sample was dried under vacuum at 30 °C (Speedvac, Eppendorf Vacuum Concentrator Plus/5301, Eppendorf, Leicestershire, UK). The extracts thus obtained were reconstituted in 0.5 mL of 50% methanol containing 0.1% of formic acid, centrifuged (20,000 g, 10 min, RT), filtered through 0.22 µm PTPE membranes (Thermo Fisher Scientific, Courtaboeuf, France) and then filtered through 0.22 µm PTPE membranes (Thermo Fisher Scientific, Courtaboeuf, France). The filtrate was collected in 1.5 mL LC/MS certified sample vials.

All analysis were performed using a Thermo Scientific liquid chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS, connected to a quadrupole-orbitrap Q Exactive hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), which was equipped with a heated-electrospray ionization probe (HESI-II). Chromatographic separations were performed using an Acquity UPLC BEH C18 column (2.1×100 mm, 1.7 µm) (Waters). The column was maintained at 40 °C and eluted under the following conditions: 5% B for 1 min, linear gradient from 5 to 100% in solvent B for 9 min, isocratic at 100% B for 2 min, and return to initial conditions, 5% B for 3 min. A flow rate of 0.5 mL/min was used. Eluent A was 0.1% formic acid in water and eluent B was 0.1% formic acid in methanol. Injection volume was 5 µl.MS detection was performed using a quadrupole hybrid mass spectrometer Orbitrap Q Exactive (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionisation source (HESI). HESI source parameters in positive mode were spray voltage, 3.5 kV; S-lens RF level, 50; capillary temperature, 320 °C; sheath and auxiliary gas flow, 60 and 25, respectively (arbitrary units); and probe heater temperature, 400 °C. Instrument control and data acquisition was carried out using Xcalibur v.4.3 software. Spectral data were acquired in full scan (FS) at a resolution of 70,000 (full-width half-maximum, FWHM at m/z 200) for MS1 and a data dependent acquisition MS2 method was acquired at resolution 70,000 and 17,500 (FWHM at m/z 200) for Full Scan and Product Ion Scan, respectively, fragmenting the five most abundant precursor ions per MS scan (Top5). Full Scan MS and data dependent acquisition MS2 methods were acquired in positive mode and mass range used for both experiments was 70–1,050 m/z. Three biological replicates of each ripening stage and three of the quality control (QC) mixture were analysed. QC samples were prepared using equal volumes of all samples and injected after every third sample to ensure continuous quality and promote data confidence. In addition, the QC samples were analysed in a data-dependent manner (dd-MS2/dd-SIM).

The acquired data were exported from Xcalibur software and were deposited in https://doi.org/10.21228/M83F09. Data treatment, alignment (with a maximum shift of 0.1 min and a mass tolerance of 5 ppm), peak selection, deconvolution, normalization and annotation were performed using Compound Discoverer v3.1 (Thermo Fisher Scientific, Bremen, Germany). A relative comparison among the areas of the chromatographic peaks of the MS1 precursor was employed in the compound quantification step. The hypothetical chemical formula for each feature was predicted, and the metabolite annotation was developed using a ddMS2 similarity search preferably (and formula or exact mass) in mzCloud and ChemSpider. Finally, using InChIKey codes for each putatively annoted compound, we classified them into chemical families (Superclass, class and subclass) using ClassyFire (https://cfb.fiehnlab.ucdavis.edu/) [57].

Data processing and statistical analysis

To deal with missing values (which can affect multivariate statistics) only the "consistent spots" (which were those present in the three sample type) were considered, according to [58, 59]. For the statistical analysis of the proteomics data, first the normal distribution of data was checked by the Shapiro-Wilk test. Some variables violate normality, so a Kruskal-Wallis non-parametric test was used to verify if there were differences among the groups (ripening stages). In order to determine which groups were different from others, post-hoc Dunn test was conducted. In the R environment (R Core Team, 2018; https://www.R-project.org/), a principal component analysis (PCA) two-dimensional biplot illustrating relationships between experimental samples and protein spots was created to illustrate the protein pattern. When working with multivariate data sets, the biplot graphical representation is useful to show and explain the relationships between variables and observations, which are represented as dots and rays, respectively. The variables (proteins) are represented by rays, and the lengths of the rays are directly proportional to the variance of the matching protein present in the two components that are being shown [58]. The link between two vectors represents the distance from each other.

For the metabolites, three biological replicates were used for all processing and statistical procedures in the environment RStudio 2023.09.1+494 running under R v4.3.1. Firstly, neutral masses were evaluated to avoid duplicities while retaining the most intense peaks,

quantification data matrix was abundance balanced by *AvgIntensity* method, then processed using Random Forest algorithm (with a threshold of 0.35) and filtered based on consistency with a threshold of 0.5 using pRocessomics package (available on web direction: https://github.com/Valledor/pRocessomics.wiki.git*)*. Principal Component Analysis with all samples and annotated matrix was performed. To determine which metabolites were characteristic of each maturation stage, we performed (1) Venn diagram, (2) Kruskal-Wallis non-parametric test and (3) calculation of fold-changes between stages with respect to T0. Regarding starch, sugars, phenolic compounds, free amino acids, flavonoids and photosynthetic pigments the Shapiro-Wilk test did not show a significant departure from normality, hence a parametric test (ANOVA with Tukey post-hoc comparison, $p<0.05$) was used to verify whether they significantly change during ripening.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05677-2.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

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Author contributions
M.T., C.G. and J.J.-N. conceptualized and designed the study. D.Z. sampled the plant matrices under analysis. M.T., M.A.R., M.T.-P., T.H. performed the samples analyses. D.Z. and M.T.P. performed the statistical data analysis. All authors jointly interpreted data. M.T. wrote the first draft of the manuscript. A.P. and P.S. prepared figures and tables. All authors contributed to manuscript critical evaluation, revision, read, and approved the submitted version.

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Data availability
Metabolomic data that support the findings of this study have been deposited at Metabolomic Workbench, (PR001877) available at link http://dx.doi.org/10.21228/M83F09 . Proteomic data that support the findings of this study are available from the corresponding authors on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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