Research Square

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Proteomics and metabolomics analysis of Cormus domestica (L.) fruits and the valorisation of an ethnobotanical heritage of culinary and medicinal uses in Mediterranean area.

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Research Article

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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 provide data on its chemical composition, nutritional and nutraceutical properties.

Results

Proteomic and metabolomic analyses were performed at three ripening stages: 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 *C. domestica* fruit metabolomic and proteomic profiling during the post-harvest process showed in the study, fills an information gap and has enabled the molecular and phytochemical characterisation of this erosion-endangered fruit. Data support the nutritional and nutraceutical value of this species.

1 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 rural 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. Unfortunately, the natural spread of this species has progressively eroded, and cultivation for fruit production is now even more limited [4]. Service tree 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. Ripe service tree fruits suitable for consumption are brown or yellowish coloured and ripen in September-October. The average weight of a service tree fruit is 25 g and its diameter can reach a maximum of 4.5 cm [5–7].

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 [8–12]. During the ripening process there are drastic biochemical changes that transform them into palatable products for consumers. The changes typically observed include 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 [13, 14].

Although the ripening process generally makes fruits more palatable, the Service Tree 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 [15]. At this stage, the fruit can be eaten as it is or used to make jam, juice, fruit wine, or cider. Although the service tree 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 [6, 16]. To date, there are few manuscripts in the scientific literature analysing the metabolomic profile of this peculiar fruit during the post-harvest phases[15, 17], while there are no data about the proteomic profile during the same process. Therefore, given the traditional process that precedes the consumption of this fruit, and the lack of information regarding its proteomic and metabolomic profile, the aim of this work is to fill these gaps to characterise and valorise this ancient fruit.

2 Results

2.2 *Cormus domestica* (L.) post-harvest proteomic profiles

As shown in Fig. 1A and 1B, proteomic analysis allowed us to identify 643 proteins (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 abundance of the identified proteins as for the extraction yield, decreased on average as the process progressed (T0 > T1 > T2) (Table 1).

Table 1
Average 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 ($p < 0.05$) between the different post-harvest stages observed (Supplementary File1-2).

	T0	T1	T2
Extraction Yeld µg/0,25g sample	201	209	175
Identified Proteins	638	559	469
Univocal proteins	77	3	2
Statistically significantly different			
T0 vs T1	115	95 up	20 down
T0 vs T2	225	186 up	39 down
T1 vs T2	139	103 up	29 down

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), and with Mercator4 V2.0 (https://mapman.gabipd.org/app/mercator)[18] and are depicted in Fig. 2B. Most proteins were involved in carbohydrates, amino acids, protein and lipid metabolism, cell wall and cytoskeleton reorganisation. Several proteins involved in cell wall modifiction, and thus with the 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 the early stages, with a peak at the usual consumption stage for this fruit (T1). The enzyme malate dehydrogenase (A0A498I601, A0A498I8M1, A0A540KIX2) was consistently abundant in the three observed stages (T0, T1, T2), whereas fructose-bisphosphate aldolase (A0A5N5HXN8, A0A540KUY8) was more abundant in T2 and showed the opposite trend to glucose-1-phosphate adenylyltransferase (A0A498HC29) and glucose-6-phosphate 1-dehydrogenase (A0A5N5GTK5). Regarding the acidity

lowering 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 most abundant in the early stages of post-harvest process T1 > T0 and was then absent 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 Protein involved in protein homeostasis. Also, some peculiar proteins related to the physiological stress induced by the ripening process in the fruit, such as abscisic stress ripening protein (A0A498JY74) that accumulates in T2, remorin (A0A498JK49) that showed a peak in abundance in T1, and spermidine synthase (A0A5N5HGV6) that instead maintained a constant trend as the process progressed.

Statistical analysis, performed by means of a two-factor ANOVA followed by Tukey's test ($p < 0.05$), allowed us to filter out 254 differentially abundant proteins between the three maturation stages considered, i.e. between the freshly harvested *C. domestica* fruit (T0), after 14 days (T1) and 28 days (T2) of post-harvest ripening (Table 1, Supplementary File 2). The PCA results in Figs. 2 clearly show the degree of homogeneity of the biological replicates taken into analysis (T0, T1, T2) and the good separation of the samples at their maturation stages based on the significant differential protein expression. A PCA with the top fifty proteins that more contribute to the variability of the stages under analysis is presented in Supplementary Fig. 4.

2.3 *Cormus domestica* (L.) metabolomic profiles under post-harvest ripening

The fruit of *C. domestica* (L.) traditionally undergoes a post-harvest phase in which the chemical and physical characteristics of the fruit change considerably, making it more palatable for consumption. 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 (T3), 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 allowed the identification of 4377 features in positive ionisation mode, of which 2334 with putative annotation (Supplementary File 4). A principal component analysis (PCA) allowed us to observe a good clustering of the replicates, from which it can also be seen that the metabolites of the habitual stage of consumption as such of the fruit (T1) are clearly differentiated from both the immature stage (T0) and the over-ripening stage (T2). Table 2 shows the main metabolites characterising the T1 stage of ripening of the *C. domestica* (L) fruit (Fig. 3B). The main metabolites characterising the T0 and T2 stages, in which the fruit is not routinely consumed, are presented in Supplementary Table 1, and prominent among them are Chlorogenic acid,4-Coumarylalcohol, Caffeic acid, Citric acid and Pipecolic acid. These were filtered maintaining a variable

consistency of 0.25. The metabolites were then classified using ClassyFire (http://classyfire.wishartlab.com/) to subdivide them into Superclass/Class/Subclass. As shown in Fig. 3C, the metabolic profile of the three ripening stages under analysis undergone consistent alterations, and the profile of the intermediate stage (T1), have 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 times under analysis (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 under analysis, and which classes were the most represented. As can be seen, the T1 consumption phase 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 are unique compounds peculiar to each class, the trend in class abundance remains almost constant at T0 and T1 and then decreases at T2 (Supplementary File 4). A statistical analysis was performed on the 1752 variables thus selected, obtaining 606 statistically significant differentially abundant metabolites.

The class of organoheterocyclic compounds is the most represented in the usual *C. domestica* fruit consumption stage. To this superclass belong 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 (T1) 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, a peak in abundance was also observed at the T1 stage. In fact, while for some compounds, such as glucose and maltose, a constant 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 is 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 the *C.domestica* fruits were oleamide and hexadecanamide, two compounds well known for their beneficial effects on human health due to their anti-inflammatory properties. To the same class of metabolites belong several terpenoids found in all post-harvest ripening phases analysed, known for their antioxidant, antineoplastic and anti-inflammatory properties, 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, reach a peak in abundance at the preferential consumption stage(T1), in which the aromatic bouquet is enriched with compounds such as borneol and capsidiol. An interesting result is the presence of endocannabinoids (2-Arachidonoyl glycerol) most abundant at the fruit harvest stage (T0). The class of lysergic acids and derivatives belonged to several compounds that exhibited different abundance trends. Among these, the metabolites most representative of the harvest stage (T0) are chlorogenic acid and caffeic acid, whose values then decrease 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 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 belong 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.

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

LOADINGS	Compound	Class/Subclass
0.5887615	5-hydroxy-4-methoxy-5,6-dihydro-2H-pyran-2-one	Pyranones and derivatives
0.23985723	Oleamide	Fatty Amides
0.18086692	2-Hexadecylpyridine	Pyridines and derivatives
0.17225204	D-(+)-Proline	Amino acids, peptides, and analogues
0.12401163	(4-Methylphenyl)-2-thienylmethanone	Carbonyl compounds
0.11938738	(R)-4-Dehydropantoate	Fatty Acyls
0.11405736	4-(Trifluoromethoxy)phenylacetic acid	Phenol ethers
0.1022925	acetoacetic acid	Lysergic acids and derivatives
0.09725218	2-Hydroxy-2_4-pentadienoate	Fatty Acyls
0.08819292	2-(1-Piperidinylmethyl)cyclododecanone	Piperidines
0.07726014	Piptamine	Phenylmethylamines
0.07171227	Trihexyl 2-(butyryloxy)-1,2,3-propanetricarboxylate	Tetracarboxylic acids and derivatives
0.05542785	Bis-D-fructose2'_1:2_1'-dianhydride	Carbohydrates and carbohydrate conjugates
0.05464245	2-ACETYL-3,5-DIMETHYLBENZO(B)THIOPHENE	Benzothiophenes
0.05400113	13(S)-HOTrE	Fatty Acyls
0.05279933	1-Dodecyl-2-pyrrolidinone	Fatty Acyls
0.05179841	Resorcinol	Benzenediols
0.04893773	Rickiol C	Macrolides and analogues
0.04674569	Dimethylpyruvic acid	Short-chain keto acids and derivatives
0.04599191	D-(+)-Glucose	Carbohydrates and carbohydrate conjugates
0.04594392	(R)-Malate	Hydroxy acids and derivatives
0.03920299	(2R_3S)-2_3-Dimethylmalate	Hydroxy acids and derivatives
0.03880485	Hexadecanamide	Fatty amides
0.0359764	Bis(2,2-dihydroxyethyl) hydrogen phosphate	Non-metal phosphates

LOADINGS	Compound	Class/Subclass
0.03574935	3-Hydroxyaminophenol	N-phenylhydroxylamines
0.02860971	Sulcatol	Fatty alcohols
0.02798536	Myristamine oxide	Aminoxides
0.02795987	2,4-Xylidine	Xylenes
0.02615869	(3aS,4R,5S,7aR)-4,5-dihydroxy-7-methyl-3a,4,5,7a-tetrahydrobenzo[1, 3]dioxol-2-one	Carbonic acid diesters
0.02530938	Gephyronic acid (hemiketal)	Terpene glycosides

3 Discussion

Although much information is available for other fruits belonging to the Rosaceae family regarding physiological and biochemical changes due to the ripening process, little is known about the postharvest ripening process of *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[19]. The ripening process is a genetically programmed, irreversible and highly regulated process involving changes in the fruit structure 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 change mainly due to the chloroplasts turning into chromoplasts and the accumulation of neo-synthesised pigments, softening of the pulp due to the alteration of the cell walls, sweetening due to the accumulation of sugars and the reduction of sour components, and the production of various metabolites that contribute to the attainment of aroma and flavour [13, 20–22]. Traditionally, *C. domestica* fruits are harvested fully developed, and eaten after a maturation 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[17]. The pronounced colour change, usually observed during the fruit ripening [23] is not observed in *C. domestica* fruit, which, as shown by analyses of photosynthetic pigment content and in the sample photos shown, does not accumulate carotenoids during ripening, but rather takes on a brownish colour mainly due to the natural oxidative processes involving the fruit in post-harvest ripening[24], and as shown by the accumulation of enzymes involved in oxidative stress found in our samples. 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[14]. 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 act 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 [25]. These enzymes, like others that act on hemicelluloses (XET) and non-enzymatic proteins such as expansin, accumulate 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* makes it particularly soft in consistency, but this, together with the accumulation of sugars and oxidative processes, significantly reduces its shelf life [14]. 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 [11, 26]. The accumulation of antioxidant enzymes and soluble antioxidant compounds such as flavonoids has also been observed in the fruit of the service tree. In particular, the accumulation of quercetin-3β-D-glucoside, taxifolin, fustin and coumarin increase the nutraceutical power of this ancient fruit [27, 28], which has a general anti-inflammatory effect and can modulate the lipid peroxidation involved in various 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[20]. 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 [29, 30]. Similarly, Remorin, a protein involved in the regulation of several physiological processes, shows a peak in abundance two weeks after service tree fruit harvest, and its contribution in regulating the ripening process is known from the literature. Other proteins involved in stress and energy metabolism such as Small Heat Shock Proteins (sHSPs) were found to be abundant in the ripening *C. domestica* fruit. These chaperones have been found in several proteomics studies related to abiotic and developmental stress response that have been characterised in several ripening fruits [31, 32]. 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 [33–35]. As expected, also in the post-harvest ripening process of the service tree fruit followed in this experiment, a marked conversion of starch to soluble sugars was observed and the levels of the enzyme sucrose synthase remain high until the end of the ripening process. The main soluble sugars in sorbola-like fruits such as apples or pears are sucrose, fructose and glucose, which is also particularly abundant in the sorbola stage of consumption; as are some sugar alcohols such as sorbitol [9].

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. 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. Citrate synthase and Malic enzyme accumulate in the service tree fruit during ripening, causing a reduction in malate and contributing to the decline in fruit acidity [36]. Several peculiar metabolites characterize the habitual stage of fruit consumption. Some of these, particularly abundant such as piptamine and resorcinol, are known for their antimicrobial and antifungal properties. 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. 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 provide 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, confer fresh and citrus notes. The abundance of camphor, pulegone and borneol, on the other hand, adds intense minty notes, while benzophenone adds soft, fresh notes with touches of fruit and flowers. These compounds not only impart the final aroma to the fruit but also have antimicrobial and anti-inflammatory properties[13, 37, 38].

4 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 in order to characterise both the biological process and the organoleptic and nutraceutical peculiarities of this ancient fruit. The metabolomic and proteomic profile of the *C. domestica* fruit during the post-harvest process, shown in the study, fills an information gap and enabled its molecular and phytochemical characterisation. The combined analysis of proteins and metabolites revealed the activation of the main physiological processes related to the process that makes the fruit edible, such as the softening of the walls, the conversion of starch into simple sugars and the accumulation of secondary metabolites. The latter were of particular interest because, in addition to characterising the aromatic bouquet of the fruit under analysis, they also confirmed the nutraceutical properties already known and inherited from in rural popular culture.

5 Methods

5.1. Experimental workflow and Plant Material

Cormus domestica (L.) fruits postharvest process was followed using metabolomic and proteomic approaches at different stages (Fig. 4). For proteomics, in situ digestion of single-band SDS fractionated protein extracts were performed for each sample (freshly harvested inedible fruit T0, fruit in the traditional stage of consumption at incipient state of decomposition T1, fruit in advanced decay T2) to monitor the differential protein abundance throughout the process. For metabolomics, metabolites were extracted by 80% acetone extraction and supernatants were analysed using a quadrupole hybrid mass spectrometer Orbitrap at the same stages.

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. Carmine Guarino identifed all samples. 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 [39]. The harvested fruits (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 (T1). Ripening continued for a further 2 weeks until the fruit had completely turned brown (T2).

5.2 Proteomics analysis

5.2.1 Protein extraction

Three fruits for each time 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[40]. After precipitation in ammonium acetate in methanol and three washes with acetone, the pellet was dried under vacuum and solubilised in a solubilisation buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 40mM DTT and 0.5% IPG buffer) and centrifuged to remove insoluble material (12,000g 3min 4°C). Using the Bradford method and bovine serum albumin as a standard, the protein concentration obtained was then estimated [41].

5.2.2 SDS page

The extracted and solubilised proteins (80 µg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel and the Mini-PROTEAN 8.6 × 6.7 cm2 Bio-Rad cell. The 80 V run 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 mm3 and transferred individually into 1.5 ml tubes for digestion with 12.5 ng µL -1 sequential grade trypsin from Promega (Madison, WI, USA) [42].

5.2.3 Shotgun Proteomic Analysis, LC-MS/MS 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). For most analyses, 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. total run time 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.

5.2.4 Protein identification and quantitation

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). Global imputation values were set to. To account for post-translational modifications and chemical labeling, 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.

5.2.5 Statistical Analyses of the data

For the statistical analysis of the proteomics data, RStudio was used and the statistical significance of the abundance of the identified proteins was checked by means of two-factor ANOVA followed by Tukey's test assuming a p value < 0.05 (ref) as significant. 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 (Zuzolo et al., 2020). The link between two vectors represents the distance from each other. 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 Meunier et al. (2007) and Valledor and Jorrín (2011)[43, 44].

5.3 Metabolomics analysis

5.3.1 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 milligrams of sample using 600 microlitres 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 microlitres of ethanol:water (4:1).

5.3.2 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[45]. 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. For flavonoids, instead, a general blank with 80% ethanol was used and the absorbance was measured at 415 nm. 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.

5.3.3 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 (20,000g, 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 x 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 5–100% 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 ºC.

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 http://dx.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 compound, we classified them into chemical families (Superclass, class and subclass) using ClassyFire (https://cfb.fiehnlab.ucdavis.edu/)[46].

5.3.4 Data Processing and Statistical Analysis

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) as missing value imputation algorithm, 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.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

MT, CG and JJN conceptualized and designed the study. DZ sampled the plant matrices under analysis. MT, MR, MTP, TH conducted the samples analyses. DZ and MTP performed the statistical data analysis. All authors jointly interpreted data. MT wrote the first draft of the manuscript. All authors contributed to manuscript critical evaluation, revision, read, and approved the submitted version.

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Not applicable

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Figures

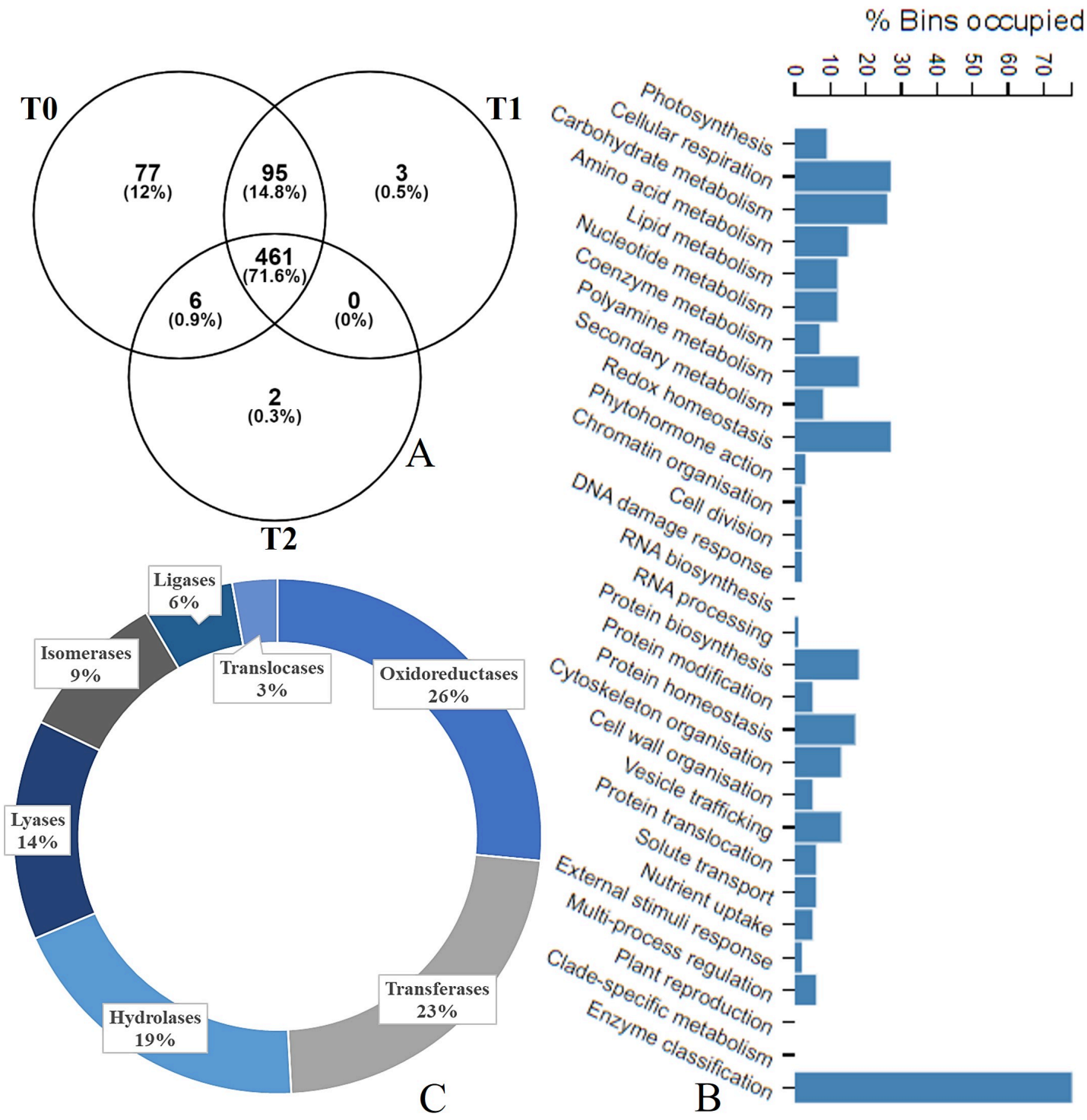


Figure 1

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

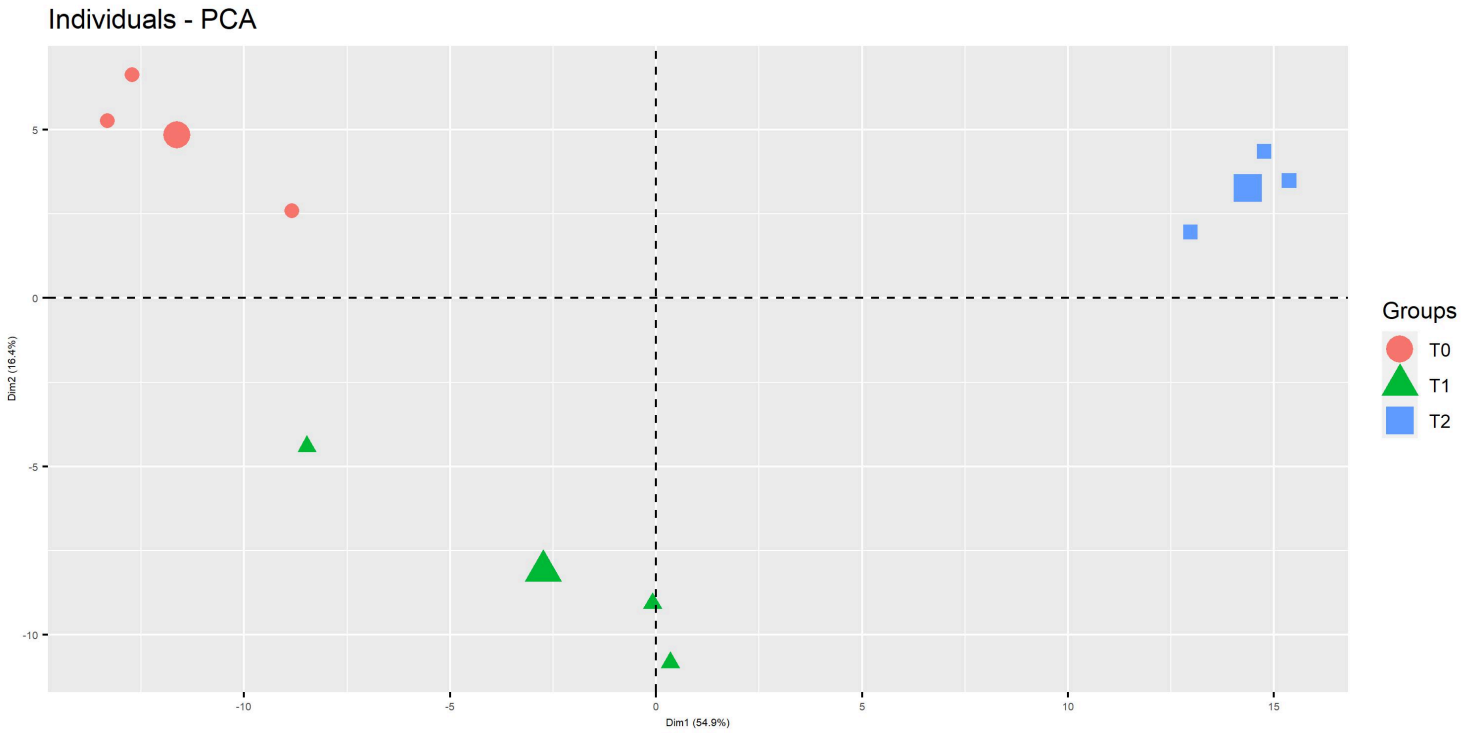
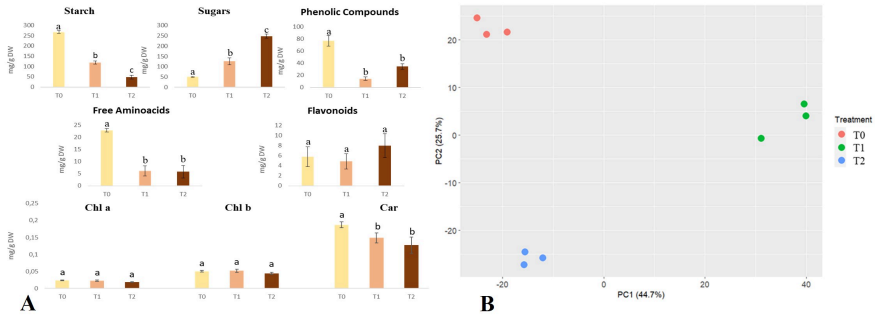
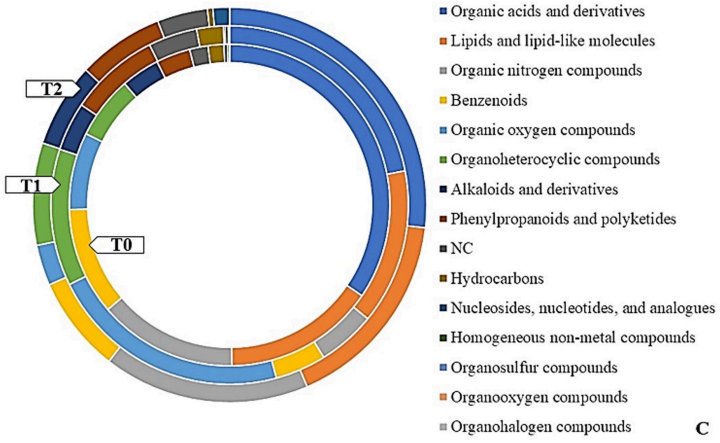
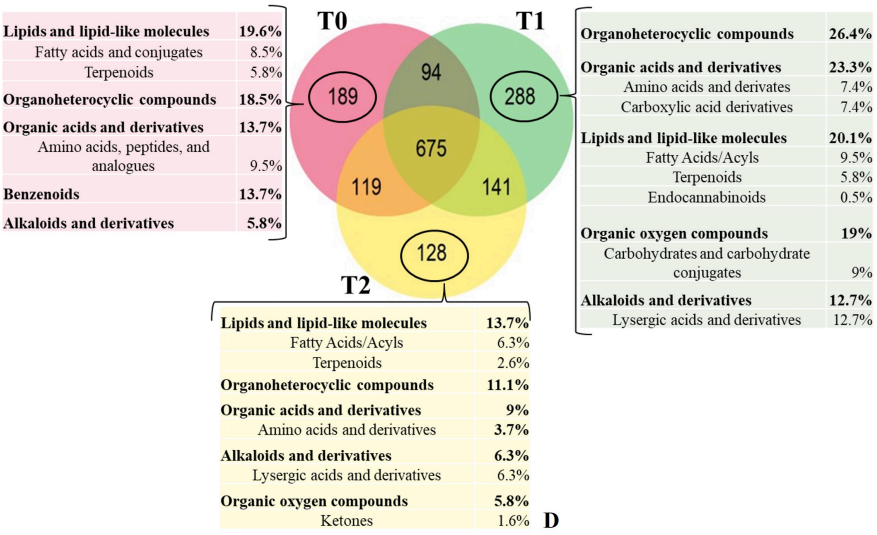


Figure 2

Two-dimensional principal component analysis (PCA) biplot showing clusters of samples (three replicates for each ripening stage under analysis) based on their similarity. Mean point is also plotted.







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%

Figure 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 taken into analysis (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). B: PCA analysis was performed to visualise the three datasets each consisting of the three replicates for each ripening stage under

analysis. The PCA shows a good clustering of the replicates and the clear difference in terms of metabolites between the three times T0, T1 and T2. C: Metabolites macro-clustering with relative percentage abundances in *S.domestica* fruits during the post-harvest ripening process. D. Venn diagram showing unique or shared metabolites in multiple stages with fucus on metabolites present exclusively in one of the three stages (T0,T1,T2) and most abundant classes in percentage. (Supplementary File4).

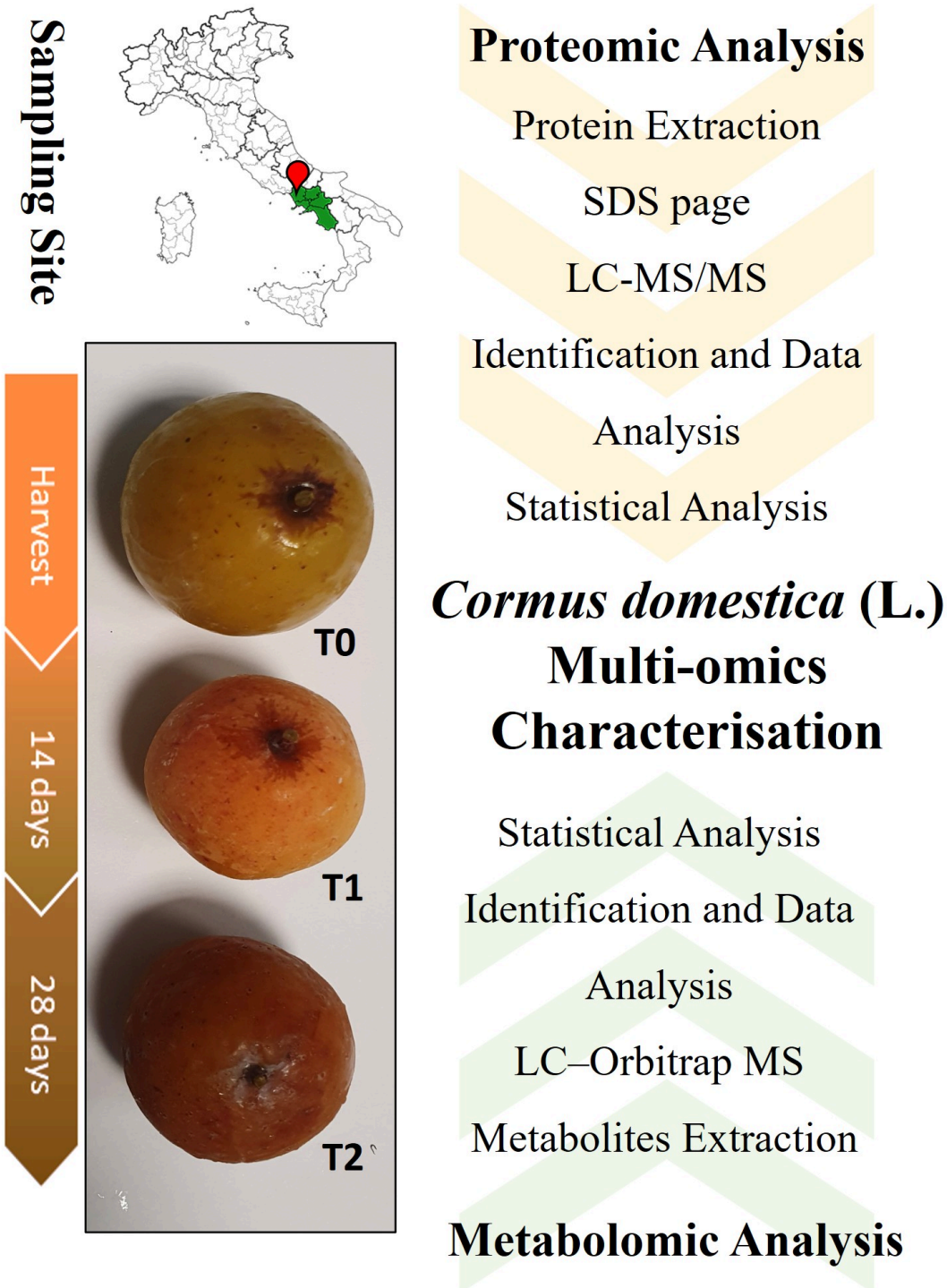


Figure 4

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.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.jpg
- SupplementaryFigure2.jpg
- SupplementaryFigure3.jpg
- SupplementaryFigure4.jpeg
- SupplementaryFile1.xlsx
- SupplementaryFile2.xls
- SupplementaryFile3.xlsx
- SupplementaryFile4.xlsx
- SupplementaryTable1.docx