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Assessment of flesh texture in Peach (*Prunus persica* L. Batsch)

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Contents

1. Introduction

We now know that the peach tree (*Prunus persica* L. Batsch) is native of China, in the Tarim basin north of the Kunlun mountains, but its origin was uncertain until the nineteenth century.

The name, literally "Persian plum", reveals the prior putative origin--Persia. De Candolle was the first to refuse the "persian hypothesis". He observed that, if peach had really been native of Persia, we should find mentions of the fruit in Xenophon's works (e.g. Cyropaedia) and in Hebrew and Sanskrit writings, but we do not. So he supposed that Romans called peach "*persica*" because they just received it from Persia, where in turn it had arrived along the silk trading routes in the 2nd or 3rd century B.C. (De Candolle, 1883).

The argument that peach originated in China receives strength by two evidences. The first is that in China there exists the highest variability of peach trees and, in accord with (Vavilov, 1951), the center of diversification coincides with the center of the origin of plants. The second is the anthropologic evidence, based on the presence of the peach tree in Chinese mythology and folklore since antiquity (1000 years before it first appeared in any European writing).

Thus, after arriving in what are now Italy and France, the peach spread from Western to Eastern Europe, in territories that were part of the Roman Empire. Later, after the discovery of America, the Spanish brought the peach initially to Mexico, whence it spread to New Mexico, Arizona and California. More or less in the same years, the peach was also planted in Florida, again by the Spanish, and about a century and a half later in Louisiana by the French (Hedrick, 1917).

In addition to the ‘‘Spanish channel’’, the peach found another route to the New World, as it was also brought there from China, through the London markets, at about 1850. From crosses between Chinese peaches (‘Chinese Clings’) and ‘‘local’’ peach trees, originated cultivars such ‘Elberta’, one of the most important cultivars of its time worldwide, and ‘J. H. Hale’ (Hedrick, 1917). These two varieties were to become the basic cultivars used in peach breeding, which prompted the criticism that the genetic base of the peach is very narrow, stemming largely from the ‘Chinese Cling’ (Scorza, R. et al., 1985) .

In the nineteenth century, commercial peach growing began in the United States and, to meet the growing demand of the market, institutional breeding was started in the subsequent century. In this way, the number of the peach cultivars greatly increased, reaching 2181 in 1917 (Hedrick, 1917), 6000 in 1994 (Okie, 1994) and more the 8000 at the end of the past century (Okie, 1998). American peaches and nectarines were successful and were tested all around the world (Italy, France, Spain, Hungary and Chile). Their success was so great that about 60% of Italian peach production is based on American cultivars, and even new cultivars developed by Italian breeders are mostly first generation hybrids of American cultivars (Faust and Timon, 2011). In a certain

sense, America may thus be considered the second cradle of the peach. Fig. 1 shows origin and diffusion of the peach.

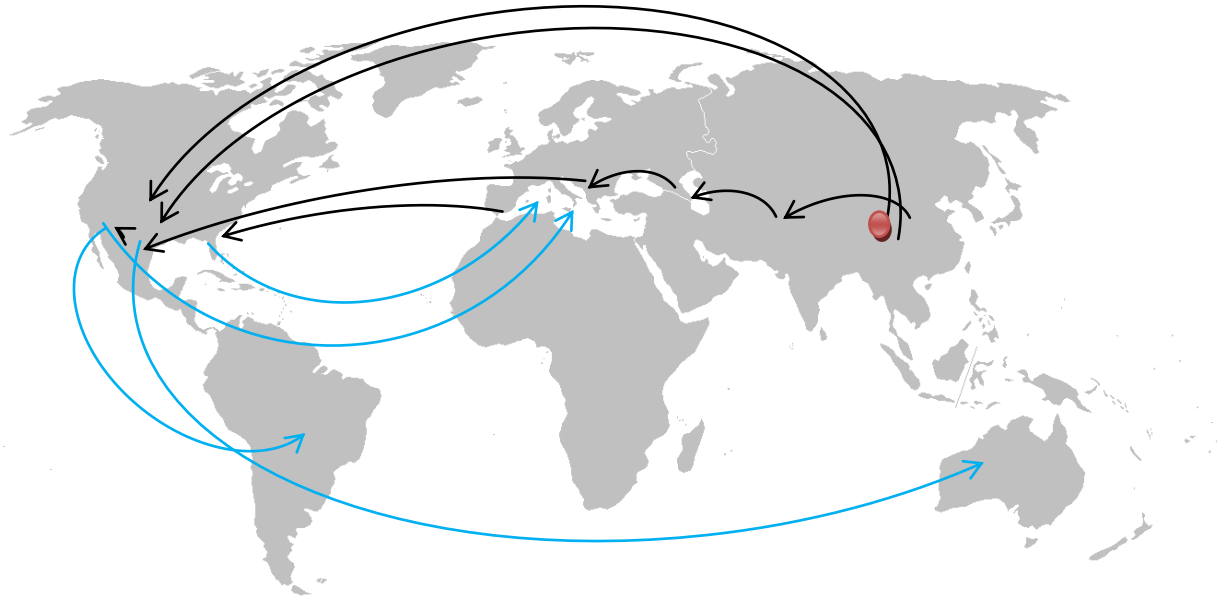


Fig.1 Origin and diffusion of the peach (*Prunus Persica* L. Batsch)

1.1. Economic aspects

Italy has a long history of peach cultivation, due to the very early introduction of the fruit in the Italian peninsula, which probably occurred in the first centuries B.C..

The early establishment and the easy propagation by seed led to the development of a wealth of peach germoplasm in terms of fruit type and season of ripening, and it has been estimated that more than 20% of the 2400 accessions in the Italian peach collections are of Italian origin (Bellini et al., 1990).

Probably for the same reasons Italy is the first peach producer in Europe and, providing 8% of all the world peaches, ranks second in the world, although Italy does not come even close to China, the leading producer (fig. 2). For what concerns exports, Italy was the first exporter in the world until 2003, when it was surpassed by Spain, and now maintains consistently the second place (fig. 3).

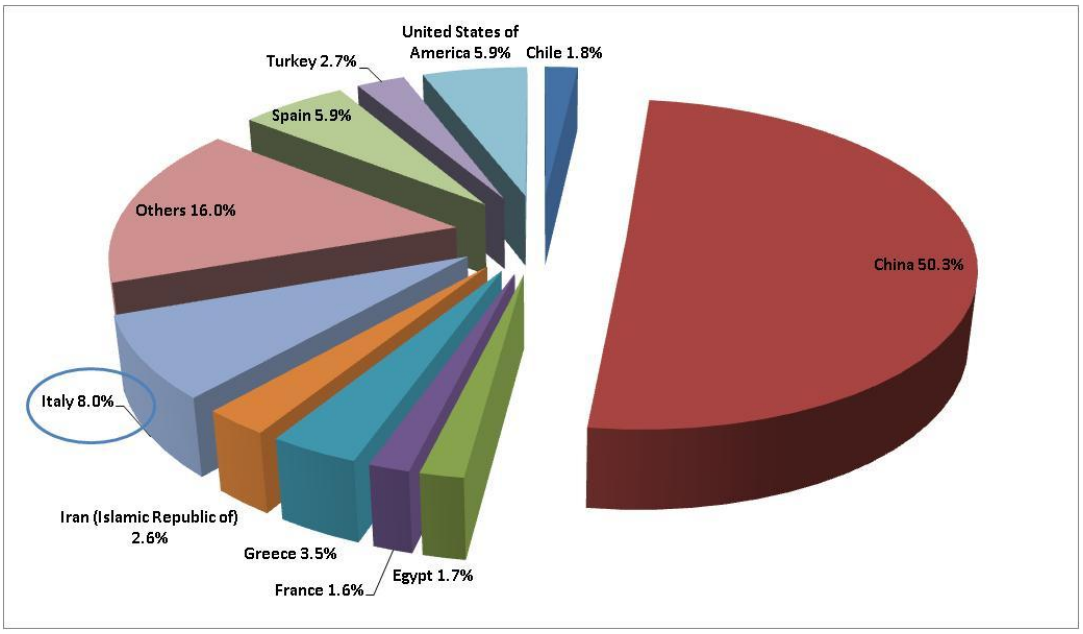


Fig. 2 World peach production years 2008-2010 (FAO)

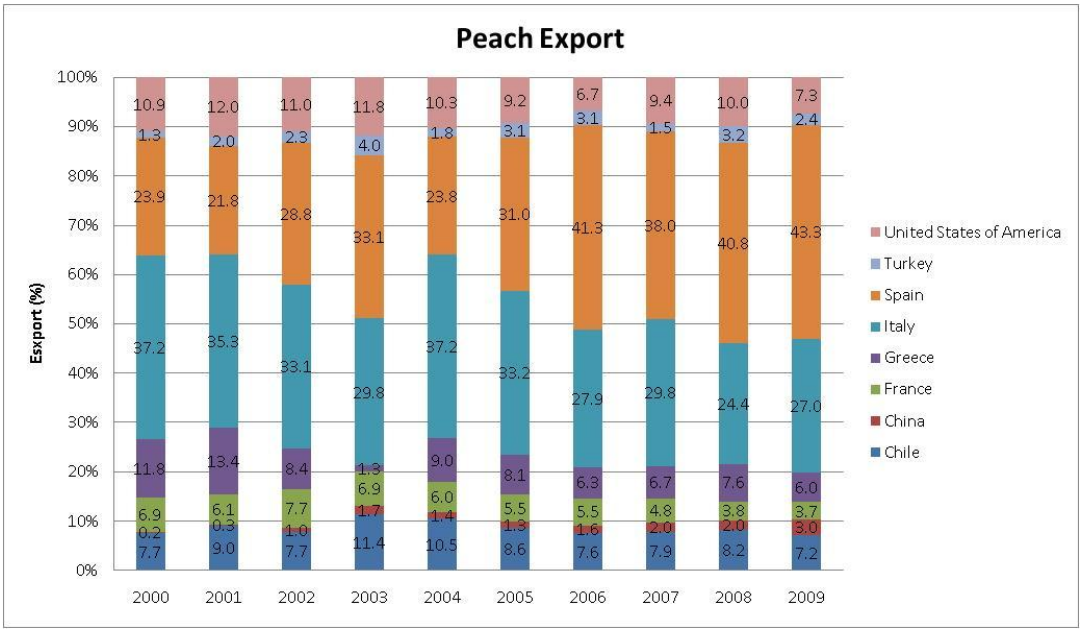


Fig. 3 World peach export years 2000-2009 (FAO)

These premises notwithstanding, this sector is lately experiencing a severe crisis, and some Regions of Italy have asked the Ministry of Agriculture to set up a fund to help them face the crisis. Part of the responsibility is to be found in globalization: the reduction or elimination of the import tariffs (between UE and regions of Maghreb and Masherek) increased competition and lowered peach prices. But the greatest responsibility is

undoubtedly to be found in the chain of production and distribution, which often affects the quality of the product.

In fact, each component of the production and distribution chain, from grower to final consumer, demands fruits with no or very few defects, but each component in this chain has its own peculiar opinion regarding the absence of defects. In this way the importance of each one of the various quality traits inevitably becomes highly variable.

The grower, for example, is interested in a high yield, in fruits of big size, in a high resistance to disease, and in uniform ripeness. The "packer", the carrier, the distributor and the wholesaler attach greater importance to flesh firmness, which leads undoubtedly to practical advantages: ease of transport and storage, and prolonged "shelf life" of the fruit.

Retailers, for their part, attach higher importance to features such as color, size and texture.

Finally, the consumer is more attracted by characteristics such as size, skin color, and texture, but also by sugar content, acidity and aroma, which together define the concept of quality of the fruit.

The latter characteristics however, are often not heeded by the other members of the chain (Abbott, 1999) and, as a consequence, more often than not, the only possible outcome from such a situation is bad quality fruits.

In addition, if it is in the growers' interest to sell at the most profitable moment (which often means selling still unripe fruits), consumers will end up buying a product that is unsatisfactory from both the organoleptic and the nutraceutical points of view (the latter being a strongly felt aspect as well). This situation inevitably leads to consumer disaffection to peach in their choice of fruit, with a consequent reduction in demand both in the large and in the small distribution, accumulation of unsold production, and lowering of prices.

To return to the levels the peach industry attained a few years ago, it is no longer sufficient merely to use a more rational management of quality (prevention and treatment of diseases, know-how of producers, harvesting techniques and storage), but it is necessary to identify new varieties that have characteristics that come as close as possible to current market needs.

Breeders' efforts have been focused on this aim and have led to the development of some new cultivars, such as 'Rich Lady', 'Vistarich', 'Diamond Ray' and the nectarine 'Big Top'. These new cultivars, firmer and crisper, melt more slowly, are less susceptible to bruising during handling and show a longer shelf life than their relatives 'Redhaven', 'Glohaven', 'Indipendence'. These characteristics allow the grower to harvest at a later time ensuring a better fruit, in contrast to the "old ones" that cannot achieve the maximum quality, because they have to be harvested earlier in order to prevent mechanical damages, short postharvest life and decay when shipped.

1.2. Botany

Prunus persica (L.) Batsch is a diploid (2n = 16) species selfcompatible autogamous species.

It belongs to the *Rosaceae* family, which includes many important fruit species that provide tasty, healthy and economically relevant fruits like almond, apple, apricots, cherry, pear, plums and strawberries. Among these species peach is probably the most variable. There are several types of peaches, which differ in the characteristics of the fruit (beaked, round, extremely flat shaped; white, red, or yellow fleshed; melting or non-melting flesh; clingstone or freestone; hairy or smooth skin), seed (sweet or bitter), flower (large, medium, or small; single or double; red, pink, white, or mixed; and single or grouped on spurs), growth habit of the tree (columnar, upright, spreading, spur type, short internode dwarf, evergreen), leaves (narrow, wide, red or green, with reniform or globose glands on the petioles, or eglandular), buds (hairy or smooth), in the requirement for various environmental factors (short or long chilling requirement, extreme hardiness, or late blooming), and in the resistance to various diseases (Faust and Timon, 2011).

1.3. Fruit texture and different flesh phenotype

Fruit texture is one of the traits that contribute to the variability of the peach fruit and it is strongly affected by the composition of the cell wall. It is characterized by differences in flesh softening and flesh adherence to the pit and it defines industry market classes (fresh market or canning).

So far three different flesh phenotypes are known: Melting (M), Non Melting (NM) and Stony Hard (SH), even if not all is understood in terms of genetic determination and biochemical pathways during the final ripening stage (Layne and Bassi, 2008).

Melting peaches (MF) are mostly used for fresh market. They are not suitable for processing, because they tend to form ragged edges when sliced. At maturity their flesh becomes soft and fibrous. They can be freestone (FMF), if the stone is easy to separate from the flesh, or clingstone (CMF), if the flesh adheres to the stone. Features of this flesh phenotype are the development of ethylene, the rather high amount of water-insoluble pectins and of Ca bound to the cell wall (Mignani et al., 2006) and the prominent softening in the last stage (IV) of ripening, until a complete melting. Actually it's possible to consider this phenotype a macro-group. Inside of it, in fact, there is some variability in firmness and there are different rates of softening (*soft*, *medium*, *firm* and *very, very firm* Melting). This variability results by point mutations in the gene sequence, which give a less efficient product and a subtle difference in phenotype (Pflieger et al., 2001). 'Big Top' and 'Rich Lady' described above, for example, are *very, very firm* (or Slow Softening, or Slow Melting) fleshed. Their flesh is firm and crisp (more closer to "Stony Hard" then to "Melting flesh") and melt only when fully ripe, so that they can be harvested later, ensuring a fragrant and flavorful fruit. The Melting trait (M) has been described to be dominant over the Non Melting (m).

Non Melting (NM) peaches have a firm, non-melting flesh. This character is the most intense and most simply inherited (one recessive mendelian gene) form of firmness, and it is closely associated (genetically linked) with the genetic clingstone character (Sherman et al., 1990). The peculiar flesh texture (they don't show the "melting phase") allows the fruit to be harvested at the preferred tree-ripe stage, transported and processed, often at high temperature, without appreciable loss in fruit integrity. For this reason they are usually grown for canning purpose, and are also called "*canning* peaches", although industrial use is not the only one. In fact their peculiar capacity to provide "tree-ripe" fruit to the consumer has also guaranteed Non-Melting peaches a market share of fresh market in Europe (Spain and Southern Italy) and Central and South America. Some other important features are the lack of the red overcolor, the low acidity and the rubbery consistency that they exhibit during the senescence stage, due to the loss of water. Further, they develop ethylene between stage III and IV, but in higher quantities then in Melting phenotype, and exhibit a very high total pectin content (Mignani et al., 2006). The lack of "*melting phase*" is due to the loss of endopolygalacturonase (endoPGase) activity, an

enzyme that plays a central role in cell wall degradation. Non Melting" peaches are homozygous recessive (mm). They are all clingstone non-melting flesh (CNMF).

Stony hard phenotype (SH) is the least known by Western consumers and surely is the most puzzling phenotype. In the past, in fact, there has been some confusion around this flesh phenotype and now it is still very difficult to discriminate from NM or very firm, unripe M phenotypes. It was first described by Yoshida in 1976 (Yoshida, 1976), more than forty years after the first mention of Melting and Non Melting types in scientific literature. Peaches belonging to this group are very firm and crisp. Water-insoluble pectins in cell wall are rather high, as Ca is bound to them, although its content is variable (Bassi et al., 1998). Like the "Non Melting", they never melt, but become rubbery in senescence. The only feature that stands out from the "Non Melting" phenotype is the inability to synthesize ethylene during ripening and this is the reason why they soften slowly. This feature, that at first sight can seem a "defect" in relation to traditional flesh peaches, actually offers big potential applications. In fact, even if at the moment the appeal around Stony Hard peaches is not economically relevant, their retaining the peculiar texture of the flesh, even after being cut, can make them suitable for the IV range, a new way of fruit eating that is becoming much more sought after by consumer. This type of flesh is determined by the recessive omozygosis at "*Hd*" locus (*hd/hd*) (Scorza and Sherman, 1996).

Stony Hard is believed to result from a mutation in ethylene production (Haji et al., 2001; Haji et al., 2003). In particular, the non-induction of PpACS1, an ACC synthase isogene during ripening, prevents the conversion of S-adenosyl-l-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene. Therefore, it is presumed that there are individual plants with "Melting" and "Non-Melting" genotypes among the plants showing the "Stony Hard" phenotype (Haji et al., 2005). This is proved by the fact that ethylene can be stress-induced, as by storage below 10°C (Tatsuki et al., 2006; Begheldo et al., 2008), but in a different way depending on whether the peaches are genotypically "Melting" or "Non Melting". So it's clear that "Stony Hard" trait is epistatic to the melting flesh/non melting flesh trait (Haji et al., 2005).

A classification of peach fruit flesh phenotypes is reported in tab. 1.

Table 1 Classification of peach fruit flesh phenotypes from chemical analysis and sensory evaluation at physiological maturity (Layne and Bassi, 2008)

		Pectins[a]			
Flesh texture	Firmness	Soluble	Insoluble	Calcium[a]	Ethylene[b]
Melting					
Soft	Low	+++	+	++	++
Firm	High	+++	++	+++	++
Very, very firm	Very high	++	++	++	+
Stony Hard	Very high	+	+++	++	-/(+)
Non Melting	Very high	+++	+++	+++	+++

a Flesh content; no clear-cut threshold between different phenotypes.

b Amount produced by whole fruits.

c Similar to the Stony Hard, but produces ethylene and becomes melting in the very last stages of ripening (e.g. 'Big Top' nectarine)

2. Aim of the research

The Italian peach compartment is nowadays experiencing a period of crisis. The cause of this crisis can be traced to a chain of production and distribution which is too attentive to the satisfaction of the individual components of the chain (e.g. large fruit size for the growers, fruit firmness for the packers, fruit color, size and texture for retailers) and not sufficiently attentive to the overall fruit quality at the end of the chain, on the table of the final consumers. The development of new cultivars belonging to the Slow Melting phenotype, characterized by a firmer texture even at full ripening and softening more slowly (so that they can be harvested at a riper stage), definitely could help the fruit sector. Until now, the recognition of this new flesh phenotype was entrusted only to operator experience, which is not very reliable and allows recognition of the flesh phenotype only after obtaining the fruit, therefore some years after planting. An early determination, as it could be achieved through molecular markers, would lead to an early selection of fruit phenotypes, thus saving time, money and space. The objective of this research is to find possible physical or chemical differences in different flesh phenotypes, in order to characterize flesh texture. At the same time the expression profiles of the different flesh textures, in order to better understand which genes and pathways are involved in flesh firmness, was investigated.

To reach these goals, three different approaches were performed:

- the characterization by a non-destructive approach. With the collaboration of “Politecnico di Milano”, Time Resolved Spectroscopy (TRS) was performed on peaches belonging to different flesh phenotypes (Melting, Non Melting, Slow Melting, and Stony Hard). This technique gives information about fruit absorption and scattering properties. Scattering measurements in particular provide information concerning fruit structural characteristics, which could give useful information about the peculiar characteristics of the flesh.
- The characterization by a traditional approach. Peaches belonging to Melting, Non Melting, Slow Melting, and Stony Hard phenotypes were analyzed for weight loss, soluble solid contents (SSC), titratable acidity (TA), firmness, and expressible juice.
- The characterization by gene expression analysis. Through this method, different gene expression peach profiles belonging to Melting, Non Melting, Slow Melting, and Stony Hard phenotypes were compared at 2 different stages of ripening (fruit veraison and commercial ripening), in order to better understand which genes and pathways are involved in flesh firmness.

3. A preliminary approach to assess peach fruit texture by Time-Resolved Spectroscopy (TRS)

3.1. Introduction

Until a few years ago, a non-destructive evaluation of fruit quality meant a mere assessment of external appearance (size, shape, color and external defects). To obtain information on internal structure and properties, it was necessary to rely on destructive techniques, which were slow and prone to operational errors. Moreover, these techniques undertake obviously to restrict the analysis to a limited number of samples whose information had then to be extended to the whole batch of fruits, with the inaccuracies that can easily be imagined, given the high levels of biological variability.

The need for an assessment of the quality of each single fruit, in order to improve consumer satisfaction and thus industrial profitability, in recent years has led to a significant improvement of non-invasive techniques (Lu and Peng, 2006). The proof is that, during the last half century, scientific literature dealing with non-destructive or non-invasive analytical methods in food science and technology has been increasing exponentially (Butz et al., 2005).

Different non-destructive techniques have been developed in connection with the quality parameter to be evaluated. Firmness, for example, has been predominantly evaluated by mechanical methods including quasi-static force/deformation, impact and sonic test (Renfu Lu, 2004), while internal quality of fresh fruit, especially flavor-related quality attributes, like soluble solids content (SSC), has been evaluated by near-infrared (NIR) spectroscopy.

Near-infrared spectroscopy (NIRS) is one of the most popular techniques for quality detection of food and agricultural products because it is fast and requires little or no sample preparation (Cen et al., 2012). NIRS measurement provides an approximate quantification of absorption properties of the sample (Dahm and Dahm, 2001). Moreover attempts at measuring firmness using NIR spectroscopy have been reported, but without appreciable results (Mcglone and Kawano, 1998; Lu et al., 2000; Lu and Ariana, 2002).

The absorption coefficient (μ_a) and the reduced scattering coefficient (μ_s') may characterize adequately the optical properties of fruits and vegetables (Cen et al., 2012).

Absorption and scattering properties of the fruit give very different information about the medium. Absorption is determined by the pigments and pulp constituents that produce characteristic spectral features in the visible and near infrared region, giving information concerning the chemical composition of the fruit. Scattering is due to local variation of the refractive index inside the medium. Microscopic changes in refractive index caused by membranes, air vacuoles or organelles, in fact, deviate the photon paths and are ultimately responsible for light diffusion (Cubeddu et al., 2002). Scattering thus provides information concerning the structural

characteristics of the fruits. In order to discriminate between the different flesh phenotypes of peaches, or more generally of fruit, it is essential to know the structural characteristics of the sample and the scattering measurements could have a key role. For this reason scattering information has necessarily to be separated from the absorption information, that is not possible with the traditional optical techniques.

When a light beam (or streams of photons) impinges on a diffusive medium (the fruit), a small fraction is reflected from the surface (specular reflectance) and the majority of the light penetrates into the fruit. The penetrated light scatters and propagates in different directions in the fruit owing to the local changes in the index of refraction at the interfaces of different cellular structures. Some of the penetrated light is absorbed, some goes through the whole fruit and emerges from the opposite side (transmission), and some scatters back and reemerges from a region close to the beam incident point: this is often called diffusive reflectance. In a diffusive medium such as a fruit, in the visible and near infrared spectral region, light scattering is stronger than light absorption, so that light can be scattered many times before being either absorbed or re-emitted from the medium. This phenomenon is called multiple scattering of light (Cubeddu et al., 2002). During this process photons are also absorbed, so that the scattering profile at the surface of the fruit is influenced by, or related to, both absorption and scattering properties of the fruit (Lu and Peng, 2006). The scattering probability per unit length for non-isotropic propagation of photons is described by the reduced scattering coefficient $\mu_s' = (1 - g)\mu_s$, where g is the anisotropy factor, that is the mean cosine of scattering angle and $\mu_s=1/l_s$, l_s being the photon mean free path between successive scattering events. A direct measure of the photon path length allows to know the effect of scattering, and, since the photon path length is directly related to the photon time-of-flight in the medium, the natural choice is to perform time-resolved measurements (Cubeddu et al., 2002).

Near infrared time-resolved spectroscopy (TRS) consists in determining the delay, the attenuation and the time widening suffered by a short near infrared laser impulse propagating in a diffusive medium such as fruit pulp. The delay of the collected impulse depends on the light propagation velocity inside the medium between source and detector, and the time widening is a consequence of the different paths of the light inside the medium because of its diffusion (multiple scattering). Finally, the attenuation depends on absorption, which causes loss of photons, and on diffusion, which scatters photons along directions different from the direction under consideration (Torricelli et al., 2008).

In contrast to conventional optical methods, TRS presents several advantages. Firstly, it allows measurements of the scattering properties of the pulp independent of those of the absorption spectrum. This characteristic made this technique interesting for its potentiality to discriminate between the different flesh phenotypes in peaches. Then, TRS is also insensitive to skin color, being most sensitive to internal features. This second characteristic was demonstrated by measurements performed on different fruits with thin skins, like apples,

peaches, and nectarines. Moreover TRS measurements probe a depth of at least 2 cm within the pulp. An experiment carried on apples, peaches, tomatoes and kiwifruits demonstrated it (Cubeddu et al., 2002).

3.2. Materials and methods

The experiment is the result of a two-years collaboration with the Department of Physics, Politecnico of Milano, and in particular with Professor Alessandro Torricelli and dr. Lorenzo Spinelli.

During the first year, 2010 harvest season, a pilot experiment was carried out, in which two different wavelengths were used, but it was not sufficient to discriminate between the different types of pulp and the results are not reported.

During the second year, 2011 harvest season, the same experiment was repeated and 14 discrete wavelengths were used: 540, 580, 630, 650, 670, 690, 730, 780, 800, 830, 850, 880, 900 and 940 nm.

3.2.1. Peach samples

Peaches belonging to the four flesh phenotypes (Melting, Slow Melting, Non Melting and Stony Hard) were picked in July 2011 from both the Experimental Orchard of the "Agency for Technology Experimantation and Agroenviromental Research" (ASTRA), Azienda Agricola "Zabina" of Castel San Pietro (BO), and from the Experimental Orchard of the University of Milan, Azienda Agricola "F. Dotti" of Arcagna (LO). Peach samples were visually inspected and only those free of visual defects were selected for the experiment. During the travel, fruits were placed in peach nest trays, in order to prevent any damage. Tab. 2 reports the list of accession analyzed.

3.2.2. Ripening parameters

Immediately after harvest, a total of 30 fruits from each accession were preliminarily classified into the same ranges that were subsequently used for the shelf life experiment, basing on DA-value. A total of 330 fruits were analyzed in 2011. Each sample (fruit) was individually numbered.

Tab 2. List of all the accessions. The ones used to perform TRS approach are highlighted.

CULTIVAR	HARVEST DATE	TEXTURE TYPE	APPLICATION				
			Time Resolved Spectroscopy (TRS)	Shelf life			Transcriptomic Analysis
				2010	2011	2012	
Alice Col	13 Jul	NM	√		√	√	
BO 000200006	28 Jul	NM	√		√		
BO 010120182	11 Aug	NM					√
Iride	8 Jul	NM	√		√	√	
Oro A	29 Jun	NM					√
Ambra	15 Jul	M			√	√	
Bolero	15 Aug	M					√
Dixired	8 Jul	M	√		√		
Glohaven	22 Jul	M	√	√	√		
Redhaven	15 Jul	M	√		√	√	√
BO 05030081	28 Jul	SH			√		√
BO 05030149	28 Jul	SH			√		
Ghiaccio	21 Jul	SH	√		√		
IFF 331	30 Jul	SH	√		√		√
Big Top	15 Jul	SM	√	√	√	√	√
Rich Lady	15 Jul	SM	√	√	√	√	√
Vistarich	19 Jul	SM	√		√		

3.2.3. Imaging system

A system for TRS measurements of food quality has been completely developed at the Politecnico di Milano-Department of Physics. The schematic of the TRS setup is shown in figure 4. The light source is a supercontinuum fiber laser (SC450-6W, Fianium, UK) providing white-light picosecond pulses, adjustable in power by a variable neutral-density attenuator. A filter wheel loaded with 14 band-pass interference filters is used for spectral selection in the range 540-940 nm. Light is delivered to the sample by means of a multimode graded-index fiber. Diffuse remitted light is collected by 1 mm fiber. The light then is detected with a photomultiplier (HPM-100-50, Becker&Hickl, Germany) and the photon distribution of time-of-flight is measured by a time-correlated single-photon counting board (SPC-130, Becker&Hickl, Germany). In fig. 5 some pictures of the instruments are reported.

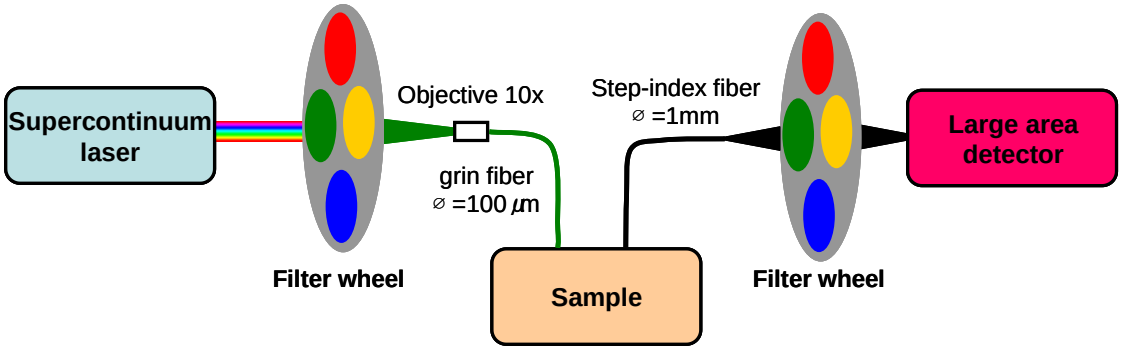


Fig. 4 Scheme of the TRS instrumental setup: PMT is for photomultiplier tube, TCSPC is for time-correlated single-photon counting electronics read-out

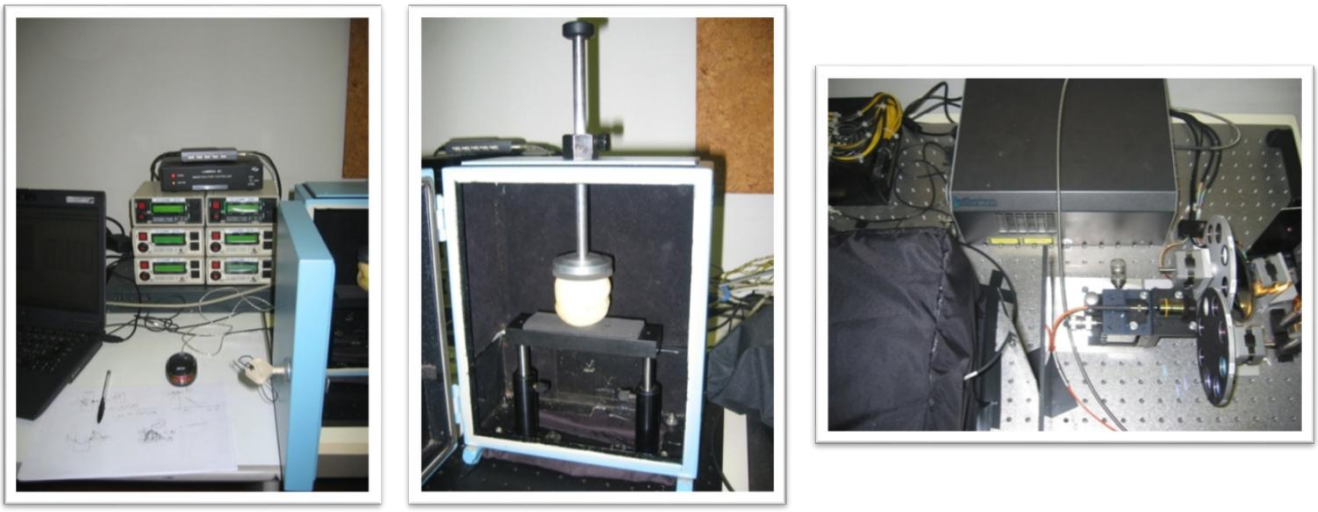


Fig.5 The system for TRS measurement developed by Politecnico di Milano

3.2.4. Data analysis

The temporal profile of the time-resolved reflectance curve was analyzed using a model for the light propagation in diffusive media based on the solution of the Diffusion Equation (Martelli et al., 2009):

$$R(\rho,t) = At^{-5/2} S(\mu_s';t) e^{-\mu_a vt}$$

where $R(\rho,t)$ is the number of photons per unit time (t) and area remitted from the tissue at a distance ρ from the injection point. ρ is the source-detector distance (or inter-fiber distance) and *v* is the speed of light in the medium. This allows for the simultaneous estimate of μ_a and μ_s'. Figure 6 shows an example of TRS curve together with the instrument response function and the fitting model adopted for the analysis.

After having retrieved the absorption and scattering coefficients at different wavelengths, concentrations of fruit constituents and structural parameters can be obtained. In particular, absorption spectra can be interpreted as a function of constituent concentrations by the Beer's law:

$$\mu_a(\lambda) = \sum_i C_i \, \varepsilon_i(\lambda) = C_{H_2O} \, \varepsilon_{H_2O} + C_{CHL-a} \, \varepsilon_{CHL-a}$$

On the other hand, structural parameters can be obtained from the scattering spectra following Mie theory:

$$\mu_s'(\lambda) \approx a \, \lambda^{-b},$$

Where *a* and *b* are two parameters linked to the structural properties of the fruit: in particular, *a* is proportional to the density of the scattering centers and *b* depends on their size.

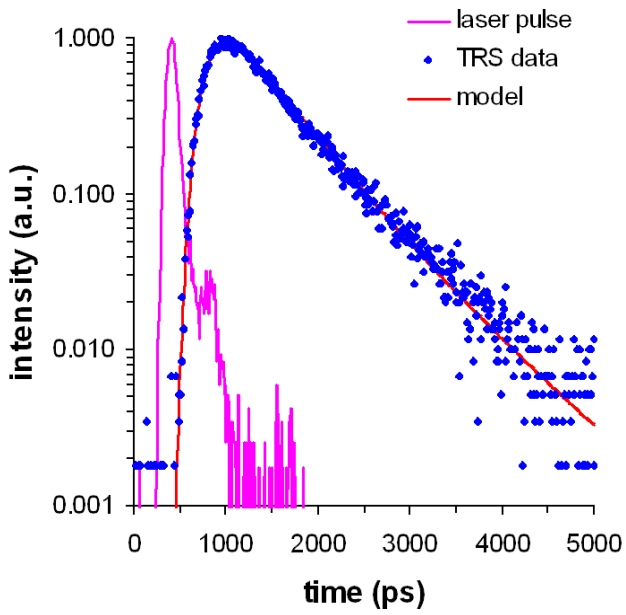


Fig.6 Example of a TRS curve (blue symbols) together with the instrument response function (pink line) and the fitting model (red line).

3.3. Results

3.3.1. Absorption

The absorption spectra were constructed by plotting the value of μ_a as a function of wavelength. Analysing the graphs obtained, it is evident that the absorption spectrum of the peaches between the wavelengths 540 and 940 nm is dominated, with few exceptions, by the anthocyanin peak around 540-580 nm (fig. 7 and 8). In particular cultivar 'Iride' scored the highest values in this range (fig. 8). This is due to the large amount of anthocyanin in the pulp of this cultivar, which is, in fact, particularly red colored.

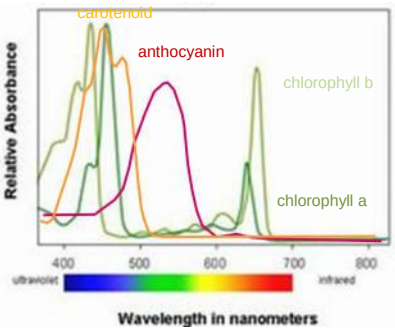


Fig. 7 Example of absorption spectrum of pigments molecules at different wavelengths

The water peak, around 970 nm, is not visible in the measurements being outside the sensitivity range of the photomultiplier used; however, it's possible to see an increasing of absorption around 940 nm in every cultivar.

A significant absorption peak at 675 nm, corresponding to chlorophyll-a, is found with the exception of cultivar 'Ghiaccio' (literally 'Ice') that is characterized by being completely depigmented and by having skin and flesh totally white cream-colored.

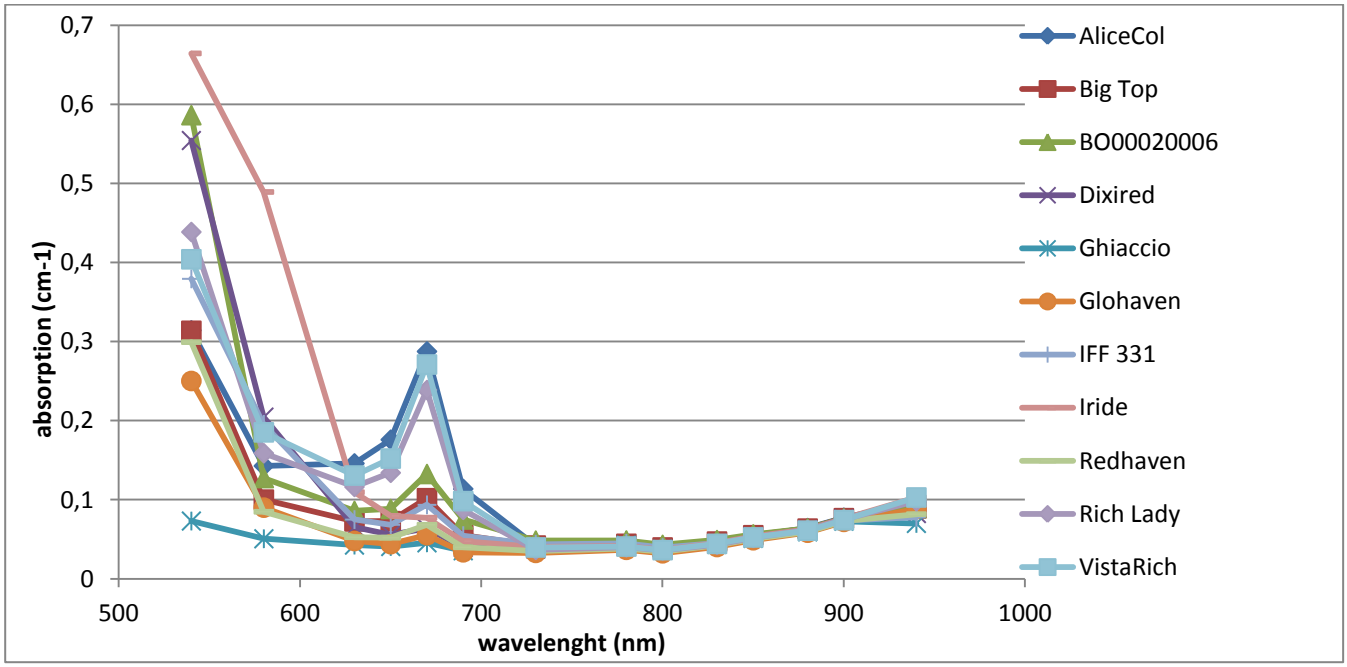
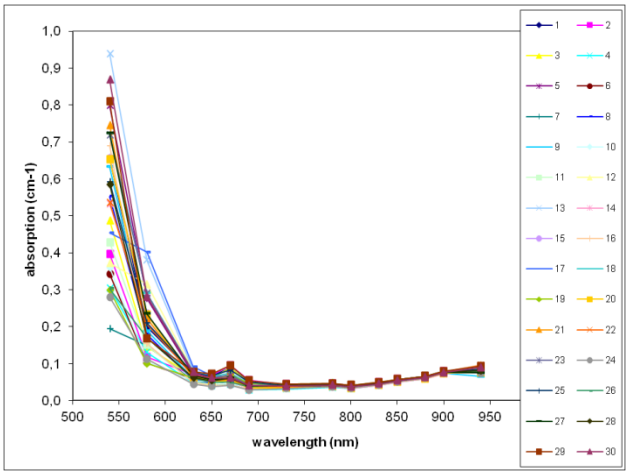


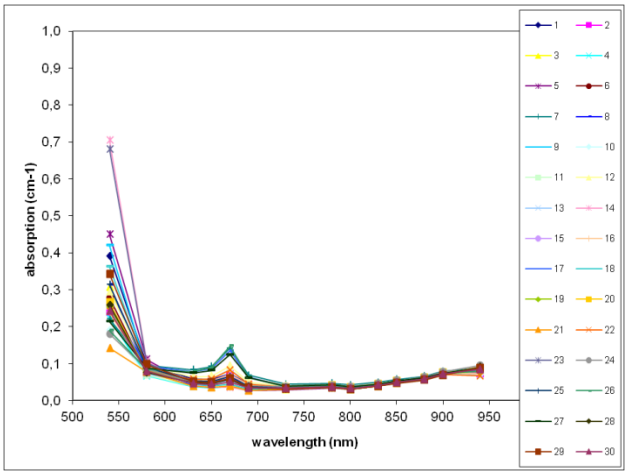
Fig.8 Summary of the absorption spectra of all the samples analyzed.

3.3.1.1. Absorption in Melting (M) phenotype

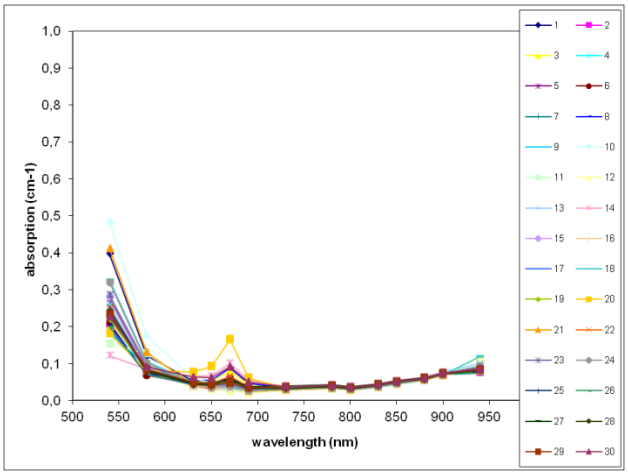
Dixired (M)



Redhaven (M)

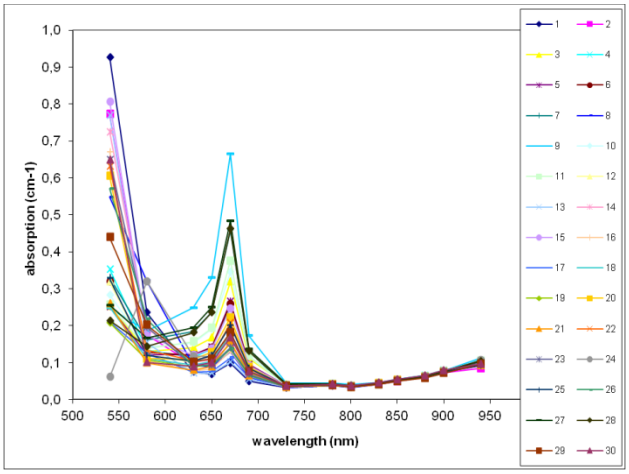


Glohaven (M)

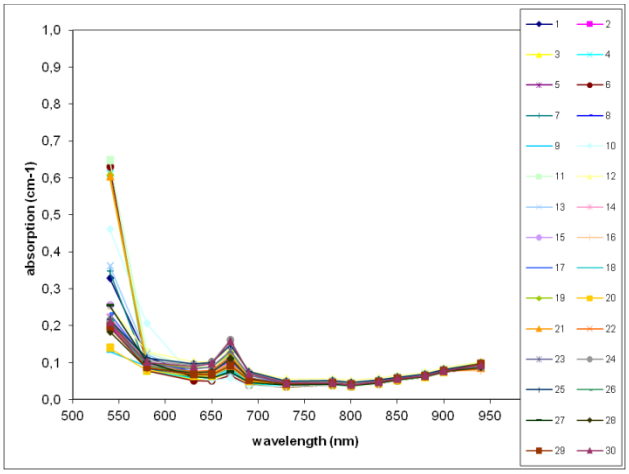


3.3.1.2. Absorption in Slow Melting (SM) phenotype

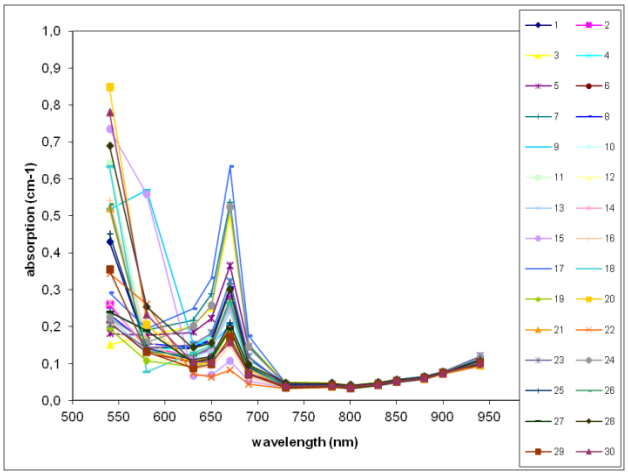
Rich Lady (SM)



Big Top (SM)

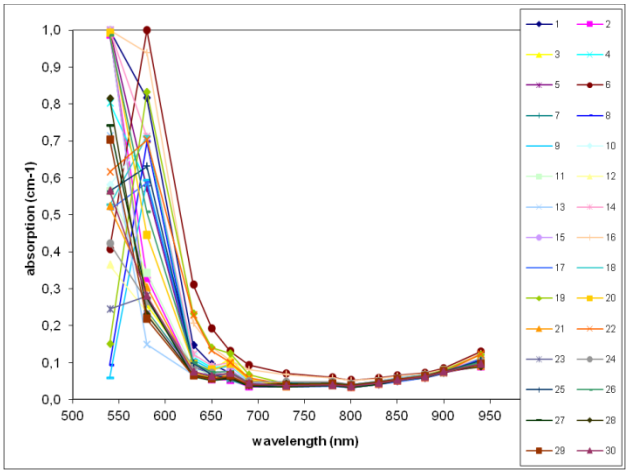


Vistarich (SM)

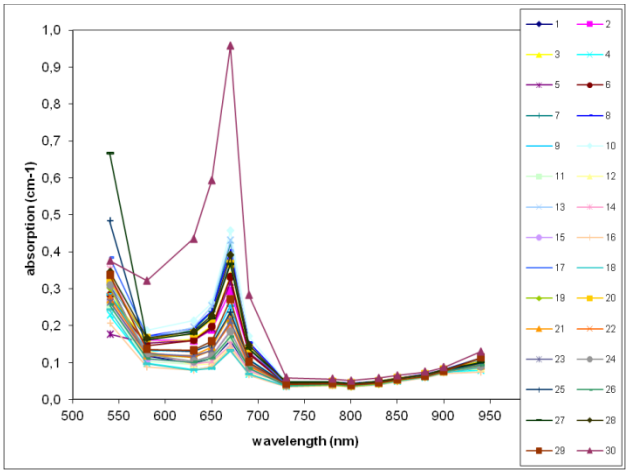


3.3.1.3. Absorption in Non Melting (NM) phenotype

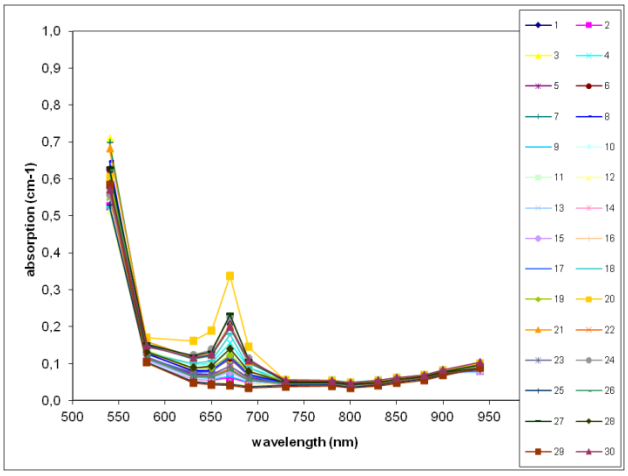
Iride (NM)



Alice Col (NM)

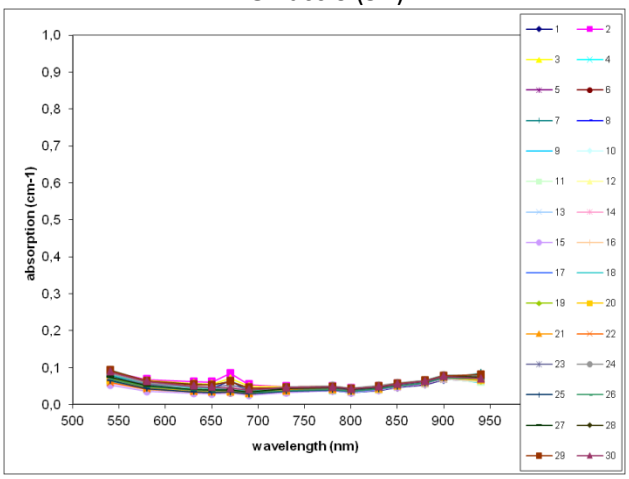


BO0020006 (NM)

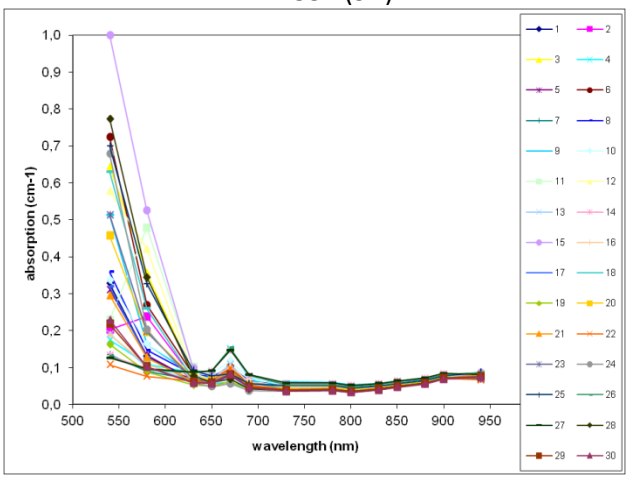


3.3.1.4. Absorption in Stony Hard (SH) phenotype

Ghiaccio (SH)



IFF331 (SH)



3.3.2. Scattering

A summary of the values of the reduced scattering coefficients based on the average of the 30 fruits for tested cultivars is showed in figure 9. Furthermore, the reduced scattering spectra for each cultivar are reported in the following pages. In general, a greater slope in the reduced scattering profile (like in 'Big Top') indicates a refraction due to smaller structures (related to parameter *b)*. 'Glohaven' shows a very high dispersion of the value of scattering, from 4 to 20 cm^{-1}. This is due to the fact that some peaches were more soft to the touch (thus riper) than others. Soft fruit in fact tend to have a broader scattering profile than firmer fruit (Peng and Lu, 2004).

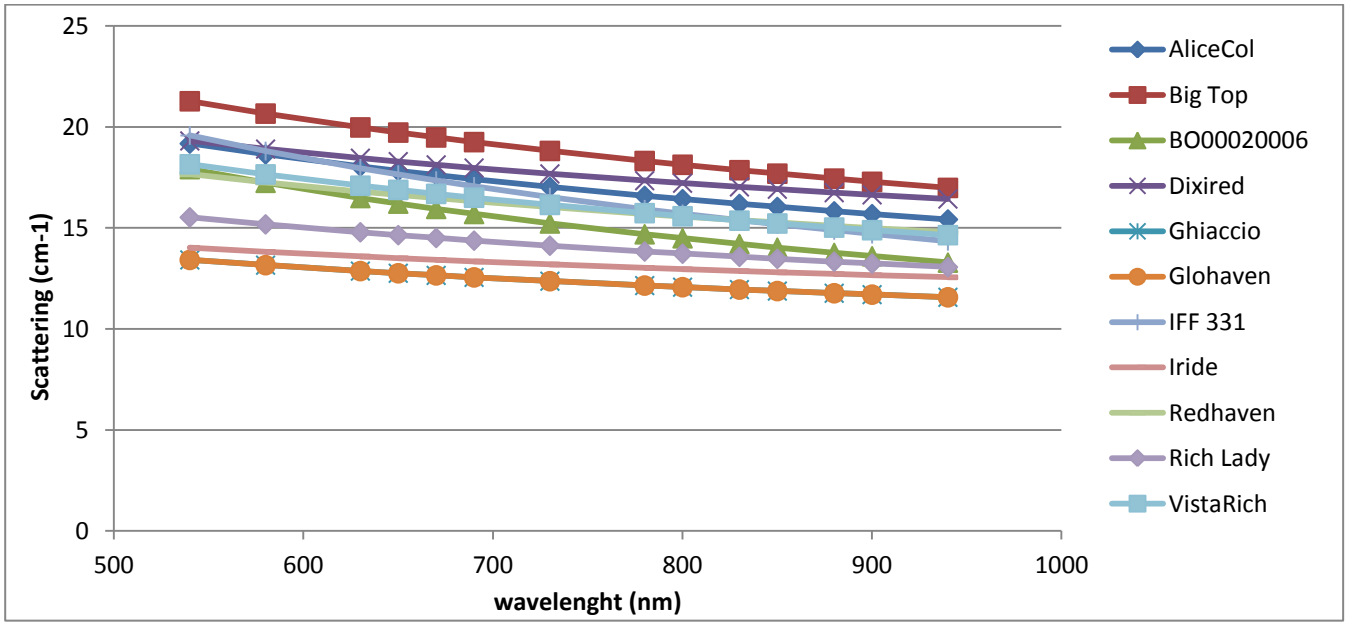
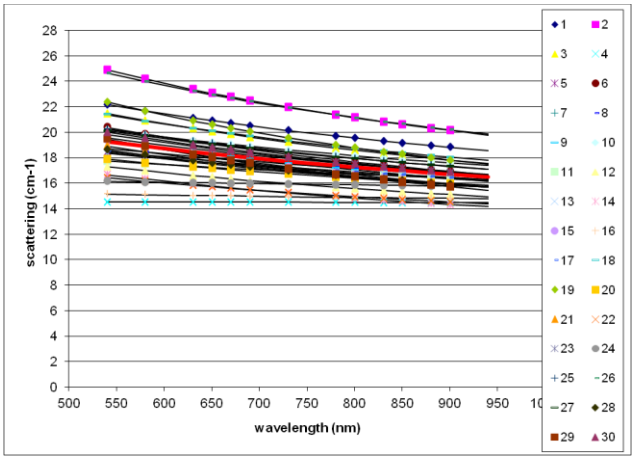


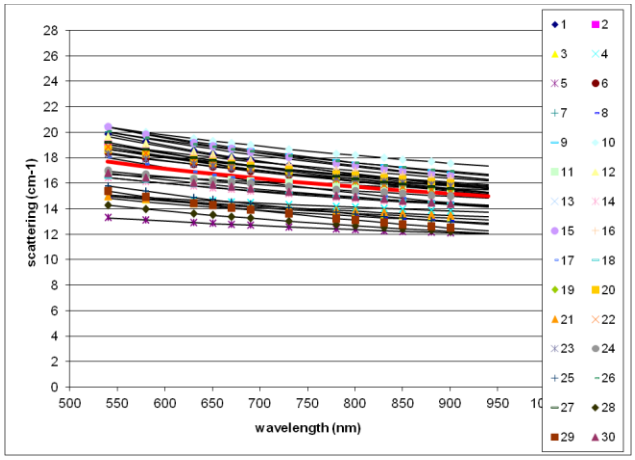
Fig. 9 Average of the scattering spectra of the 30 peach samples for tested cultivars.

3.3.2.1. Scattering in Melting (M) phenotype

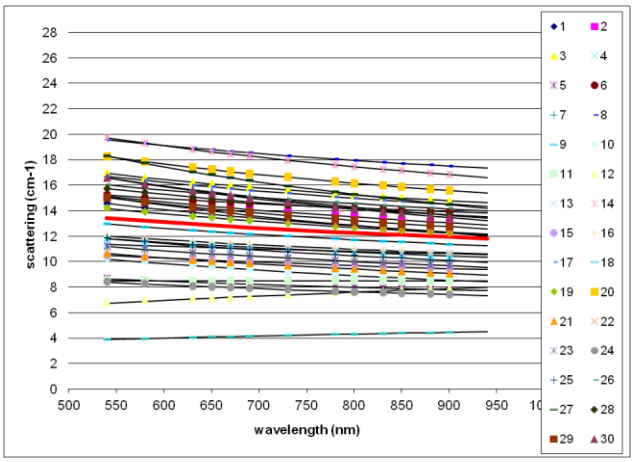
Dixired (M)



Redhaven (M)

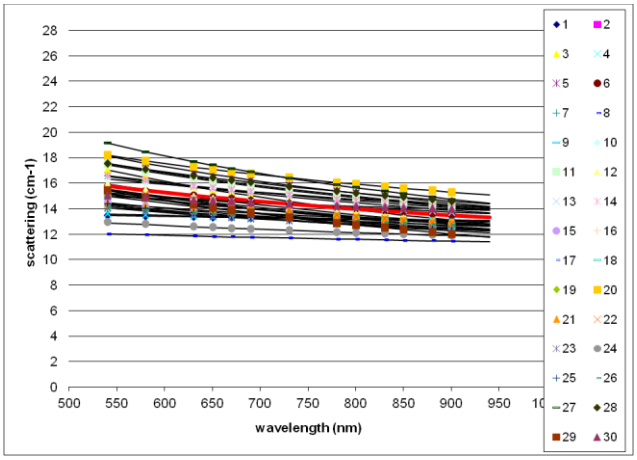


Glohaven (M)

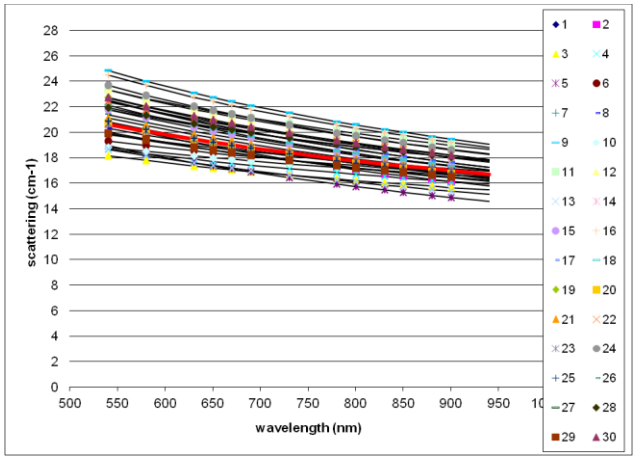


3.3.2.2. Scattering in Slow Melting (SM) phenotype

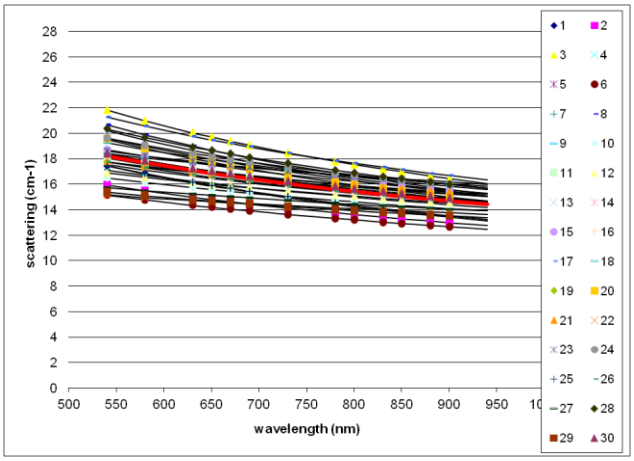
Rich Lady (SM)



Big Top (SM)

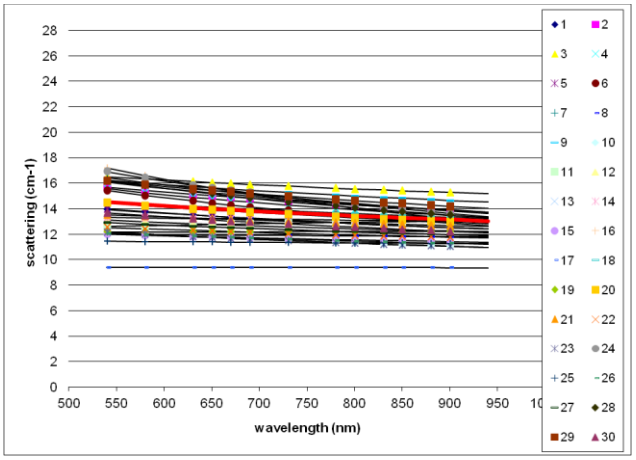


Vistarich (SM)

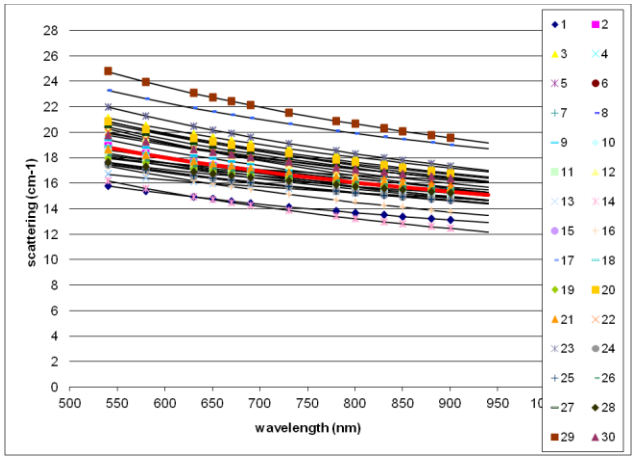


3.3.2.3. Scattering in Non Melting (NM) phenotype

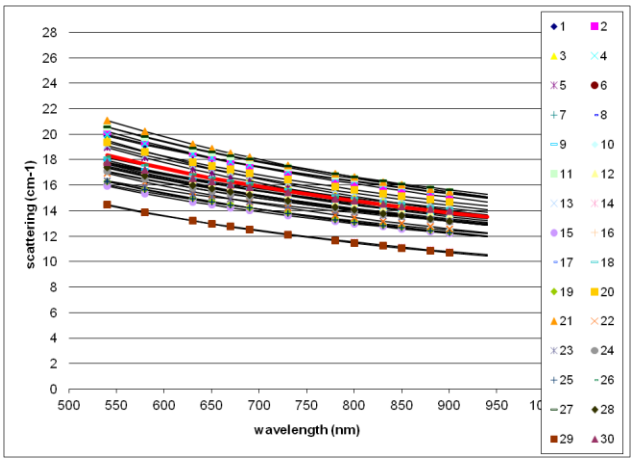
Iride (NM)



Alice Col (NM)

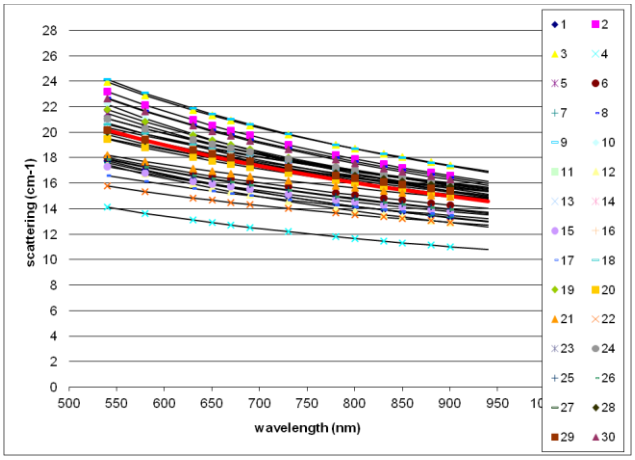


BO0020006 (NM)



3.3.2.4. Scattering in Stony Hard (SH) phenotype

Ghiaccio (SH)



IFF331 (SH)

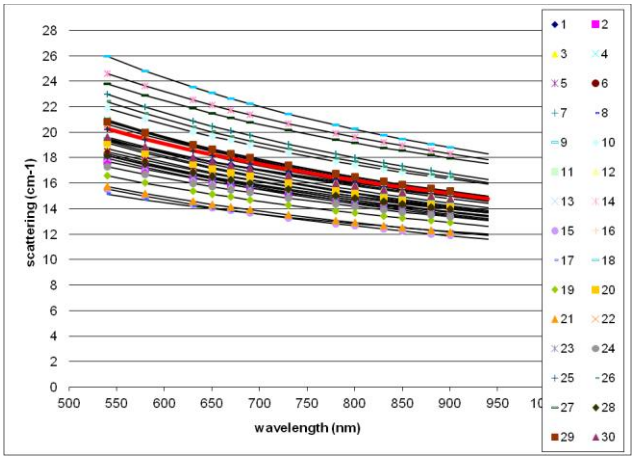


Figure 10 shows parameter *a* on the x-axis, proportional to the density of scattering centers, and parameter *b* on the y-axis, proportional to the size of the scattering centers. Analysis of the graph reveals that this technique has limited capabilities to discriminate between the peach flesh phenotypes. As a matter of fact, if one can say that Melting, Slow Melting and Stony Hard peaches are clustered in quite distinct groups, the different Non Melting peaches varieties overlap to previous cultivars.

This application therefore, although very interesting because it can cluster three flesh phenotypes out of four, cannot be considered decisive for the discrimination of the different flesh phenotypes in peach.

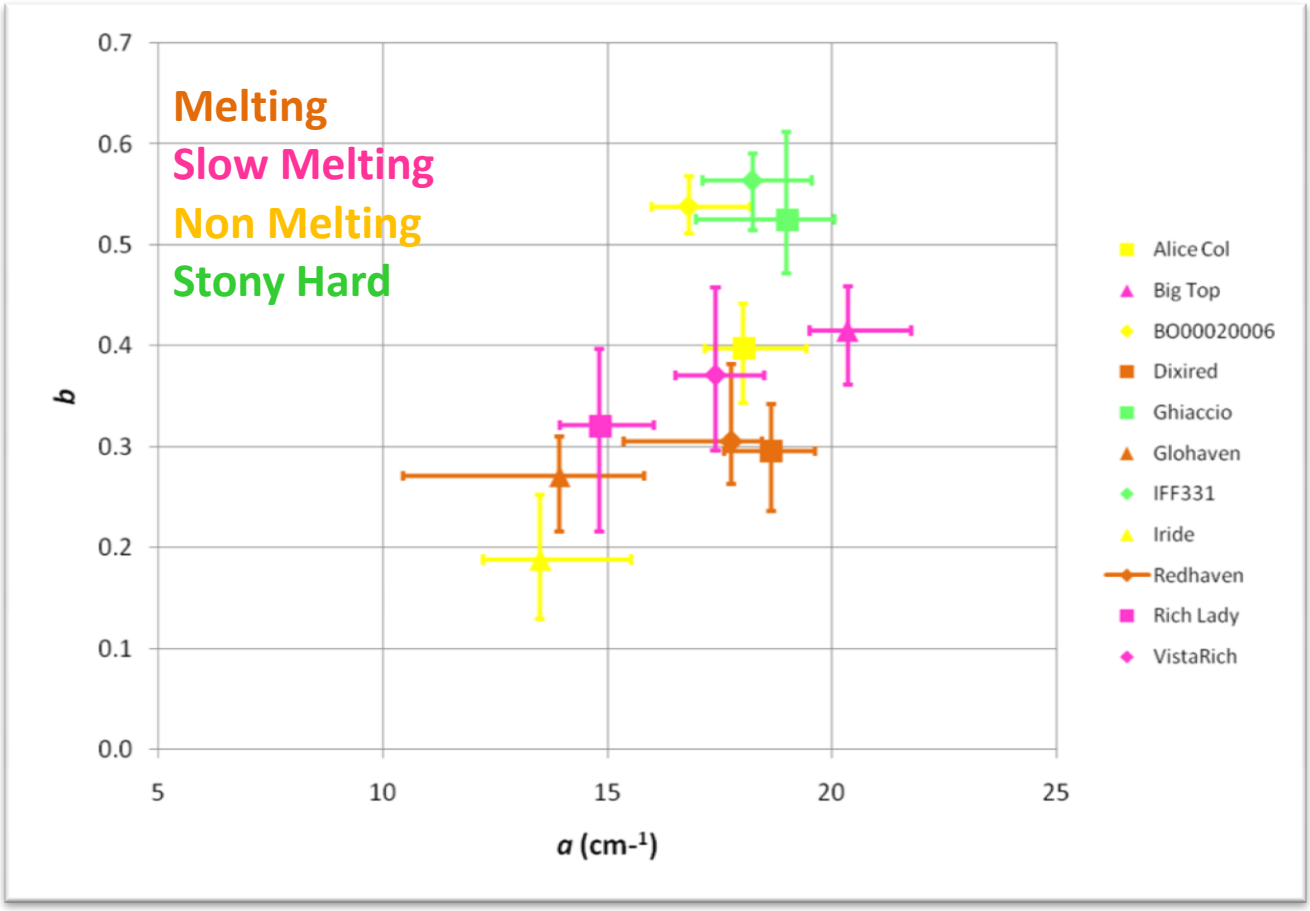


Fig. 10 Power to discriminate of TRS technique between different flesh phenotypes

3.4. Conclusions

The possibility of applying the TRS technique to assess the texture phenotype of peach fruit has been tested.

The TRS technique allowed to separate the effects of absorption and scattering properties.

The absorption spectrum is dominated by peaks around 550 nm, which it is close to the anthocyanins'peak. A second peak is visible at 675 nm, which corresponds to the peak of chlorophyll-a. This peak gives an idea about the fruit ripeness (a higher peak corresponds to less ripe fruit). With the exception of the cultivar 'Iride', particularly rich in anthocyanins, and 'Ghiaccio', a cultivar totally depigmented, the absorption spectra of all the samples are similar.

For what concerns scattering properties, by considering the two Mie parameters, *a* and *b*, it's possible to assert that this technique has limited capabilities to discriminate between the different flesh phenotypes. In fact it can discriminate three flesh phenotypes (Melting, Slow Melting and Stony hard) out of four.

4. Shelf life characterization of peach fruit differing in flesh type

4.1. Introduction

Peaches and nectarines are highly perishable; they ripen and deteriorate quickly at room temperature (Lurie and Crisosto, 2005). To slow down the ripening process and defer decay development, and thus lengthen shelf life, storage at low temperature is used (Robertson et al., 1990). Temperatures below 8°C for more then 2-3 weeks may lead to chilling injury (Lill et al., 1989). In particular peaches show maximum defect development at 2.2 to 7.6°C (Anderson and Penney, 1975; Crisosto et al., 1996). This range of temperature is also known as "killing temperature zone". It has been reported that symptoms are more severe in unripe than in ripe fruit (Ben-Arie and Lavee, 1971).

During shelf life, and more so in extended storage, different quality parameters like weight, firmness, expressible juice, titratable acidity (TA), and soluble solid content (SSC) can change. These changes are the physiological response of peaches and nectarines to shelf life, and the way in which these changes take place can depend also on their texture. For example, peaches (particularly Melting flesh cultivars) are more susceptible to chilling injury, if compared to nectarines or to the firmer Non-Melting cultivars (Lurie and Crisosto, 2005). Melting flesh cultivars and Non Melting Flesh cultivars, in fact, differ in enzymatic capacity for pectic degradation and an association between the development of mealiness and the metabolism of pectic substances has been established (Lester et al., 1996).

Changes in firmness are in a certain way peculiar to a given flesh phenotype. Firmness tends to decrease less in Non Melting than in Melting cultivars. From a genetic point of view, the reason for this behavior lies in the reduced capacity of Non Melting cultivars to degrade cell walls, as they lack the endo-form of polygalacturonase (PG) (Pressey and Avants, 1978). Stony Hard also remain firmer then Melting, not producing ethylene as the result of reduced expression of the gene 1-aminocyclopropane-1-carboxylic acid syntase 1 [Pp-ACS1] (Begheldo et al., 2008; Tatsuki et al., 2006). Non Melting and Stony Hard cultivars thus soften to 16N or higher, while Melting fruits soften to below 8N firmness (Lurie and Crisosto, 2005). Slow Melting, on the other hand, soften like Melting, but with a certain delay.

Soluble solids content (SSC), while being very important for the quality of the fruit (like TA, it is associated with taste), does not change significantly with texture or storage, but it varies widely depending on maturity, cultivar and agronomic conditions (Robertson et al., 1990). A deficit irrigation treatment, for example, induces higher fruit soluble solids concentration and thus a higher retail value (Crisosto et al., 1994; Parker et al., 1991).

Expressible juice is usually used as indicator of woolliness: the amount of juice extracted decreases as the fruit becomes more woolly. Flesh mealiness occurs after fruits are exposed to low temperatures. In these conditions occurs an imbalance between polygalacturonase (PG) and pectin esterase (PE) activity, leading to large pectin

molecules with low esterification. This kind of pectins, aided by calcium in the cell wall, forms a gel which binds free water and cause wooliness symptoms (Ben-Arie and Sonego, 1980). Clingstone peaches, and thus all the Non Melting cultivars, do not develop flesh mealiness during cold storage, although they could be susceptible to other manifestations of chilling injury, such as loss of flavor and development of flesh browning (Crisosto et al., 1999).

Achieving a characterization of the different flesh phenotypes under different conditions leads to finding objective differences between the different flesh phenotypes. Taking advantage of these differences, in the past the Melting cultivars were chosen for fresh consumption, and Non Melting genotypes for refrigerated storage and long distance transportation. Today, however, it is necessary to find the genetic determinants underlying these and other differences, and to link them clearly to the flesh phenotypes. Indeed, this is the only way to the development of molecular markers useful for peach breeding. Peach breeding would undoubtedly benefit from the ability to identify a given flesh phenotype at the seedling stage, which would be possible with marker-assisted selection (MAS).

4.2. Materials and Methods

4.2.1. Plant Material

The experiments were carried out with a total of fourteen peaches accessions [*Prunus persica* (L.) Batsch] belonging to the four different flesh phenotypes, harvested in three seasons from 2010 to 2012 (tab.3). Fruits were picked between June and August from the Experimental Orchard, Azienda Agricola "Zabina" sited in Castel San Pietro (BO) and from the Experimental Orchard of the University of Milan, Azienda Agricola "F. Dotti" in Arcagna (LO).

Fruits of each genotype were harvested in different part of the crown of the tree (lower, medium and upper) in order to have the complete range of fruit ripeness.

One hundred sixty-five fruits were harvested for each accession. Peaches were divided into three maturity stages based on I_{AD}, thus divided into lots for daily analysis, so that each lot had the full I_{AD} range. For each accession, five lots with fifteen fruits were obtained. Each lot was composed by five fruits classified as less mature, five as medium mature and five as more mature. Out of the total of one hundred sixty-five fruits for each accession, seventy-five were held at 20°C, fifteen were put into 4°C storage for two weeks and seventy-five were put into 4°C storage for three weeks. After removing from cold storage, fruits were subsequently held at 20°C during the daily analysis. Every day one lot of fruit was taken for measurement, and fruit quality traits (I_{AD}, fruit weight, firmness, expressible juice) were evaluated. Titratable acidity (TA) and soluble solid content (SSC) were measured on the first and the last day (fifth) of each treatment. Shelf life evaluation was conducted for fruits after harvest and after two and three weeks cold storage.

Tab.3 List of all the accessions. The ones used to perform Shelf life analysis are highlighted.

CULTIVAR	HARVEST DATE	TEXTURE TYPE	APPLICATION				
			Time Resolved Spectroscopy (TRS)	Shelf life			Transcriptomic Analysis
				2010	2011	2012	
Alice Col	13 Jul	NM	√		√	√	
BO 000200006	28 Jul	NM	√		√		
BO 010120182	11 Aug	NM					√
Iride	8 Jul	NM	√		√	√	
Oro A	29 Jun	NM					√
Ambra	15 Jul	M			√	√	
Bolero	15 Aug	M					√
Dixired	8 Jul	M	√		√		
Glohaven	22 Jul	M	√	√	√		
Redhaven	15 Jul	M	√		√	√	√
BO 05030081	28 Jul	SH			√		√
BO 05030149	28 Jul	SH			√		
Ghiaccio	21 Jul	SH	√		√		
IFF 331	30 Jul	SH	√		√		√
Big Top	15 Jul	SM	√	√	√	√	√
Rich Lady	15 Jul	SM	√	√	√	√	√
Vista Rich	19 Jul	SM	√		√		

4.2.2. I_{AD}

The index of absorbance difference (I_{AD}) was measured on two sides of each fruit at harvest and daily during the analysis. The lower value of I_{AD} was taken as expression of the maturity stage of the fruit. These measurements were performed using the DA-Meter (fig. 11), a portable spectrometer that measures absorbance difference between two wavelengths near the chlorophyll-a absorption peak:

Fig. 11 DA-Meter spectrometer

$$I_{AD} = A_{670} - A_{720}$$

where A_{670} and A_{720} are the absorbance values (A) at the wavelengths of 670 and 720 nm respectively.

4.2.3. Weight loss

At harvest time and at the day of analysis, each peach was weighted with the digital Mettler PM4600 DeltaRange Balance to determine weight loss.

4.2.4. Titratable acidity (TA) and soluble solid content (SSC)

Fruits were cut and stored at -20°C until the analysis. In order to highlight the cultivar juice characteristics and to eliminate the variability due to the maturity stage, a sample for each maturity stage was taken from each lot, pooled and passed through an electric juicer (Moulinex, Vitae France) for the measurement of soluble solids content (SSC) and titratable acidity (TA).

The SSC was measured by a digital refractometer (Techniquip, BrixStix).

The TA was determined by titration of 5 mL juice with 0.1 N NaOH and expressed as percentage of malic acid (Crison, Compact Titrator).

4.2.5. Firmness

Firmness measurements were taken on the day of analysis after skin removal on both cheeks using a digital penetrometer (Andilog Centor) fitted with an 8-mm diameter plunger (fig.12). The data were acquired by the RSIC bundle software and outputted to an Excel sheet through the RS232 connection.

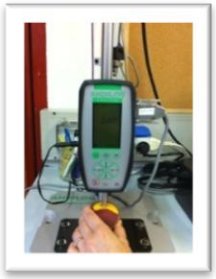


Fig. 12 Andilog Centor penetrometer

4.2.6. Expressible juice

The amount of expressible juice was determined by removing a tissue plug (1 cm long cylinder) from each fruit with a 1 cm diameter cork borer after the skin had been removed. Each cylinder was placed in a 5 mL syringe with no needle and forced through the luer hub to achieve gentle homogenization. The homogenate was collected in an Eppendorf centrifuge tube, weighed and centrifuged (10,000 rpm x 10'). The proportion of juice in relation to the total flesh was calculated and expressed as percentage (Lill and Van Der Mespel, 1988). This value is considered to be the expressible juice content. In fig. 13 pictures of some step of the expressible juice analysis are reported.

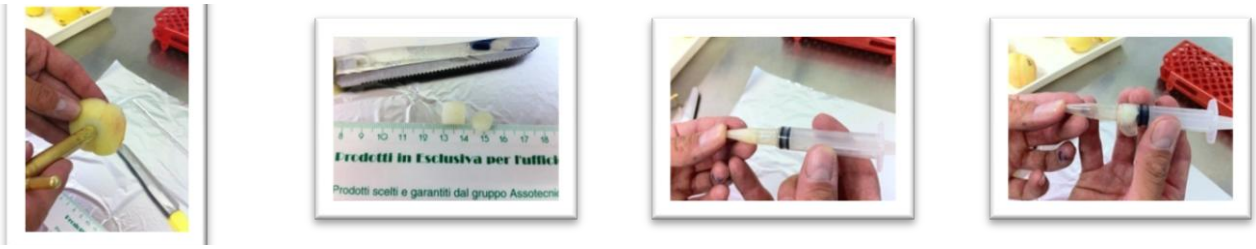


Fig. 13 Some steps of the expressible juice analysis

4.2.7. Statistical data analysis

The data of the chemical and physical analysis were tested for differences between flesh phenotypes using the one-way analysis of variance (ANOVA; general linear model) using IBM SPSS statistic software. The differences between flesh phenotypes were tested with Tukey's high significance difference (HSD) test at the 0.05 significance level. The means and the standard deviations are also reported.

4.3. Results

4.3.1. Weight loss

Figure 14 shows weight loss trend for each flesh phenotypes at physiological ripening during 5 days at 20°C with and without previous cold storage. Weight loss of all the flesh phenotypes was higher in samples held at 20°C after 3 weeks at 4°C.

In samples held without previous cold storage, weight loss increased day after day. It was statistically different in Stony Hard (which lost less weight) and in Slow Melting (which lost the most weight), but it couldn't discriminate between Non Melting and Melting phenotypes (tab 4). The only flesh phenotype that was statistically different from the others during each one of shelf life five days, was Slow Melting which scored the greatest lost weight (tables 5 to 9).

At commercial ripening and in unripe fruits differences between the phenotypes were less stressed and the phenotypes overlap each others (tables 10-11).

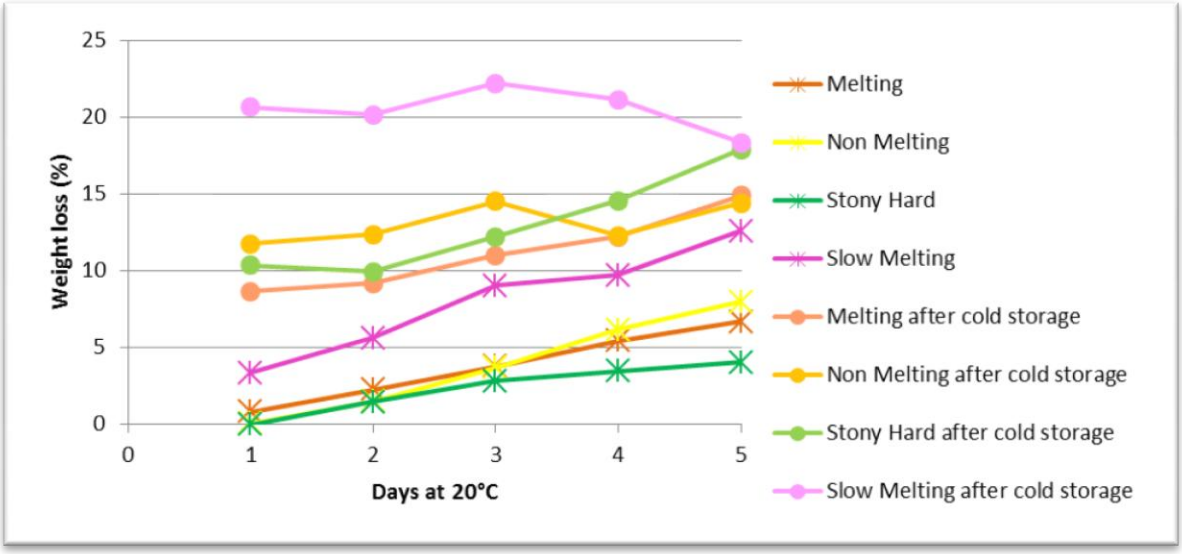


Fig. 14 Weight loss (%) trends without/with previous cold storage at physiological ripening (2011-2012)

Tab. 4 Weight loss (%) at physiological ripening[d] without cold storage (2011-2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
SH	204	2,4		
NM	188		3,8	
M	388		3,9	
SM	192			8,7
Sig.		1,0	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 16.059.
a. Uses Harmonic Mean Sample Size = 222.139.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Physiological ripening without cold storage

Tab. 5 Weight loss (%) at physiological ripening[d] without cold storage (2011-2012)-Day 1

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
SH	39	,0		
NM	37	,1		
M	69		,8	
SM	26			3,4
Sig.		1,0	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 1.257.
a. Uses Harmonic Mean Sample Size = 37.871.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 1, Physiological ripening without cold storage

Tab. 6 Weight loss (%) at physiological ripening[d] without cold storage (2011-2012) -Day 2

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SH	39	1,5	
NM	37	1,5	
M	76	2,3	
SM	35		5,6
Sig.		,3	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 3.871.
a. Uses Harmonic Mean Sample Size = 42.374.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 2, Physiological ripening without cold storage

Tab. 7 Weight loss (%) at physiological ripening[d] without cold storage (2011-2012) -Day 3

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SH	39	2,8	
NM	43	3,7	
M	78	3,8	
SM	36		9,0
Sig.		,4	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 7.999.
a. Uses Harmonic Mean Sample Size = 44.695.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 3, Physiological ripening without cold storage

Tab. 8 Weight loss (%) at physiological ripening[d] without cold storage (2011-2012) -Day 4

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	2	3
SH	44	3,5		
M	88	5,4	5,4	
NM	39		6,2	
SM	47			9,7
Sig.		,9	,8	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 16.677.
a. Uses Harmonic Mean Sample Size = 49.378.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 4, Physiological ripening without cold storage

Tab. 9 Weight loss (%) at physiological ripening[d] without cold storage (2011-2012) -Day **5**

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	2	3
SH	43	4,0		
M	77		6,7	
NM	32		8,0	
SM	48			12,6
Sig.		1,0	,5	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 20.865.
a. Uses Harmonic Mean Sample Size = 45.287.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 5, Physiological ripening without cold storage

Tab. 10 Weight loss (%) at commercial ripening[d] without cold storage (2011-2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
SH	20	1,7		
M	21	2,6	2,6	
SM	129		4,3	4,3
NM	74			5,6
Sig.		,7	,3	,5

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 15.232.
a. Uses Harmonic Mean Sample Size = 33.646.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Commercial ripening without cold storage

Tab. 11 Weight loss (%) in unripe fruits[d] without cold storage (2011-2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
M	10	1,7	
SM	49	1,9	1,9
NM	56		3,3
Sig.		,9	,1

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 4.309.
a. Uses Harmonic Mean Sample Size = 21.697.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Unripe fruits without cold storage

Concerning samples held at 20°C after 3 weeks at 4°C, weight loss at physiological stage greatly exceeded that of samples without cold storage (tab. 12). It discriminated, in the first four days, only for the Slow Melting phenotype, which lost more than 20% of weight (tables 13-17). No statistical differences were found between the four flesh phenotypes at commercial ripening (tab. 18) and in the unripe fruits (data not shown).

Tab. 12 Weight loss (%) at physiological ripening[d] after cold storage (2011-2012)

TukeyHSD[a,b,c]

		Subset	
Flesh Type	N	1	2
M	311	10,9	
SH	66	12,6	
NM	143	13,1	
SM	192		20,5
Sig.		,1	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 57.020.
a. Uses Harmonic Mean Sample Size = 130.855.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Physiological ripening

Tab. 13 Weight loss (%) at physiological ripening[d] after cold storage (2011-2012) -Day 1

TukeyHSD[a,b,c]

		Subset	
Flesh Type	N	1	2
M	68	8,7	
SH	13	10,3	
NM	26	11,7	
SM	38		20,7
Sig.		,4	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 52.068.
a. Uses Harmonic Mean Sample Size = 25.574.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 1, Physiological ripening after cold storage

Tab. 14 Weight loss (%) at physiological ripening[d] after cold storage (2011-2012)-Day 2

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
M	70	9,2	
SH	15	9,9	
NM	36	12,4	
SM	41		20,1
Sig.		,3	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 51.973.
a. Uses Harmonic Mean Sample Size = 30.048.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 2, Physiological ripening after cold storage

Tab. 15 Weight loss (%) at physiological ripening[d] after cold storage (2011-2012) -Day 3

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
M	64	11,0	
SH	15	12,2	
NM	35	14,5	
SM	38		22,2
Sig.		,2	1,00

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 45.458.
a. Uses Harmonic Mean Sample Size = 29.159.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 3, Physiological ripening after cold storage

Tab. 16 Weight loss (%) at physiological ripening[d] after cold storage (2011-2012) -Day 4

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
M	65	12,2	
NM	24	12,3	
SH	15	14,6	
SM	41		21,1
Sig.		,7	1,00

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 63.535.
a. Uses Harmonic Mean Sample Size = 27.007.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 4, Physiological ripening after cold storage

Tab. 17 Tests of Between-Subjects Effects[b]

Dependent Variable:Weight loss (%) at physiological ripening[d] after cold storage (2011-2012) Day 5

Source	Type III Sum of Squares	df	MeanSquare	F	Sig.
Corrected Model	321,7[a]	3	107,2	1,6	,2
Intercept	19276,3	1	19276,3	293,5	,0
FleshType	321,7	3	107,2	1,6	,2
Error	6829,9	104	65,7		
Total	35125,7	108			
Corrected Total	7151,6	107			

a. R Squared = .045 (Adjusted R Squared = .017)

b. Day = 5, Physiological ripening after cold storage

Tab. 18 Tests of Between-Subjects Effects[b]

Dependent Variable: Weight loss (%) Commercial ripening after cold storage(2011-2012)

Source	Type III Sum of Squares	df	MeanSquare	F	Sig.
Corrected Model	127,9[a]	3	42,6	,75	,5
Intercept	1426,6	1	1426,6	25,1	,0
FleshType	127,9	3	42,6	,75	,5
Error	6715,9	118	56,9		
Total	24238,3	122			
Corrected Total	6843,7	121			

a. R Squared = .019 (Adjusted R Squared = -.006)

b. Commercial ripening after cold storage

Tab. 19 Weight loss (%) as recorded for the four different flesh phenotypes with or without cold storage

	NO PREVIOUS STORAGE				3 WEEKS STORAGE AT 4°C			
Dayat 20°C	**Flesh Type**	**Mean**	**N**	**Std. Deviation**	**Flesh Type**	**Mean**	**N**	**Std. Deviation**
1	M	0.8b	69	1.3	M	8.7a	68	4.0
	NM	0.1a	37	0.3	NM	11.7a	26	4.2
	SH	0.0a	39	0.0	SH	10.4a	13	2.2
	SM	3.4c	26	1.8	SM	20.7b	38	12.5
	Total	0.9	171	1.6	Total	12.5	145	8.7
2	M	2.3a	76	1.8	M	9.2a	70	4.1
	NM	1.5a	37	1.0	NM	12.4a	36	4.7
	SH	1.5a	39	0.4	SH	9.9a	15	2.1
	SM	5.6b	35	3.6	SM	20.1b	41	12.5
	Total	2.6	187	2.5	Total	12.7	162	8.4
3	M	3.8a	78	2.2	M	11.0a	64	5.3
	NM	3.7a	43	1.4	NM	14.5a	35	3.3
	SH	2.8a	39	0.9	SH	12.2a	15	2.1
	SM	9.0b	36	5.5	SM	22.2b	38	11.1
	Total	4.5	196	3.5	Total	14.7	152	8.1
4	M	5.4ab	88	3.1	M	12.2a	65	5.1
	NM	6.2b	39	3.6	NM	12.3a	24	3.2
	SH	3.5a	44	1.6	SH	14.6a	15	2.7
	SM	9.7c	47	6.8	SM	21.1b	41	13.2
	Total	6.1	218	4.6	Total	15.0	145	8.8
5	M	6.7b	77	3.4	M	14.9	44	4.4
	NM	8.0b	32	3.9	NM	14.4	22	3.1
	SH	4.0a	43	1.1	SH	17.9	8	3.7
	SM	12.6c	48	7.6	SM	18.3	34	13.1
	Total	7.7	200	5.5	Total	16.1	108	8.2

4.3.2. Titratable acidity (TA)

Titratable acidity was evaluated on samples held at 20°C with and without cold storage on the first day and on the last (fifth) day of storage.

In samples held at 20°C without cold storage there were no consistent difference in TA (%) between Melting, Non Melting and Slow Melting flesh phenotypes, on both the first day and the last day of storage. Stony Hard instead, was different from the others flesh phenotypes, having the lowest TA value (tables 20-21).

A similar behavior was observed in samples held at 20°C after cold storage (tables 22-23).

Titratable acidity in samples stored at 20°C after 3 weeks at 4°C, compared with that of samples held at 20°C without cold storage, was lower in all the flesh phenotypes but not in Stony Hard (fig. 15).

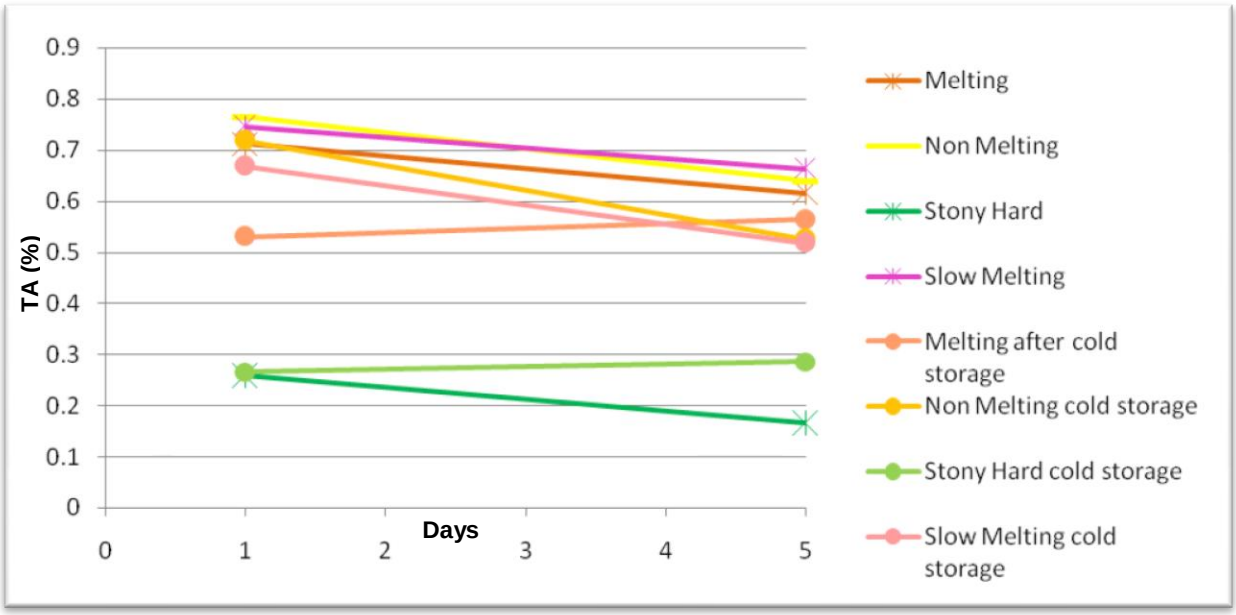


Fig. 15 TA (%) trend without/with previous cold storage (2011-2012)

Tab. 20 TA (%)[d] without cold storage (2011-2012) -Day 1

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SH	12	,3	
M	18		,7
SM	15		,8
NM	15		,8
Sig.		1,0	,9

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .031.
a. Uses Harmonic Mean Sample Size = 14.694.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day at 20°C= 1 without cold storage

Tab. 21 TA (%)[d] without cold storage (2011-2012) -Day **5**

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SH	12	,2	
M	18		,6
NM	12		,6
SM	15		,7
Sig.		1,0	,9

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = .026.

a. Uses Harmonic Mean Sample Size = 13.846.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = 0.05.

d. Day at 20°C= 5 without cold storage

Tab. 22 TA (%)[d] after cold storage (2011-2012) -Day 1

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SH	6	,3	
M	15		,5
SM	15		,7
NM	12		,7
Sig.		1,0	,2

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .040.
a. Uses Harmonic Mean Sample Size = 10.435.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day at 20°C after 3 weeks at 4°C = 1

Tab. 23 TA (%)[d] after cold storage (2011-2012) -Day 5

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SH	4	,3	
SM	12	,5	,5
NM	9		,5
M	16		,6
Sig.		,1	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .029.
a. Uses Harmonic Mean Sample Size = 7.890.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day at 20°C after 3 weeks at 4°C = 5

Tab. 24 TA (%) as recorded for the four different flesh phenotypes with or without cold storage

TA (%)					
Day	Treatment	Flesh Type	Mean	N	Std. Deviation
1	20°C	M	0.7b	18	0.2
		NM	0.8b	15	0.1
		SH	0.3a	12	0.1
		SM	0.8b	15	0.3
		Total	0.6	60	0.3
5	20°C	M	0.6b	18	0.2
		NM	0.6b	12	0.2
		SH	0.2a	12	0.1
		SM	0.7b	15	0.2
		Total	0.5	57	0.3
1	20°C after 3 weeks at 4°C	M	0.5b	15	0.2
		NM	0.7b	12	0.2
		SH	0.3a	6	0.1
		SM	0.7b	15	0.2
		Total	0.6	48	0.2
5	20°C after 3 weeks at 4°C	M	0.6b	16	0.1
		NM	0.5b	9	0.2
		SH	0.3a	4	0.1
		SM	0.5ab	12	0.2
		Total	0.5	41	0.2

4.3.3. Soluble Solids Content (SSC)

Soluble Solids Content was evaluated on samples held at 20°C with and without cold storage on the first day and on the last (fifth) day of storage (fig. 16).

In both conditions there was no consistent difference in SSC (%) among all the four flesh phenotypes (tables 25-26). The SSC of peaches, in fact, is strictly related to the cultivar, agronomic conditions and maturity.

After cold storage, there was no statistical difference between the different flesh phenotypes (tables 27-28).

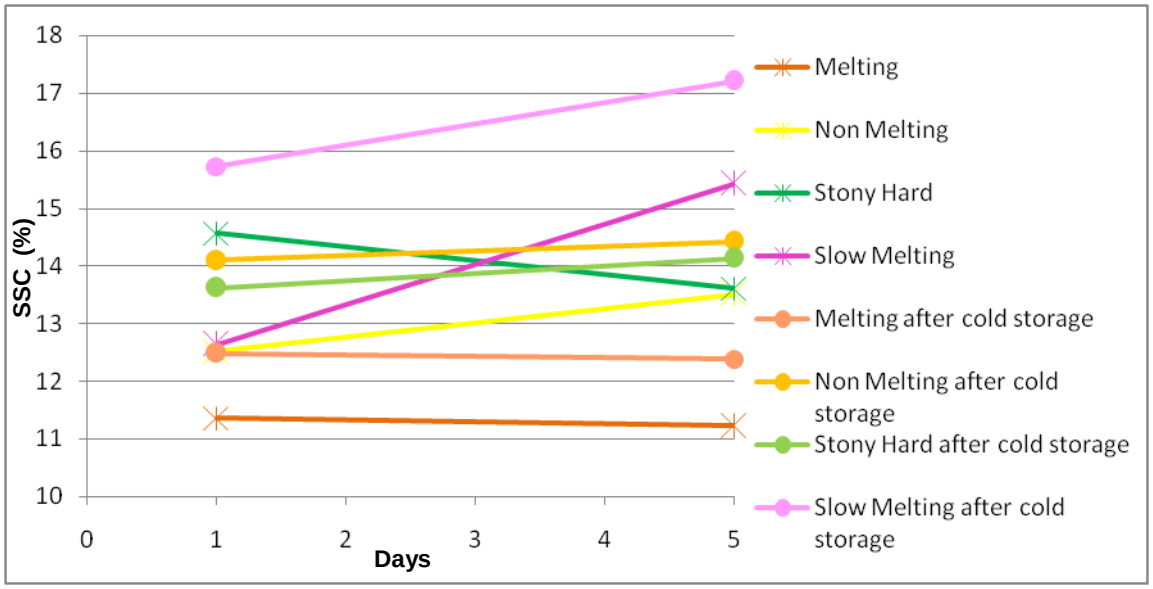


Fig. 16 SSC (%) trend without/with previous cold storage (2011-2012)

Tab. 25 SSC (%) without cold storage (2011-2012) -Day 1

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
M	18	11,4	
NM	15	12,5	12,5
SM	15	12,6	12,6
SH	12		14,6
Sig.		,6	,2

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 6.756.
a. Uses Harmonic Mean Sample Size = 14.694.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day at 20°C= 1

Tab. 26 SSC (%) without cold storage (2011-2012) -Day 5

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
M	18	11,2	
NM	12	13,5	13,5
SH	12	13,6	13,6
SM	15		15,4
Sig.		,2	,4

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 9.054.
a. Uses Harmonic Mean Sample Size = 13.846.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day at 20°C= 5

Tab. 27 Tests of Between-Subjects Effects[b]

Dependent Variable: SSC (%)after cold storage(2011-2012)Day 1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	79,6[a]	3	26,6	2,0	,1
Intercept	8156,1	1	8156,1	617,2	,0
Texture	79,6	3	26,6	2,0	,1
Error	581,4	44	13,2		
Total	10122,3	48			
Corrected Total	661,1	47			

a. R Squared = .120 (Adjusted R Squared = .060)

b. Day at 20°C after 3 weeks at 4°C = 1

Tab. 28 Tests of Between-Subjects Effects[b]

Dependent Variable: SSC (%) after cold storage(2011-2012) Day 5

Source	Type III Sum of Squares	df	MeanSquare	F	Sig.
Corrected Model	159,2[a]	3	53,1	2,8	,1
Intercept	6666,7	1	6666,7	350,0	,0
Texture	159,2	3	53,1	2,8	,1
Error	704,9	37	19,1		
Total	9380,2	41			
Corrected Total	864,0	40			

a. R Squared = .184 (Adjusted R Squared = .118)

b. Day at 20°C after 3 weeks at 4°C = 5

Tab. 29 SSC (%) as recorded for the four different flesh phenotypes with or without cold storage

SSC (%)

Day	Treatment	Flesh Type	Mean	N	Std. Deviation
1	20°C	M	11.4a	18	1.8
		NM	12.5ab	15	2.0
		SH	14.6b	12	2.3
		SM	12.6ab	15	3.9
		Total	12.6	60	2.8
5	20°C	M	11.2a	18	1.7
		NM	13.5ab	12	1.2
		SH	13.6ab	12	1.0
		SM	15.4b	15	5.4
		Total	12.7	57	2.9
1	20°C after 3 weeks at 4°C	M	12.5	15	1.9
		NM	14.1	12	1.7
		SH	13.6	6	0.8
		SM	14.9	14	5.1
		Total	13.8	47	3.2
5	20°C after 3 weeks at 4°C	M	12.4	16	2.3
		NM	14.4	9	1.6
		SH	14.1	4	1.2
		SM	15.1	10	6.1
		Total	13.7	39	3.6

4.3.4. Firmness

Fig. 17 shows firmness trend for each flesh phenotypes during 5 days at 20°C with and without previous cold storage. All the flesh phenotypes, with the exception of Stony Hard, had a similar softening trend at 20°C with and without cold storage. Firmness was higher in samples held at 20°C with previous cold storage in Non Melting and in Stony Hard, while it was lower in Slow Melting cultivars. In Melting it didn't differ in either condition.

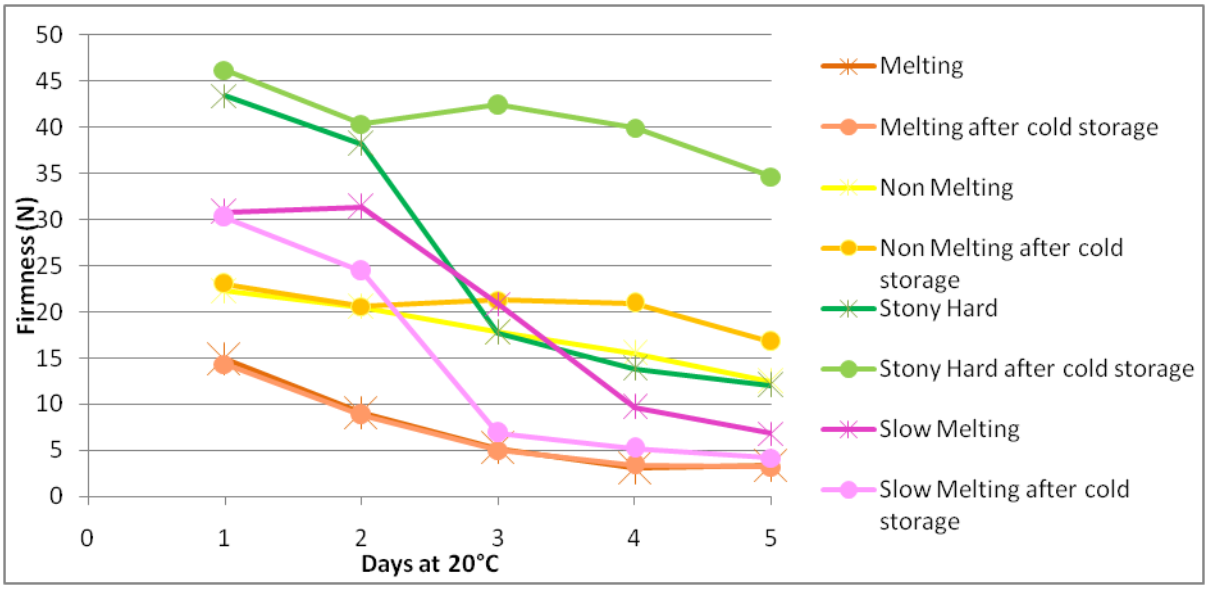


Fig. 17 Firmness trend with/without previous cold storage at physiological ripening years 2011-2012

A day by day analysis revealed that firmness was statistically different in all the flesh phenotypes on the first and second day. Only Melting flesh cultivars, being the softest, differed from the other phenotypes on each day of shelf life (tab. 31 to 35). Melting started softening on the first day and on the third it was complete melted (having a firmness value <8N). The Slow Melting phenotype started softening between the second and the third day, but only on the fifth day its firmness was lower than 8N. Non Melting and Stony Hard firmness reached 12N on the fifth day. They never melted and, at the end of the shelf life, there was no statistically significant difference between them, but they were firmer than Slow Melting and Melting.

Tab. 30 Firmness (N) at physiological ripening[d] without cold storage (2011-2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
M	387	6,8		
NM	187		17,8	
SM	192		17,9	
SH	204			24,5
Sig.		1,0	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 136.253.
a. Uses Harmonic Mean Sample Size = 221.707.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Physiological ripening

Tab. 31 Firmness (N) at physiological ripening[d] without cold storage (2011-2012)-Day 1

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3	Subset 4
M	69	14,9			
NM	37		22,2		
SM	26			30,8	
SH	39				43,4
Sig.		1,0	1,0	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 128.059.
a. Uses Harmonic Mean Sample Size = 37.871.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 1

Tab. 32 Firmness (N) at physiological ripening[d] without cold storage (2011-2012)-Day 2

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	2	3	4
M	75	9,0			
NM	37		20,5		
SM	35			31,4	
SH	39				38,2
Sig.		1,0	1,	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 116.607.
a. Uses Harmonic Mean Sample Size = 42.295.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 2

Tab. 33 Firmness (N) at physiological ripening[d] without cold storage (2011-2012)-Day 3

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	2
M	78	5,2	
SH	39		17,7
NM	43		17,8
SM	36		20,9
Sig.		1,0	,4

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 78.627.
a. Uses Harmonic Mean Sample Size = 44.695.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 3

Tab. 34 Firmness (N) at physiological ripening[d] without cold storage (2011-2012)-Day 4

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
M	88	3,1		
SM	47		9,6	
SH	44			13,9
NM	38			15,5
Sig.		1,0	1,0	,4

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 28.621.
a. Uses Harmonic Mean Sample Size = 48.970.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 4

Tab. 35 Firmness (N) at physiological ripening[d] without cold storage (2011-2012)-Day 5

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
M	77	3,4		
SM	48		6,8	
SH	43			12,0
NM	32			12,4
Sig.		1,0	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 18.371.
a. Uses Harmonic Mean Sample Size = 45.287.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 5

In samples held at 20°C after 3 weeks at 4°C firmness discriminated between the different flesh phenotypes (tab. 36). Stony Hard was the phenotype that retained its firmness better. Melting was the softest.

Tab. 36 Firmness (%) at physiological ripening[d] after cold storage (2011-2012)

TukeyHSD[a,b,c]

		Subset			
Flesh Type	N	1	2	3	4
M	311	7,3			
SM	192		14,4		
NM	144			20,7	
SH	66				41,2
Sig.		1,0	1,0	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 109.838.
a. Uses Harmonic Mean Sample Size = 131.063.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Physiological ripening after 3 weeks at 4°C

Analyzing firmness day by day, Slow Melting was not statistically different from Non Melting for the first two days. It passed the 8N on the third day, two days in advance compared with Slow Melting without cold storage. From the third day it became not statistically different from Melting. Non Melting and Stony Hard were statistically different mutually and from the others phenotypes from the third days (tab 37 to 41).

Tab. 37 Firmness (N) at physiological ripening[d] after cold storage (2011-2012) -Day 1

TukeyHSD[a,b,c]

		Subset		
Flesh Type	N	1	2	3
M	68	14,30		
NM	27	23,01	23,01	
SM	38		30,27	
SH	13			46,22
Sig.		,06	,15	1,00

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 150.598.
a. Uses Harmonic Mean Sample Size = 25.809.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 1 after cold storage

Tab. 38 Firmness (N) at physiological ripening[d] after cold storage (2011-2012) -Day 2

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
M	70	8,8		
NM	36		20,6	
SM	41		24,4	
SH	15			40,4
Sig.		1,0	,5	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 122.503.
a. Uses Harmonic Mean Sample Size = 30.048.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 2 after cold storage

Tab. 39 Firmness (N) at physiological ripening after cold storage (2011-2012) -Day 3

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
M	64	5,0		
SM	38	6,8		
NM	35		21,3	
SH	15			42,4
Sig.		,6	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 29.977.
a. Uses Harmonic Mean Sample Size = 29.159.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 3 after cold storage

Tab. 40 Firmness (N) at physiological ripening[d] after cold storage (2011-2012) -Day 4

TukeyHSD[a,b,c]

		Subset		
Flesh Type	N	1	2	3
M	65	3,4		
SM	41	5,2		
NM	24		21,0	
SH	15			39,9
Sig.		,3	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 13.286.
a. Uses Harmonic Mean Sample Size = 27.007.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 4 after cold storage

Tab. 41 Firmness (N) at physiological ripening[d] after cold storage (2011-2012) -Day 5

TukeyHSD[a,b,c]

		Subset		
Flesh Type	N	1	2	3
M	44	3,2		
SM	34	4,2		
NM	22		16,8	
SH	8			34,7
Sig.		,8	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 11.232.
a. Uses Harmonic Mean Sample Size = 17.970.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Day = 5 after cold storage

When analyzing firmness of samples at commercial ripening held at 20°C without cold storage the scenario becomes more confused and only the extreme phenotypes like Melting and Stony Hard were mutually different (tab. 42). Between unripe fruits kept at 20°C with and without cold storage and between samples at commercial ripening kept at 20°C after cold storage there were no significant differences (tables 43 to 45).

Tab. 42 Firmness (N) at commercial ripening[d] without cold storage (2011-2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
M	21	15,8	
NM	74	24,2	24,2
SM	128		29,4
SH	20		33,1
Sig.		,1	,1

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 253.677.
a. Uses Harmonic Mean Sample Size = 33.629.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Commercial ripening

Tab. 43 Tests of Between-Subjects Effects[b]

Dependent Variable: Firmness(N) in unripe fruit without cold storage (2011-2012)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1347,4[a]	2	673,7	2,9	,1
Intercept	103492,4	1	103492,4	441,8	,0
FleshType	1347,4	2	673,7	2,9	,1
Error	24828,3	106	234,2		
Total	229560,6	109			
Corrected Total	26175,7	108			

a. R Squared = .051 (Adjusted R Squared = .034)
b. Unripe fruit 20°C (2011-2012)

Tab. 44 Tests of Between-Subjects Effects[b]

Dependent Variable: Firmness (N)at commercial ripening after cold storage (2011-2012)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1067,9[a]	3	356,0	1,4	,2
Intercept	14455,3	1	14455,3	57,1	,0
Flesh Type	1067,9	3	356,0	1,4	,2
Error	29858,8	118	253,0		
Total	148909,4	122			
Corrected Total	30926,7	121			

a. R Squared = .035 (Adjusted R Squared = .010)
b. Commercial ripening after 3 weeks at 4°C (2011-2012)

Tab. 45 Tests of Between-Subjects Effects[b]

Dependent Variable: Firmnessin unripe fruit after cold storage (2011-2012)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	52,2[a]	1	52,2	,4	,5
Intercept	103443,6	1	103443,6	805,4	,0
Flesh Type	52,2	1	52,2	,4	,5
Error	5265,8	41	128,4		
Total	108709,4	43			
Corrected Total	5318,0	42			

a. R Squared = .010 (Adjusted R Squared = -.014)
b. Unripe fruit 20°C after 3 weeks at 4°C (2011-2012)

Tab. 46 Firmness (N) as recorded for the four different flesh phenotypes with or without cold storage

	NO PREVIOUS STORAGE				3 WEEKS STORAGE AT 4°C			
Day at 20°C	Flesh Type	Mean	N	Std. Deviation	Flesh Type	Mean	N	Std. Deviation
1	M	14.9a	69	14.79	M	14.3a	68	13.0
	NM	22.2b	37	6.28	NM	23.0ab	27	7.1
	SH	43.4d	39	6.30	SH	46.2c	13	5.6
	SM	30.8c	26	11.98	SM	30.3b	38	15.1
	Total	25.4	171	15.88	Total	22.9	146	15.7
2	M	9.0a	75	11.24	M	8.8a	70	8.6
	NM	20.5b	37	4.87	NM	20.6b	36	7.7
	SH	38.2d	39	6.36	SH	40.4c	15	8.5
	SM	31.4c	35	16.70	SM	24.4b	41	16.7
	Total	21.7	186	15.97	Total	18.3	162	14.7
3	M	5.2a	78	7.74	M	5.0a	64	3.5
	NM	17.8b	43	4.73	NM	21.3b	35	8.9
	SH	17.7b	39	7.57	SH	42.4c	15	5.9
	SM	20.9b	36	14.51	SM	6.8a	38	3.5
	Total	13.3	196	11.06	Total	12.9	152	13.0.
4	M	3.1a	88	1.06	M	3.4a	65	0.9
	NM	15.5c	38	6.31	NM	21.0b	24	5.8
	SH	13.9c	44	7.05	SH	39.9c	15	6.8
	SM	9.6b	47	7.21	SM	5.2a	41	3.2
	Total	8.9	217	7.41	Total	10.6	145	12.3
5	M	3.4a	77	1.76	M	3.2a	44	0.6
	NM	12.4c	32	4.40	NM	16.8b	22	5.1
	SH	12.0c	43	5.98	SH	34.7c	8	8.7
	SM	6.8b	48	5.19	SM	4.2a	34	1.5
	Total	7.5	200	5.76	Total	8.6	108	9.7

4.3.5. Expressible juice

Fig. 18 shows expressible juice trend for each flesh phenotypes during 5 days at 20°C with and without previous cold storage. Expressible juice was higher in samples held at 20°C without cold storage.

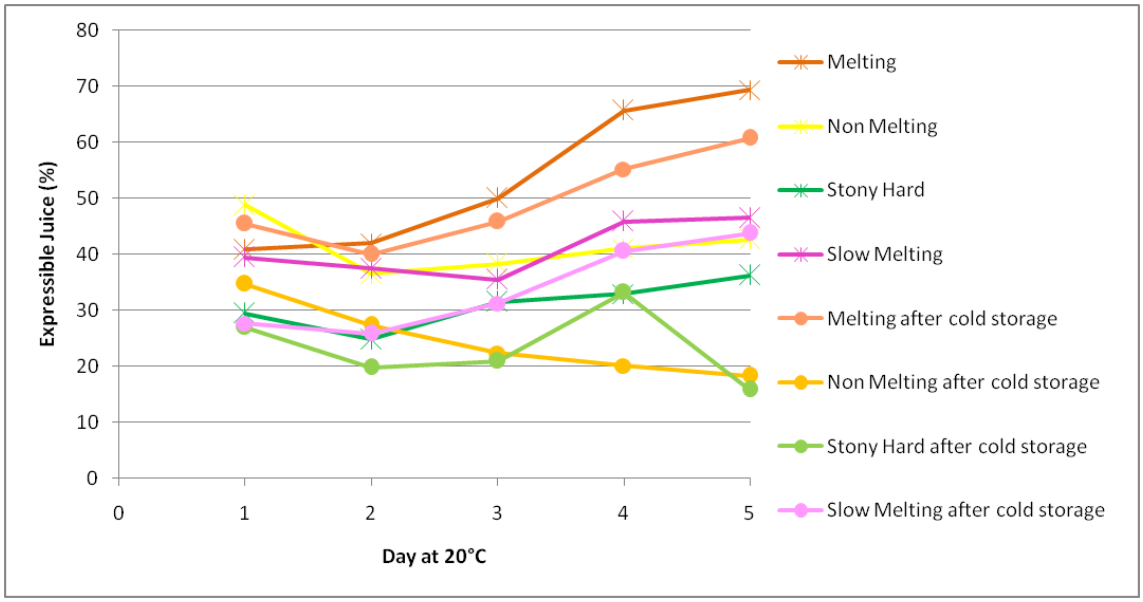


Fig. 18 Expressible Juice (%) trend with and without previous cold storage at physiological ripening years 2011-2012

In 2011, in samples at physiological ripening held at 20°C without any previous cold storage, expressible juice discriminated between all the four flesh phenotypes (tab. 47). The same result was obtained in 2012 (tab. 48). The juicier phenotype was Melting.

Tab. 47 Expressible juice (%) at physiological ripening[d](2011)

Tukey[a,b,c]

Flesh Type	N	Subset 1	2	3	4
SH	203	31,2			
NM	90		36,6		
SM	82			45,9	
M	210				53,7

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 307.077.
a. Uses Harmonic Mean Sample Size = 121.233.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Year = 2011

Tab. 48 Expressible juice (%)[d] at physiological ripening (2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2	Subset 3
SM	104	38,0		
NM	61		48,3	
M	133			58,3
Sig.		1,0	1,0	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 136.757.

a. Uses Harmonic Mean Sample Size = 89.478.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Year = 2012, physiological ripening

At commercial ripening or in unripe fruits in 2011, expressible juice couldn't discriminate between any flesh phenotypes (tab 49 and 50).

In 2012, at commercial ripening Melting and Non Melting were not statistically different and only Slow Melting was discriminated (tab. 51). In unripe fruits of the same year expressible juice couldn't discriminate between any flesh phenotypes (tab. 52).

Tab. 49 Tests of Between-Subjects Effects[b]

Dependent Variable: Expressible juice at commercial ripening without cold storage (2011)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1420,1[a]	3	473,4	2,5	,1
Intercept	177949,2	1	177949,2	945,6	,0
FleshType	1420,2	3	473,4	2,5	,1
Error	28417,4	151	188,2		
Total	315011,5	155			
Corrected Total	29837,6	154			

a. R Squared = .048 (Adjusted R Squared = .029)
b. Year = 2011, Commercial ripening

Tab. 50 Tests of Between-Subjects Effects[b]

Dependent Variable: Expressible juice inunripe fruits without cold storage (2011)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1093,11	2	546,6	2,1	,13
Intercept	44881,3	1	44881,3	174,8	,0
Flesh Type	1093,1	2	546,6	2,1	,13
Error	17199,4	67	256,7		
Total	87618,4	70			
Corrected Total	18292,5	69			

a. R Squared = .060 (Adjusted R Squared = .032)
b. Year=2011, Unripe fruit

Tab. 51 Expressible juice (%)[d] at Commercial ripening (2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SM	34	30,3	
NM	41		44,8
M	7		46,3
Sig.		1,00	,9

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 77.917.

Tab. 50 Tests of Between-Subjects Effects[b]

Dependent Variable: Expressible juice inunripe fruits without cold storage (2011)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1093,11	2	546,6	2,1	,13
Intercept	44881,3	1	44881,3	174,8	,0
Flesh Type	1093,1	2	546,6	2,1	,13
Error	17199,4	67	256,7		
Total	87618,4	70			
Corrected Total	18292,5	69			

a. Uses Harmonic Mean Sample Size = 15.255.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Year = 2012

Tab. 52 Tests of Between-Subjects Effects[b]

Dependent Variable: Expressible juice inunripe fruit without cold storage (2012)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	62,5[a]	2	31,3	1,2	,3
Intercept	16084,7	1	16084,7	612,1	,0
Flesh Type	62,5	2	31,3	1,2	,3
Error	1024,8	39	26,3		
Total	59753,4	42			
Corrected Total	1087,4	41			

a. R Squared = .058 (Adjusted R Squared = .009)
b. Year = 2012, Unripe fruit

Concerning samples held at 20°C after 3 weeks at 4°C in 2011, expressible juice at physiological ripening divided flesh phenotypes in two different group: Non Melting-Stony Hard, the less juicy and Slow Melting-Melting the most juicy (tab. 53). At commercial ripening and in unripe fruits it couldn't discriminate between the different flesh phenotypes (data not shown).

In 2012, expressible juice in samples at physiological ripening held at 20°C after cold storage discriminated for Melting phenotype (tab. 54). At commercial ripening and in unripe fruits expressible juice didn't discriminate between the different flesh phenotypes (data not shown).

Tab. 53 Expressible juice (%)[d] after cold storage at physiological ripening (2011)

TukeyHSD[a,b,c]

FleshType	N	Subset 1	Subset 2
NM	72	20,9	
SH	66	24,1	
SM	82		44,7
M	187		46,8
Sig.		,6	,8

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 255.245.
a. Uses Harmonic Mean Sample Size = 85.868.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Year = 2011, physiological ripening

Tab. 54 Expressible juice (%)[d] after cold storage physiological ripening (2012)

TukeyHSD[a,b,c]

Flesh Type	N	Subset 1	Subset 2
SM	110	25,3	
NM	71	29,1	
M	123		51,1
Sig.		,2	1,0

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 201.207.
a. Uses Harmonic Mean Sample Size = 95.830.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.
d. Year = 2012, Physiological ripening after cold storage

Tab. 55 Expressible juice (%) as recorded for the four different flesh phenotypes with or without cold storage (2011)

	NO PREVIOUS STORAGE				3 WEEKS STORAGE AT 4°C			
Day at 20°C	Flesh Type	Mean	N	Std. Deviation	Flesh Type	Mean	N	Std. Deviation
1	M	35.4	32	25.4	M	45.2	43	13.0
	NM	45.7	17	5.8	NM	31.5	14	21.2
	SH	29.5	39	13.7	SH	27.0	13	5.6
	SM	42.5	5	4.5	SM	39.7	14	8.4
	Total	35.2	93	18.4	Total	39.2	84	14.9
2	M	33.9	35	25.8	M	36.0	45	20.2
	NM	31.5	18	7.8	NM	19.4	21	14.4
	SH	24.8	38	10.7	SH	19.8	15	8.3
	SM	32.8	14	6.9	SM	30.5	18	7.2
	Total	30.0	105	17.1	Total	29.0	99	17.4
3	M	44.7	34	29.7	M	43.6	41	21.9
	NM	29.4	18	8.0	NM	11.7	14	7.8
	SH	31.5	39	11.8	SH	21.0	15	9.1
	SM	39.2	13	6.5	SM	39.5	11	13.2
	Total	36.4	104	19.8	Total	33.3	81	21.4
4	M	66.0	59	6.2	M	56.9	40	14.4
	NM	36.4	20	12.5	NM	22.3	15	10.0
	SH	33.0	44	14.0	SH	33.2	15	4.6
	SM	49.1	25	8.1	SM	56.3	18	8.2
	Total	49.3	148	17.8	Total	46.8	88	18.1
5	M	70.8	50	5.7	M	62.3	18	8.7
	NM	41.0	17	7.0	NM	19.5	8	8.5
	SH	36.3	43	10.5	SH	15.9	8	7.5
	SM	54.2	25	9.6	SM	53.1	21	7.6
	Total	53.0	135	17.2	Total	45.8	55	20.2

Tab. 56 Expressible juice (%) as recorded for the four different flesh phenotypes with or without cold storage (2012)

	NO PREVIOUS STORAGE				3 WEEKS STORAGE AT 4°C			
Day at 20°C	Flesh Type	Mean	N	Std. Deviation	Flesh Type	Mean	N	Std. Deviation
1	M	48.8	22	15.0	M	46.0	25	15.8
	NM	54.0	10	4.8	NM	38.3	13	10.1
	SM	38.7	21	10.9	SM	20.7	24	11.5
	Total	45.7	53	13.4	Total	34.6	62	17.3
2	M	53.0	26	14.3	M	47.1	25	14.8
	NM	48.0	8	9.9	NM	38.4	15	14.3
	SM	40.5	21	7.3	SM	22.2	23	9.2
	Total	47.5	55	12.7	Total	36.0	63	16.8
3	M	56.2	29	8.4	M	49.9	23	15.1
	NM	49.0	15	10.8	NM	29.8	20	12.0
	SM	33.4	23	14.3	SM	27.9	27	10.5
	Total	46.8	67	15.0	Total	35.7	70	16.0
4	M	64.8	29	6.9	M	52.5	25	17.0
	NM	46.6	17	6.6	NM	16.4	9	10.2
	SM	42.2	22	9.4	SM	28.3	23	14.2
	Total	52.9	68	12.9	Total	37.0	57	20.6
5	M	66.5	27	6.1	M	59.7	25	11.8
	NM	45.1	11	4.6	NM	17.6	14	12.6
	SM	35.2	17	18.6	SM	28.8	13	13.3
	Total	52.6	55	18.1	Total	40.6	52	22.5

4.4. Conclusions

The current comparison of peaches belonging to different flesh phenotypes included fruit ripening at 20°C with or without a previous cold storage at 4°C for three weeks. Analyses were conducted in three seasons (2010-2011-2012). The first was a pilot year, and for this reason its results were not included in this work.

All the analyzed samples were divided into stages of ripening in order to compare samples at the same physiological stage. The stages of ripening were: physiological ripening, commercial ripening and unripe fruit.

A clearer discrimination between the different flesh phenotypes was visible at physiological ripening as the result of changes in the gene expression. In previous ripening stages, like commercial ripening or in unripe fruit, these changes were still in progress and it was not possible to see them with a phenotypic approach.

As the aim of this experiment was to find a trait capable to discriminate between all the different flesh phenotypes, it is possible to conclude that only expressible juice could reach this goal.

Weight loss couldn't discriminate between the different flesh phenotypes. In both evaluated conditions Slow Melting was the phenotype which lost more weight.

Both TA and SSC are mostly related to cultivar, agronomic conditions and maturity, so they cannot discriminate between the different flesh phenotypes.

A general analysis of samples firmness at physiological ripening and after keeping at 20°C without cold storage didn't reveal any difference between Non Melting and Slow Melting phenotypes. Melting and Stony Hard cultivars were different from all the others, being respectively the softest and the firmest phenotype. Firmness seemed capable to discriminate between the different flesh phenotypes at day 1 and 2 in samples at physiological ripening held at 20°C without cold storage. Firmness also discriminated between the different flesh phenotypes in samples at physiological ripening after three weeks at 4°C, but this result is not encouraging, as it comes from cultivars not suitable for cold storage.

In general, Melting and Slow Melting softened to the lowest firmness value during shelf life after cold storage, while Stony Hard and Non Melting reached the lowest value of firmness after shelf life at 20°C without cold storage. In both the shelf life conditions Non Melting and Stony Hard confirmed their ability to retain their firmness better then Melting and Slow Melting.

Expressible juice discriminated between all the flesh phenotypes in samples at physiological ripening in 2011. The same result was confirmed in 2012, thus strengthening the hypothesis that this trait could really be the key factor discriminating between the different flesh phenotypes. Further studies are needed to determine whether this trait can be used as phenotyping method to be applied in QTL analysis with the aim of finding molecular markers associated to this character.

5. Gene expression profile comparison of peach fruit textures

5.1. Introduction

Transcriptome analysis is increasingly used as a means to phenotype an organism, be it animal or vegetal. This technique relies on the fact that the mRNA population specifies a cell's identity and helps to govern its present and future activities. A correlation between mRNA levels and amount of protein produced has been demonstrated, making the expression value a good index of gene functionality. Only recently the ultra-high-throughput sequencing of cDNA has come into use to measure transcriptome composition and to find new exons or genes, thanks to the improvement of genomics technology (Mortazavi et al., 2008).

The beginning of genomics is conventionally set at the time of the development of automated sequencing methods, known as Sanger sequencing. This approach lead to the publication of the first plant genome sequence, that of *Arabidopsis thaliana*, in 2000. From that moment on, many steps have been taken, thanks also to the reduction of costs stemming from the improvements in sequencing platforms (Hamilton and Buell, 2012).

Starting from the Sanger platform, passing through the "Whole-Genome Shotgun Sequencing" (WGS), firstly of small microbes and then of fragmented genomes of large eukaryotes like *Drosophila melanogaster* (Adams, 2000) and the methods based on "reduced representation sequencing", in few years researchers came to what it is called "Next Generation Sequencing" (NGS). Roche 454, SoLiD (Life Technologies), and Illumina are the most used commercial platforms developed from 2005 to date. Continuous improvements in these technologies lead to significantly increased throughput, with reduced error rate and increased read length, making these methods economically feasible for a wide range of plant species.

To use RNA-Seq technology (fig. 19) means to use the latest and most powerful tool for characterizing transcriptomes (Wang et al., 2009). Unlike array-based approaches, in fact, RNA-Seq provides information on transcripts that are expressed at very low levels, limited only by the total number of reads that are generated (Marioni et al., 2008). Nevertheless, several sequence artifacts, including read errors (base calling errors and small insertions or deletions), poor quality reads and primer/adaptor contaminations are quite common in the NGS data, which can impose significant impact on the downstream sequence processing and analysis. The quality of data is very important for various downstream analyses, such as sequence assembly, single nucleotide polymorphisms identification (SNP naming) and gene expression studies (Patel and Jain, 2012). In order to ensure quality and to prevent erroneous conclusions, a quality check and filtering of NGS data before processing is needed. Most of the programs available for downstream analyses do not provide such tools, and

therefore these sequence artifacts must be removed before the analyses. To this end, many computer programs have been developed and are being continually improved. To trim data by quality and to filter them from contamination, for example, tools like ERNE-filter (Extended Randomized Numerical alignEr) or rNA have been developed. These programs were developed by researchers at the Istituto di Genomica Applicata and at the University of Udine (http://erne.sourceforge.net/; Vezzi et al., 2012). Only after quality filtering it is possible to proceed with the subsequent step, the reads alignment, which can be easily performed if a reference genome is available. However, in the absence of a sequenced genome, the transcript *de-novo* assembly is still possible, although not advisable.

For the aligning of data from next generation sequencing experiments, many software packages are available too. Some of the most popular are BWA (http://bio-bwa.sourceforge.net), Bowtie (http://bowtie-bio.sourceforge.net; Langmead et al., 2009), Bowtie2 (Langmead and Salzberg, 2012), Stampy (Lunter and Goodson, 2011), Novoalign (www.novocraft.com/main/index.php), Tophat (Trapnell et al., 2009) and ERNE. The differences between these software packages are often subtle and generally a scientist has to make a choice by balancing many parameters which depend mainly on their reliability combined with speed, efficiency and available computing resources. These programs provide alignment to the genome in a quality-aware mode, alignment of reads across splice sites, and statistical assessment of transcript abundances (Hamilton and Buell, 2012). Aligning sequencing reads to a reference genome is not only crucial, but it is often the slowest step in the most of comparative genomics pipelines (Langmead and Salzberg, 2012). This happens because the aligner must determine each read's likely point of origin with respect to a reference genome (Li and Homer, 2010). Aligned reads are very informative of the sample being sequenced. Mismatches, insertions and deletions in the alignments can identify polymorphism between the sequenced sample and the reference genome. Reads that align outside annotated genes are often strong evidence of new protein-coding genes and noncoding RNAs. However not all the mentioned software packages are capable to perform *De novo* transcript assembly. Alignments can also be used to accurately quantify gene and transcript expression, because the number of reads produced by a transcript is proportional to its abundance (Trapnell et al., 2012). One of the main objectives in most transcriptome studies is to quantify differences in expression across multiple samples, in order to capture differential gene expression, and to identify sample-specific alternative splicing isoforms and their differential abundance. Differential expressions can be assessed by different software packages, like R and Bioconductor (http://bioconductor.org/).

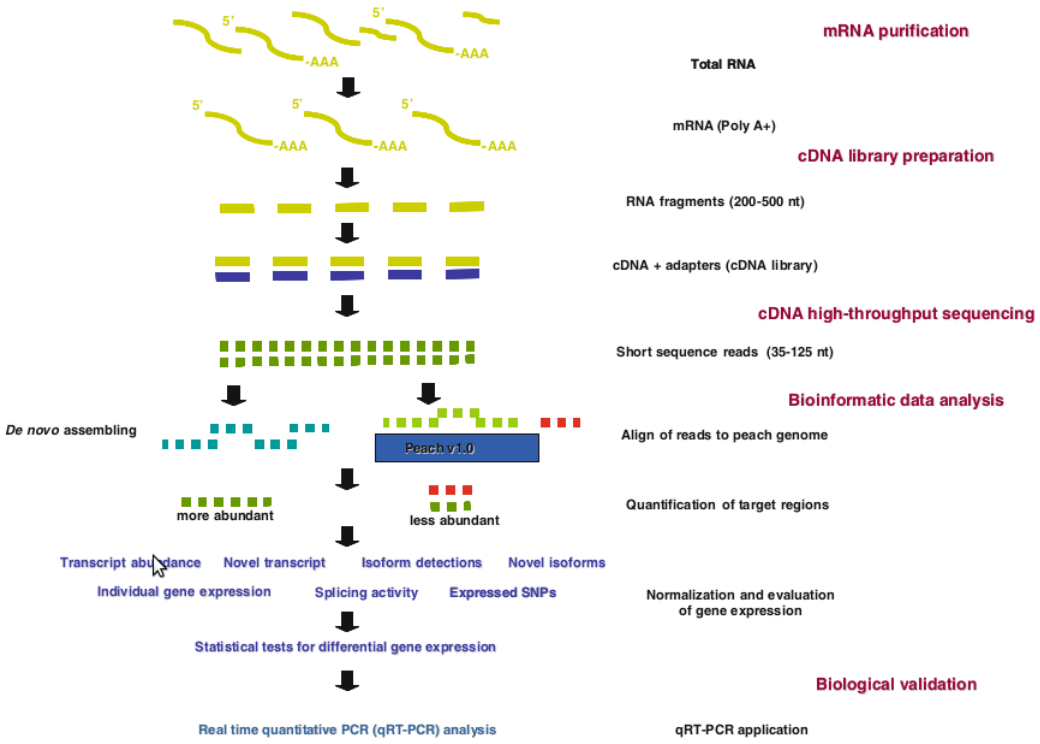


Fig.19 Summarized overview of RNA-Seq technology using Illumina Genome Analyzer mRNA (Poly A+) purified (enriched) from total RNA is fragmented, ligated to specific adaptor sequences, and retrotranscribed to convert to a cDNA library. Short sequence reads are generated from the cDNA library. These reads can be mapped on a reference genome (the reference *Prunus persica* Peach genome v1.0) using efficient alignment software. Part of these reads can be aligned to previously annotated sequences in the reference genome (shown in green). In addition, some reads without a match to the reference map are shown in red (unmatched reads). Another alternative strategy consists of *de novo* assembling to produce anew genome-scale transcriptional map. Later, a bioinformatic analysis of the date must be performed in other to describe the gene expression level, the abundance of transcripts, the presence of novel transcripts or the isoform detection. In the following steps data must be analyzed in order to describe gene expression levels, to individuate novel transcripts and to perform the detection of alternative isoforms. Finally, the putative candidate genes expressed differentially can be validated by qRT-PCR. From Martínez-Gómez et al., 2011. Adapted to *Prunus* analysis from Blencowe et al., 2009; Wang et al., 2009; Nagalakshmi et al., 2010;Costa et al., 2010; Haas and Zody, 2010.

The release of the first draft of the peach genome (*Prunus persica* genome v1.0) by the International Peach Genome Initiative (IPGI) (Sosinski et al., 2010; Arús et al., 2012) has thus opened new possibilities for genomics and transcriptomics in peach (and in related species as well). Peach is a model plant for the *Rosaceae* family due to its small genome size of 230 Mbp (approximately twice the size of *Arabidopsis*) with eight haploid chromosomes (Arumuganathan and Earle, 1991; Ahmad et al., 2011). An advantage of using the peach genome as reference is the high synteny among *Prunus* genomes (Jung et al., 2009). This synteny has been described, in a certain way, also in *Rosaceae* family (Dirlewanger et al., 2004; Arus et al., 2006; Shulaev et al., 2008; Cabrera et al., 2009; Illa et al., 2011). Thanks to these features it is possible to deepen our knowledge of the molecular basis and of the expression of the most important agronomic traits (Leader, 2005).

The availability of the peach genome then, surely fosters genomic applications to plant breeding (Arús et al., 2012). Analysis of the recently released peach genome sequence has contributed to clarify some aspects concerning the flesh softening (melting/non-melting, *M/m*) traits and the flesh adhesion (freestone/clingstone *F/f*) traits (Peace et al., 2005; Verde et al., 2005; Dirlewanger et al., 2006; Ogundiwin et al., 2009; Cantín et al., 2010), corroborating the hypothesis of two genes (endopolygalacturonase genes) controlling these traits. In fact in the distal region of G4 between nt 22,649,519 and nt 22,687,159 two endoPG genes have been manually annotated in the Peach V1.0 assembly (IPGI www.peachgenome.org). Further refining of the data stemming from the peach genome sequence, combined with the use of NGS technology like RNA-seq, will enhance our understanding of the structural and functional aspects of peach and *Prunus* genetics and will facilitate the connection between genotype and phenotype. Searching for markers or candidate genes for genomic regions already identified as containing genes/QTLs of interest will be possible, thus enabling the accurate prediction of phenotypes for new genes, or the advantageous replacement of markers currently used for MAS. Furthermore, the increased gene knowledge will also facilitate the search of natural or induced mutations at genes of interest, identifying novel variations that can be integrated into elite cultivars to improve their disease resistance, their adaptation to changing environment, and the quality or health properties of the fruit (Arús et al., 2012).

5.2. Materials and Methods

5.2.1. Plant material

The plant material came from the "Centro Ricerche Produzioni Vegetali" (CRPV) Experimental Orchard, Azienda Agricola Zabina sited in Castel San Pietro (BO). Peach trees were grown under integrated pest management growing systems.

Instead of using two "classical" biological replicates, two different cultivars for each flesh phenotype (Melting, Non Melting, Stony Hard and Slow Melting) were used. For each cultivar two fruits from three different trees were collected.

With this approach an attempt was made to reduce the "noise" due to the different genetic background (cultivars) and maximize the similarities due to flesh phenotype. For each class, four time points were collected relying on phenotypic assay, in order to assure sample homogeneity. The first time point was "pit hardening", identifiable by a lag in the fruit diameter growing curve. The second was "fruit veraison" identifiable both by skin color change and DA-meter value. The third was "commercial ripening" and the fourth "physiological ripening", both assessed using DA-meter, an instrument that measure the difference in absorbance (index of absorbance difference, $I_{AD,}$) between two wavelengths near the chlorophyll-a absorption peak, 670 and 720 nm (Ziosi et al., 2008). In the table 57 the I_{AD} values for each time step are reported. Once fruit were collected they were immediately peeled, sliced in wedges, quickly frozen using liquid nitrogen, lyophilized and stored at -20°C until further use.

Total mRNA was extracted and sequenced using Illumina RNA-Seq technology (HiSeq 2000) at IGA Technology Service (Udine, Italy). The samples were multiplexed six per lane and sequenced for a length of 50 bp single read.

In tables 58 and 59 a list of accessions analyzed to perform gene expression analysis is reported.

Tab. 57 I_{AD} values for each time step

PHYSIOLOGICAL STAGE	TIME STEP CODE	I_{AD}
Fruit veraison	TS1	0.8- 0.6
Commercial ripening	TS2	0.6- 0.3
Physiological ripening	TS3	<0.3

Tab 58. List of all the accessions. The ones used to perform gene expression analysis are highlighted.

CULTIVAR	HARVEST DATE	TEXTURE TYPE	APPLICATION				
			Time Resolved Spectroscopy (TRS)	Shelf life			Transcriptomic Analysis
				2010	2011	2012	
Alice Col	13 Jul	NM	√		√	√	
BO 000200006	28 Jul	NM	√		√		
BO 010120182	11 Aug	NM					√
Iride	8 Jul	NM	√		√	√	
Oro A	29 Jun	NM					√
Ambra	15 Jul	M			√	√	
Bolero	15 Aug	M					√
Dixired	8 Jul	M	√		√		
Glohaven	22 Jul	M	√	√	√		
Redhaven	15 Jul	M	√		√	√	√
BO 05030081	28 Jul	SH			√		√
BO 05030149	28 Jul	SH			√		
Ghiaccio	21 Jul	SH	√		√		
IFF 331	30 Jul	SH	√		√		√
Big Top	15 Jul	SM	√	√	√	√	√
Rich Lady	15 Jul	SM	√	√	√	√	√
Vista Rich	19 Jul	SM	√		√		

Tab 59. List of accessions analyzed to perform gene expression analysis

	PLANT CODE	TIME STEP CODE	TIME STEP	CULTIVAR	DATE OF COLLECTION
Melting	1A	TS0	pit hardening	REDHAVEN	6/9/2010
	1A	TS1	fruit veraison	REDHAVEN	7/23/2010
	1A	TS2	commercial ripening	REDHAVEN	7/23/2010
	1A	TS3	physiological ripening	REDHAVEN	7/23/2010
	1B	TS0	pit hardening	REDHAVEN	6/9/2010
	1B	TS1	fruit veraison	REDHAVEN	7/23/2010
	1B	TS2	commercial ripening	REDHAVEN	7/23/2010
	1B	TS3	physiological ripening	REDHAVEN	7/23/2010
	1C	TS0	pit hardening	REDHAVEN	6/9/2010
	1C	TS1	fruit veraison	REDHAVEN	7/23/2010
	1C	TS2	commercial ripening	REDHAVEN	7/23/2010
	1C	TS3	physiological ripening	REDHAVEN	7/23/2010
	2A	TS0	pit hardening	BOLERO	6/9/2010
	2A	TS1	fruit veraison	BOLERO	8/11/2010
	2A	TS2	commercial ripening	BOLERO	8/18/2010
	2A	TS3	physiological ripening	BOLERO	8/18/2010
	2B	TS0	pit hardening	BOLERO	6/9/2010
	2B	TS1	fruit veraison	BOLERO	8/11/2010
	2B	TS2	commercial ripening	BOLERO	8/18/2010
	2B	TS3	physiological ripening	BOLERO	8/18/2010
	2C	TS0	pit hardening	BOLERO	6/9/2010
	2C	TS1	fruit veraison	BOLERO	8/11/2010
	2C	TS2	commercial ripening	BOLERO	8/18/2010
	2C	TS3	physiological ripening	BOLERO	8/18/2010
Slow Softening	3A	TS0	pit hardening	BIGTOP	6/9/2010
	3A	TS1	fruit veraison	BIGTOP	7/7/2010
	3A	TS2	commercial ripening	BIGTOP	7/7/2010
	3A	TS3	physiological ripening	BIGTOP	7/23/2010
	3B	TS0	pit hardening	BIGTOP	6/9/2010
	3B	TS1	fruit veraison	BIGTOP	7/7/2010
	3B	TS2	commercial ripening	BIGTOP	7/7/2010
	3B	TS3	physiological ripening	BIGTOP	7/23/2010
	3C	TS0	pit hardening	BIGTOP	6/9/2010
	3C	TS1	fruit veraison	BIGTOP	7/7/2010
	3C	TS2	commercial ripening	BIGTOP	7/7/2010
	3C	TS3	physiological ripening	BIGTOP	7/23/2010
	4A	TS0	pit hardening	RICHLADY	6/9/2010
	4A	TS1	fruit veraison	RICHLADY	7/15/2010
	4A	TS2	commercial ripening	RICHLADY	7/23/2010
	4A	TS3	physiological ripening	RICHLADY	7/23/2010
	4B	TS0	pit hardening	RICHLADY	6/9/2010
	4B	TS1	fruit veraison	RICHLADY	7/23/2010
	4B	TS2	commercial ripening	RICHLADY	7/23/2010
	4B	TS3	physiological ripening	RICHLADY	7/23/2010
	4C	TS0	pit hardening	RICHLADY	6/9/2010
	4C	TS1	fruit veraison	RICHLADY	7/15/2010
	4C	TS2	commercial ripening	RICHLADY	7/23/2010
	4C	TS3	physiological ripening	RICHLADY	7/23/2010
Stony Hard	5A	TS0	pit hardening	IFF331	6/9/2010
	5A	TS1	fruit veraison	IFF331	7/23/2010
	5A	TS2	commercial ripening	IFF331	7/28/2010
	5A	TS3	physiological ripening	IFF331	7/28/2010
	5B	TS0	pit hardening	IFF331	6/9/2010
	5B	TS1	fruit veraison	IFF331	7/23/2010
	5B	TS2	commercial ripening	IFF331	7/28/2010
	5B	TS3	physiological ripening	IFF331	7/28/2010
	5C	TS0	pit hardening	IFF331	6/9/2010
	5C	TS1	fruit veraison	IFF331	7/23/2010
	5C	TS2	commercial ripening	IFF331	7/28/2010
	5C	TS3	physiological ripening	IFF331	7/28/2010
	6A	TS0	pit hardening	BO05030	6/9/2010
	6A	TS1	fruit veraison	BO05030	7/15/2010
	6A	TS2	commercial ripening	BO05030	7/23/2010
	6A	TS3	physiological ripening	BO05030	7/28/2010
	6B	TS0	pit hardening	BO05030	6/9/2010
	6B	TS1	fruit veraison	BO05030	7/15/2010
	6B	TS2	commercial ripening	BO05030	7/23/2010
	6B	TS3	physiological ripening	BO05030	7/28/2010
	6C	TS0	pit hardening	BO05030	6/9/2010
	6C	TS1	fruit veraison	BO05030	7/15/2010
	6C	TS2	commercial ripening	BO05030	7/23/2010
	6C	TS3	physiological ripening	BO05030	7/28/2010
Non Melting	7A	TS0	pit hardening	ORO-A	6/9/2010
	7A	TS1	fruit veraison	ORO-A	6/22/2010
	7A	TS2	commercial ripening	ORO-A	6/29/2010
	7A	TS3	physiological ripening	ORO-A	6/29/2010
	8A	TS0	pit hardening	BO010120182	6/9/2010
	8A	TS1	fruit veraison	BO010120182	8/11/2010
	8A	TS2	commercial ripening	BO010120182	8/11/2010
	8A	TS3	physiological ripening	BO010120182	8/11/2010
	8B	TS0	pit hardening	BO010120182	6/9/2010
	8B	TS1	fruit veraison	BO010120182	8/11/2010
	8B	TS2	commercial ripening	BO010120182	8/11/2010
	8B	TS3	physiological ripening	BO010120182	8/11/2010
	8C	TS0	pit hardening	BO010120182	6/9/2010
	8C	TS1	fruit veraison	BO010120182	8/11/2010
	8C	TS2	commercial ripening	BO010120182	8/11/2010
	8C	TS3	physiological ripening	BO010120182	8/11/2010

5.2.2. Isolation of RNA

Total RNA was obtained following the protocol of Dal Cin et al. (Dal Cin et al., 2005) with some modifications. All solutions were prepared using deionized water and autoclaved.

- Tissue (5 g) was ground to a fine powder in liquid nitrogen using a mortar and a pestle. Ground powder was mixed with 15 mL of preheated (65°C) extraction buffer (100 mMTris-HCl, pH 8.0, 100 mMNaCl, 5 mM EDTA, 2.5% [v/v] β-mercaptoethanol, 1% [w/v] SDS). Samples were incubated for 15 min at 65°C and briefly but vigorously shaken every 5 min.
- After incubation and immediately before centrifugation, 300 µL of $CaCl_2$ 0,5 M were added to each sample. Samples were then briefly shaken vigorously and incubated on ice at 4°C for 90 minutes.
- Cell debris were pelleted at 5200 rpm at RT for 30 min.

- The aqueous solution was then sequentially extracted in 1:1 volume of phenol-chloroform (twice) and 1 volume of chloroform, each time shaking to mix thoroughly then centrifuging at 5200 rpm at RT for 15 min.
- RNA was then precipitated by adding 1/10 volume of 3 M NaOAc, pH 5.2, and 0.6 volume of 2-propanol, and incubating at –80°C for 90 min. RNA was pelleted by centrifugation at 5200 rpm at 4°C for 60 min. Pellets were washed with 1 mL of 70% ethanol and vacuum dried and resuspended in max 300 µL of water.

5.2.3. Sample evaluation

Total RNA concentration was evaluated examining aliquots of samples in a Nanodrop spectrophotometer (Thermo Scientific) while the quality was assessed running samples (3 µL of RNA) on a 1% agarose gel in TAE buffer and stained with ethidium bromide.

5.2.4. Bioinformatic analysis

From the Illumina pipeline performed at IGA, FASTQ format files were obtained. The FASTQ format stores both a biological sequence and its corresponding qualities scores. Phred scores are now a *de facto* standard for representing sequencing read base qualities (Cock et al., 2010). Illumina FASTQ files used the following naming scheme:

```
<sample name>_<barcode sequence>_L<lane (0-padded to 3 digits)>_R<read number>_>set number
(0-padded to 3 digits>.fastq.gz
```

The following is an example of FASTQ file format:

```
@HISEQ1:116:D0HMNACXX:4:1101:1871:1986 1:N:0:CAGATC
GTGCTCTCTCTTTTCTTTTCTCTCTCTATCTGAAAAAGGCGACCGCCGGGN
+
BBBFFFFFHHHHHIGIJJJHIJJJJII>JGGHGBD??GHGIGFHAAABBE4
```

Where:
`@HISEQ1`: unique instrument name;
`116`: run id;
`D0HMNACXX`: flowcell barcode
`4`: flowcell name;
`1101`: tile number within the flowcell lane;
`1871`: 'x'-coordinate of the cluster within the tile;
`1986`: 'y'-coordinate of the cluster within the tile;
`(space)`
`1`: read number of a pair (1 or 2);
`N`: Y if the read is bad, N otherwise (good);
`0`: control number: is 0 when none of the control bits are on, otherwise it is an even number;
`CAGATC`: barcode sequence

5.2.4.1. Quality check and data filtering

To assess the overall quality of a run, and spot any potential problems or biases, FastQC was used. This software produces series of user-friendly graphical reports to overview sequence quality (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Based on this quality check, sequences were cleaned, trimmed and filtered. To this end, ERNE-filter with all the default options was used. After trimming, a further quality control check was performed with FastQC in order to confirm its effectiveness.

5.2.4.2. Alignment

After the *Prunus persica* genome (Phytozome: 139) was indexed (`bowtie2-build`), reads were aligned to the 'Lovell' genome using Bowtie2 at default preset parameters `--very-sensitive-local`. This is an ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences. Alignments were converted in SAM (Sequence Alignment Format) using `samtools` (http://samtools.sourceforge.net/).

```
samtools view -F4 bam-file >sam-file
```

Only aligned reads in the BAM (binary form of (SAM)) were kept (`-F4`: filter for flag #4 = unmapped reads). Finally the alignments were converted into a best hit count (raw counts).

```
cut -f3 sam-file | sort -S 2G \ uniq -c >sample_#.counts
```

5.2.4.3. Analysis of differential expression

The analysis of differentially expressed genes was performed using R, the bioconductor packages Limma (Smyth, 2005) and edgeR (Robinson et al., 2012).

Limma is a well-know package, often used for microarray data analysis, particularly suitable for handling complex experimental design, like the one performed in this experiment. It has been improved to work also with RNA-Seq data. Actually there are many other software packages capable of dealing with this kind of data. An example is Cufflinks (which works very well with data generated from TopHat, an aligner able to perform spliced alignment and the *de-novo* RNA-seq assembly), but, as it employs only a simple pairwise comparison, it wouldn't be able to unravel the complexity of a factorial design.

The counts obtained in the alignment were loaded into R software. Tables obtained were merged in order to have a single data frame. The following are the commands related to these two procedures:

```
setwd("project_directory")

table1<-read.table("sample_1.counts",header=FALSE)
colnames(table1)<-c("typeA_time1_rep1","transcript")
…
…
table16<-read.table("sample_16.counts",header=FALSE)
colnames(table2)<-c("typeA_time1_rep2","transcript")


tableA<-merge(table1,table2,by="transcript",all=TRUE)
tableB<-merge(table3,table4,by="transcript",all=TRUE)
tableC<-merge(table5,table6,by="transcript",all=TRUE)
tableD<-merge(table7,table8,by="transcript",all=TRUE)
tableE<-merge(table9,table10,by="transcript",all=TRUE)
tableF<-merge(table11,table12,by="transcript",all=TRUE)
tableG<-merge(table13,table14,by="transcript",all=TRUE)
tableH<-merge(table15,table16,by="transcript",all=TRUE)

tableX1<-merge(tableA,tableB,by="transcript",all=TRUE)
tableX2<-merge(tableC,tableD,by="transcript",all=TRUE)
tableX3<-merge(tableE,tableF,by="transcript",all=TRUE)
tableX4<-merge(tableG,tableH,by="transcript",all=TRUE)

tableY1<-merge(tableX1,tableX2,by="transcript",all=TRUE)
tableY2<-merge(tableX3,tableX4,by="transcript",all=TRUE)

tableZ<-merge(tableY1,tableY2,by="transcript",all=TRUE)

tableZ[is.na(tableZ)]<-0
countTable<-as.matrix(tableZ[,2:ncol(tableZ)])
rownames(countTable)<-tableZ[,1]
write.table(countTable,file="mycounttable.txt",sep="\t",quote=FALSE,col.names=TRUE,row.names
=TRUE)
```

After obtaining a single data frame, it was possible to perform the statistical data analysis by limma.

The experimental design (factorial design) is reported in tab 60. Two experimental dimensions (or factors) were considered: peach type and time point. Peach type consisted of M (Melting), SM (Slow Melting), SH (Stony Hard) and NM (Non Melting). The two time points were fruit veraison (T1) and commercial ripening (T2). For each peach type two cultivars were used.

Tab. 60 The experimental design obtained from the previous procedure

	M.T1	M.T2	SM.T1	SM.T2	SH.T1	SH.T2	NM.T1	NM.T2
typeM_time1_Rep_1 *[Redhaven]*	1	0	0	0	0	0	0	0
typeM_time1_Rep_2*[Bolero]*	1	0	0	0	0	0	0	0
typeSM_time1_Rep_1*[Big Top]*	0	0	1	0	0	0	0	0
typeSM_time1_Rep_2*[Rich Lady]*	0	0	1	0	0	0	0	0
typeSH_time1_Rep_1*[IFF 331]*	0	0	0	0	1	0	0	0
typeSH_time1_Rep_2*[BO05030081]*	0	0	0	0	1	0	0	0
typeNM_time1_Rep_1*[Oro A]*	0	0	0	0	0	0	1	0
typeNM_time1_Rep_2*[BO010120182]*	0	0	0	0	0	0	1	0
typeM_time2_Rep_1*[Redhaven]*	0	1	0	0	0	0	0	0
typeM_time2_Rep_2*[Bolero]*	0	1	0	0	0	0	0	0
typeSM_time2_Rep_1*[Big Top]*	0	0	0	1	0	0	0	0
typeSM_time2_Rep_2*[Rich Lady]*	0	0	0	1	0	0	0	0
typeSH_time2_Rep_1*[IFF 331]*	0	0	0	0	0	1	0	0
typeSH_time2_Rep_2*[BO05030081]*	0	0	0	0	0	1	0	0
typeNM_time2_Rep_1*[Oro A]*	0	0	0	0	0	0	0	1
typeNM_time2_Rep_2*[BO010120182]*	0	0	0	0	0	0	0	1

The experimental design matrix was generated with the following script:

```
peachTypes<-c("M","M","SM","SM","SH","SH","NM","NM","M","M","SM","SM","SH","SH","NM","NM")
timePoints<-
c("T1","T1","T1","T1","T1","T1","T1","T1","T2","T2","T2","T2","T2","T2","T2","T2")
peachDesign<-data.frame(
             rownames=colnames(countTable),
             peachType=factor(peachTypes),
             timePoint=factor(timePoints)
)
peachDesign<-as.factor(paste(peachDesign$peachType,peachDesign$timePoint,sep="."))
design<-model.matrix(~0+peachDesign)
rownames(design)<-colnames(countTable)
colnames(design)<-levels(peachDesign)
```

In order to normalize the data, the TMM (Trimmed mean of M values) was applied (Robinson and Oshlack, 2010).

```
nf<-calcNormFactors(countTable)
```

With these factors and in order to convert read counts to log2-cpm, with associated weights and ready for linear modeling, the Limma `voom` function was used. `voom` is an acronym for mean-variance modelling at the observational level. Log-counts typically show a decreasing mean-variance trend. This function estimates the mean-variance trend for log-counts, then assigns a weight to each observation based on its predicted variance. The weights are then used in the linear modelling process to adjust for heteroscedasticity.

```
normTable<-voom(countTable,design,plot=TRUE,lib.size=colSums(countTable)*nf)
```

The Multi Dimensional Scaling (macro differences) was then plotted. This function is a variation of the usual multidimensional scaling (or PCA). The distance between each pair of samples (columns) is the root-mean-square deviation (Euclidean distance) for the top 500 genes. This plot gives an idea as to whether the samples, grouped for flesh type, cluster together as expected.

```
plotMDS(normTable,labels=peachTypes,col=c(rep("blue",8),rep("red",8)))
title("MDS of RNASeq data")
legend("topleft",legend=c("Time point 1","Time point 2"),text.col=c("blue","red"))
```

To point out the contrasts we were interested in (differences between time points for the same flesh type, between flesh type at T1, between flesh type at T2, and differences of differences), these procedures were used:

```
contrast.matrix<- makeContrasts(
```

differences between time points for the same flesh type

```
      M.T2-M.T1,
      SM.T2-SM.T1,
      SH.T2-SH.T1,
      NM.T2-NM.T1,
```

differences between flesh type at T1

```
      M.T1-SM.T1,
      M.T1-SH.T1,
      M.T1-NM.T1,
      SM.T1-SH.T1,
      SM.T1-NM.T1,
      SH.T1-NM.T1,
```

differences between flesh type at T2

```
      M.T2-SM.T2,
      M.T2-SH.T2,
      M.T2-NM.T2,
      SM.T2-SH.T2,
      SM.T2-NM.T2,
      SH.T2-NM.T2,
```

Interaction terms: specific time point reactions (difference of difference)

```
      (M.T2-M.T1)-(SM.T2-SM.T1),
      (M.T2-M.T1)-(SH.T2-SH.T1),
      (M.T2-M.T1)-(NM.T2-NM.T1),
      (SM.T2-SM.T1)-(SH.T2-SH.T1),
      (SM.T2-SM.T1)-(NM.T2-NM.T1),
      (SH.T2-SH.T1)-(NM.T2-NM.T1),
```

levels=design)

The differential expression (on the base of the contrast previously defined) was estimated by fitting a linear model to the data. To test the differential expression, a linear model was fitted to the data in order to estimate the differential expression on the base of the contrast previously defined (Smyth, 2005). The central idea of this package is to fit a linear model to the expression data for each gene. Empirical Bayes and other shrinkage

methods are used to borrow information across genes making the analyses stable. The first step is to fit a linear model, which fully models the systematic part of the data. The data so obtained are adjusted for multiple testing obtaining as result a log2 fold changes, standard errors, t-statistics and p-values. The basic statistic used for significance analysis is the moderated t-statistic, which was computed for each transcript and for each contrast. This has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes. This has the effect of borrowing information from the ensemble of genes to aid with inference about each individual gene.

```
fit<- lmFit(normTable,design)
fit<-contrasts.fit(fit,contrast.matrix)
fit<- eBayes(fit)
```

Result tables were extracted from the fit object generated after applying the contrast matrix and defined the main results of interest:

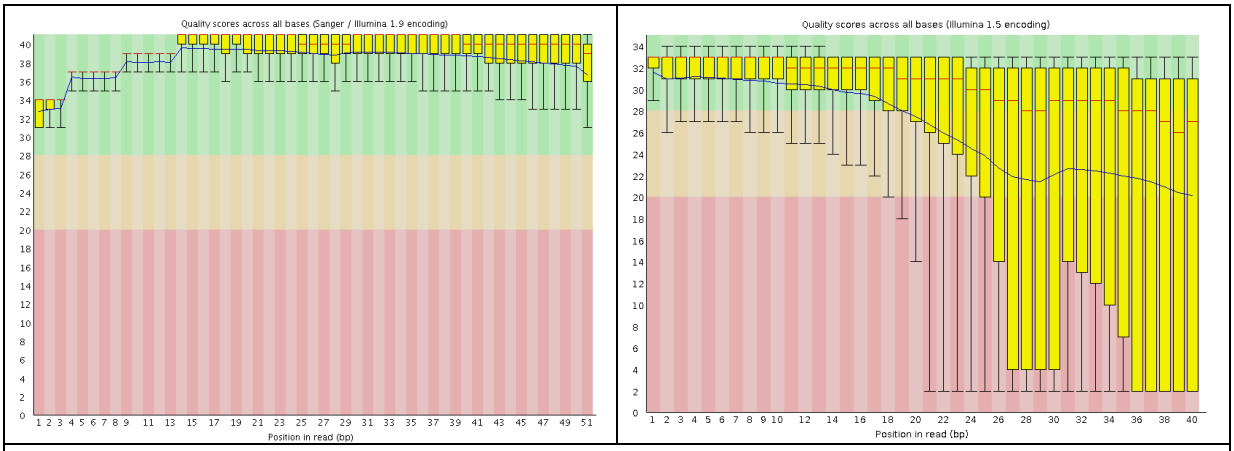
1) Which genes responded differently between the two time points
2) Which genes responded differently between the different flesh phenotype
3) Which genes responded differently between the time points and between flesh type (interaction)

```
logFC<-fit$coefficients
pvalues<-fit$p.value
adj.pvalues<-apply(pvalues,2,p.adjust,method="BH")
write.table(logFC,file="outputLog2FC.txt",quote=FALSE,sep="\t")
write.table(pvalues,file="outputPvalues.txt",quote=FALSE,sep="\t")
write.table(adj.pvalues,file="outputAdjustedPvalues.txt",quote=FALSE,sep="\t")
```

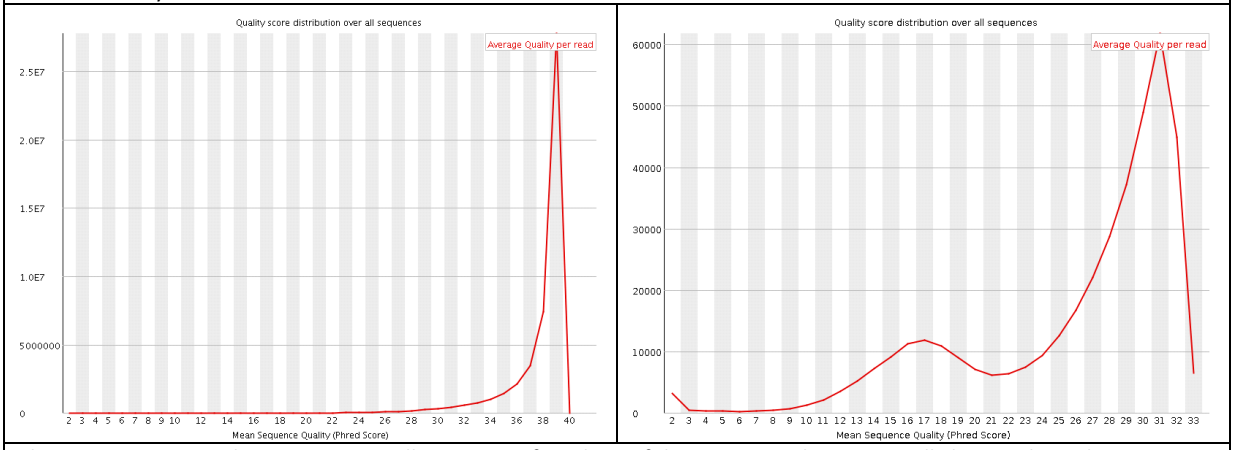
5.3. Results

5.3.1. Quality check and data filtering

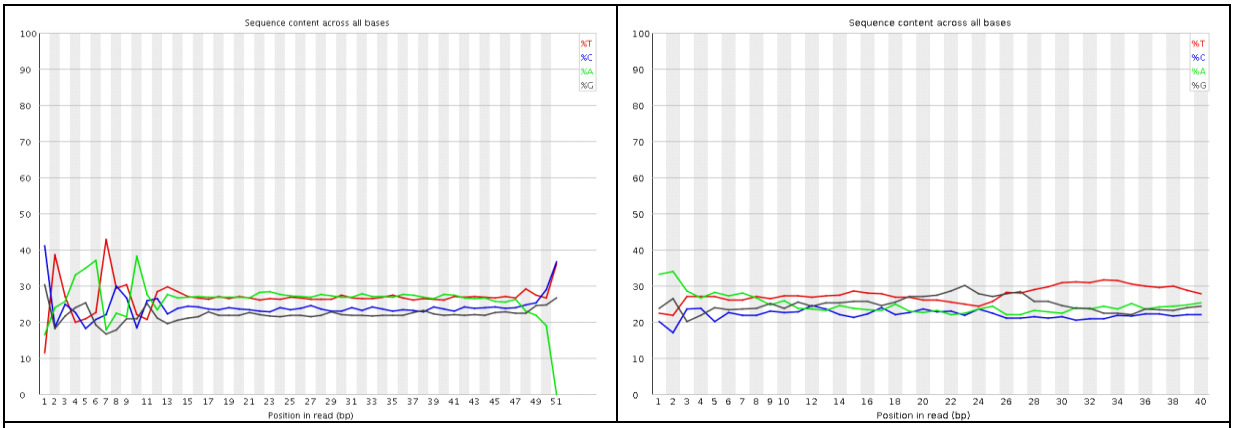
Quality control of the runs is an important step before proceeding with the assembly pipeline. Fig. 20 shows a comparison between a good run and a poor run. After the quality control, 8 out of 18 samples had to be sequenced again due to poor quality, or low read amount.



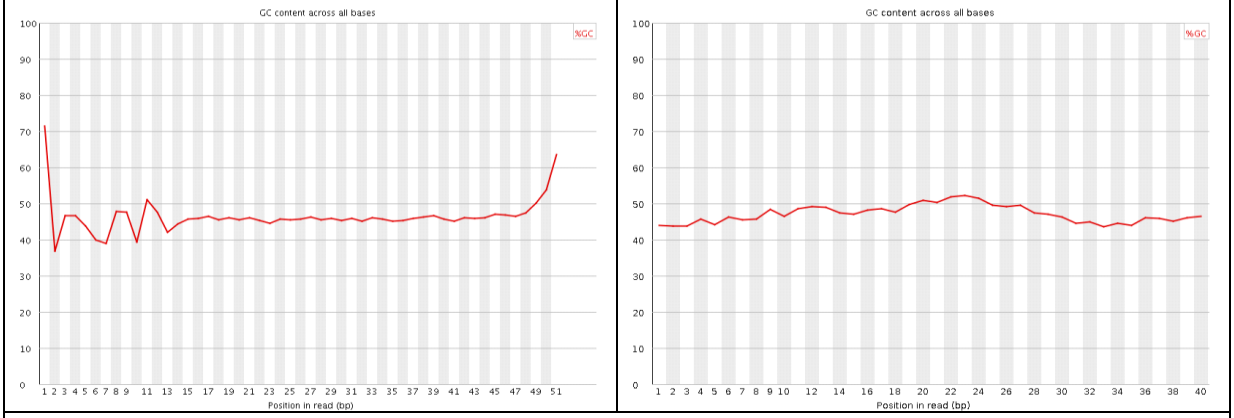
Per Base Sequence Quality: the y-axis on the graph shows the quality scores. The higher the score the better the base call. The background of the graph divides the y axis into very good quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). A simple warning will be issued if the lower quartile for any base is less than 10, or if the median for any base is less than 25. This module will raise a failure warning if the lower quartile for any base is less than 5 or if the median for any base is less than 20.



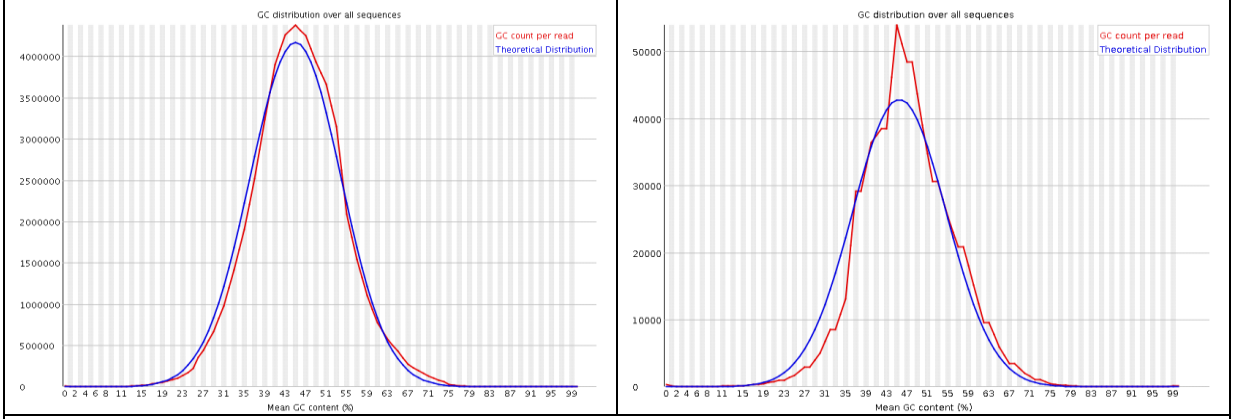
The per sequence quality score report allows to see if a subset of the sequences has universally low quality values. A warning is raised if the most frequently observed mean quality is below 27 - this equates to a 0.2% error rate. An error warning is raised if the most frequently observed mean quality is below 20 - this equates to a 1% error rate.



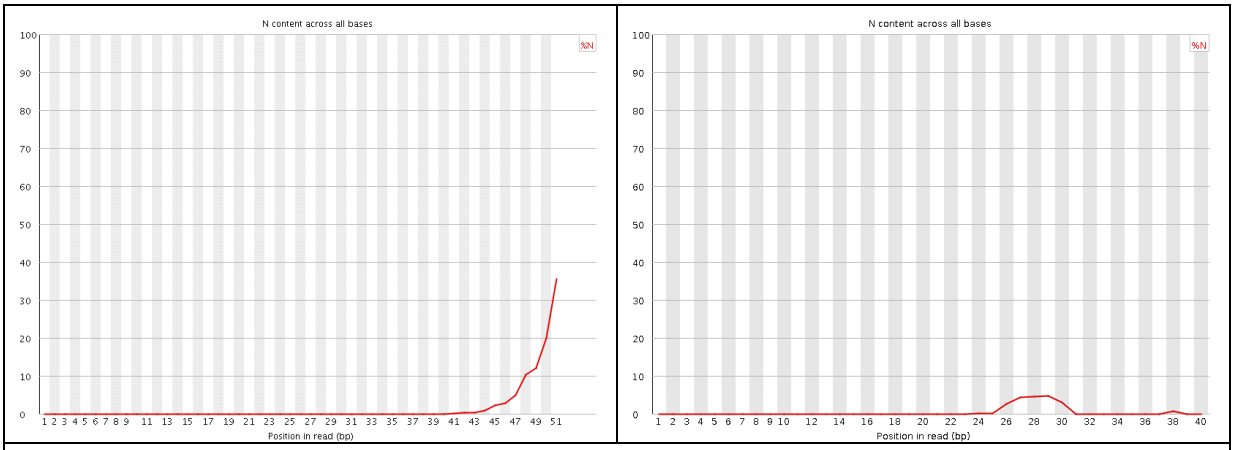
Per Base Sequence Content plots out the proportion of each base position in a file for which each of the four normal DNA bases has been called. In a random library there would be little to no difference between the different bases of a sequence run, so the lines in this plot should run parallel with each other. The relative amount of each base should reflect the overall amount of these bases in the genome, but in any case they should be hugely imbalanced from each other. This module issues a simple warning if the difference between A and T, or G and C is greater than 10% in any position. This module will signal a failure if the difference between A and T, or G and C is greater than 20% in any position.



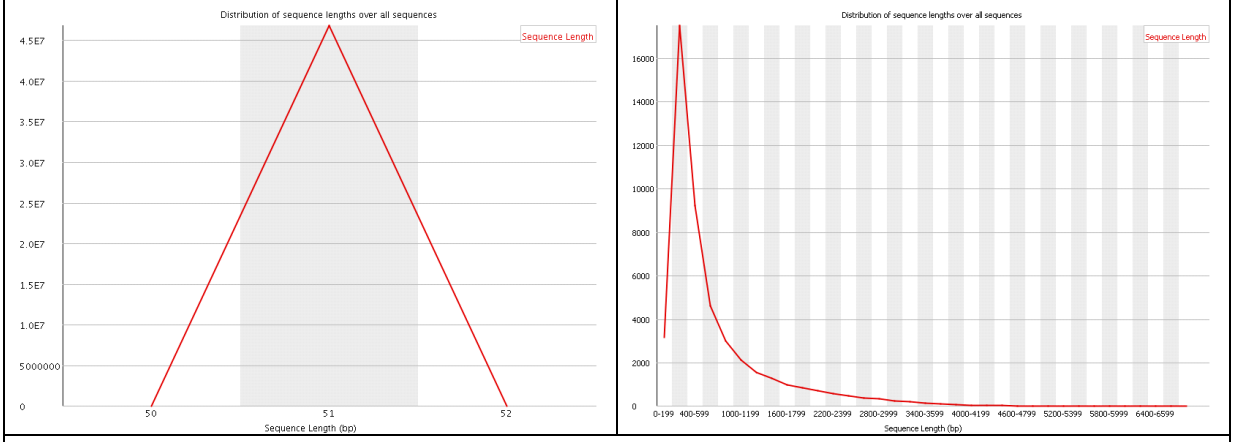
Per Base GC Content plots out the GC content of each base position in a file.
This module issues a simple warning it the GC content of any base strays more than 5% from the mean GC content. It will raise a failure warning if the GC content of any base strays more than 10% from the mean GC content.



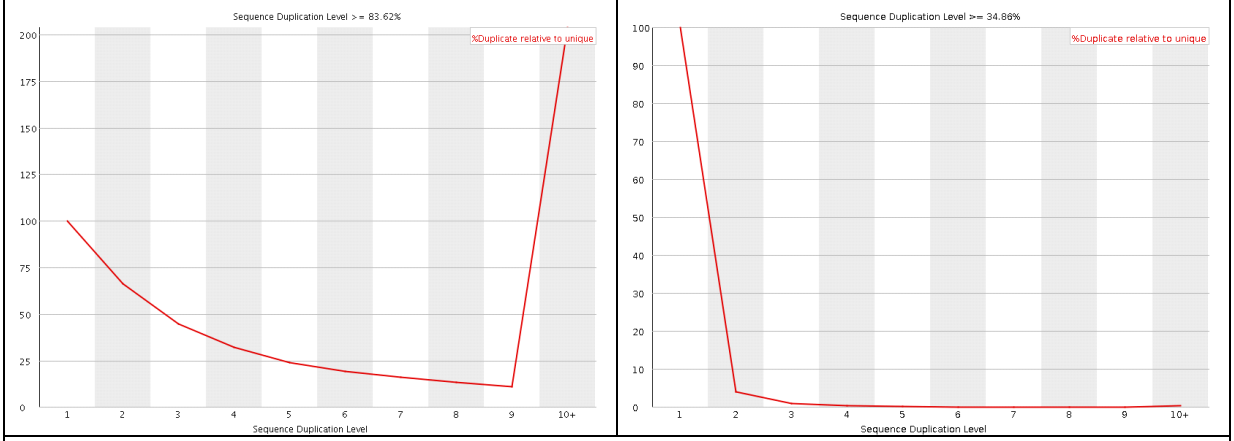
Per sequence GC content measures the GC content across the whole length of each sequence in a file and compares it to a modelled normal distribution of GC content.
A simple warning is raised if the sum of the deviations from the normal distribution represents more than 15% of the reads. This module will indicate a failure if the sum of the deviations from the normal distribution represents more than 30% of the reads.



Per base N content plots out the percentage of base calls at each position for which an N was called.
This module raises a simple warning if any position shows an N content of >5%. It will raise an error warning if any position shows an N content of >20%.



Sequence lengths distributions generates a graph showing the distribution of fragment sizes in the file which was analysed.
This module will raise a simple warning if all sequences are not the same length. It will raise an error warning if any of the sequences has zero length.



Duplicate sequences counts the degree of duplication for every sequence in the set and creates a plot showing the relative number of sequences with different degrees of duplication.
This module will issue a simple warning if non-unique sequences make up more than 20% of the total. It will issue an error warning if non-unique sequences make up more than 50% of the total.

Fig. 20 An example of a good run (on the left) and of a poor run (on the right)

After the filtering step, the reads with poor quality score were removed. Tab. 61 shows some statistics of this important step. It can be pointed out that, even in the worst sample, a maximum of 2.3% of the reads had been discarded. All the samples had an acceptable sequencing depth (measured as number of reads for sample). Following the quality control step, the samples could be judged of good quality and could be further processed.

Tab. 61 Filtering statistics

Cv	Time point	Flesh type	Tot. # of reads	Tot. # of successfully filtered reads	Tot.# of discarded reads	Tot. # of contaminated reads	Tot. length of quality filtered reads
Redhaven	Veraison	M	38594641	38377920	216430	291	1950498063
Bolero	Veraison	M	21629432	21520731	108539	162	1094208771
Big Top	Veraison	SM	17537250	17466890	70229	131	888814297
Rich Lady	Veraison	SM	20170832	20078550	92130	152	1021181951
IFF 331	Veraison	SH	20134587	20006786	127651	150	1015947032
BO 05030081	Veraison	SH	48299453	47704493	594598	362	2410977616
Oro A	Veraison	NM	34684708	33898966	785484	258	1702767218
BO010120182	Veraison	NM	36932313	36516165	415872	276	1846414103
Redhaven	Comm. Rip.	M	40720431	40406863	313262	306	2047194710
Bolero	Comm. Rip.	M	20794818	20686442	108219	157	1051921879
Big Top	Comm. Rip.	SM	27229457	27111411	117840	206	1379253493
Rich Lady	Comm. Rip.	SM	25064995	24908634	156171	190	1266184831
IFF 331	Comm. Rip.	SH	22333899	22154707	179024	168	1125168168
BO 05030081	Comm. Rip.	SH	25375882	25230142	145548	192	1282923023
Oro A	Comm. Rip.	NM	19757179	19499557	257475	147	984508894
BO010120182	Comm. Rip.	NM	24019037	23777592	241266	179	1205808293

5.3.2. Alignment

As first attempt in the alignment and data analysis, instead of TopHat, Bowtie2 was used, because of its fewer requirements in computational resources. TopHat is an aligner capable of performing spliced alignment. In the case of a read that spans two exons, TopHat would map it to both exons to splice junctions phase, while Bowtie2 (run in --very-sensitive-local setting) would soft trim until it maps to only one of the two exons, whichever gives the higher mapping score. However, taking into account the good quality of the peach genome and its relative simplicity, the loss of data would be minimal with this approach while gaining much in machine time. Bowtie2 it's also preferable due to the fact that its data suit perfectly the downstream analysis.

In figures 21 and 22 a screenshot of the alignment in stacked and packed mode is shown as it appeared using the tablet software (http://bioinf.scri.ac.uk/tablet/), a sequence assembly viewer. These figures give just an idea of the quality of the alignment.

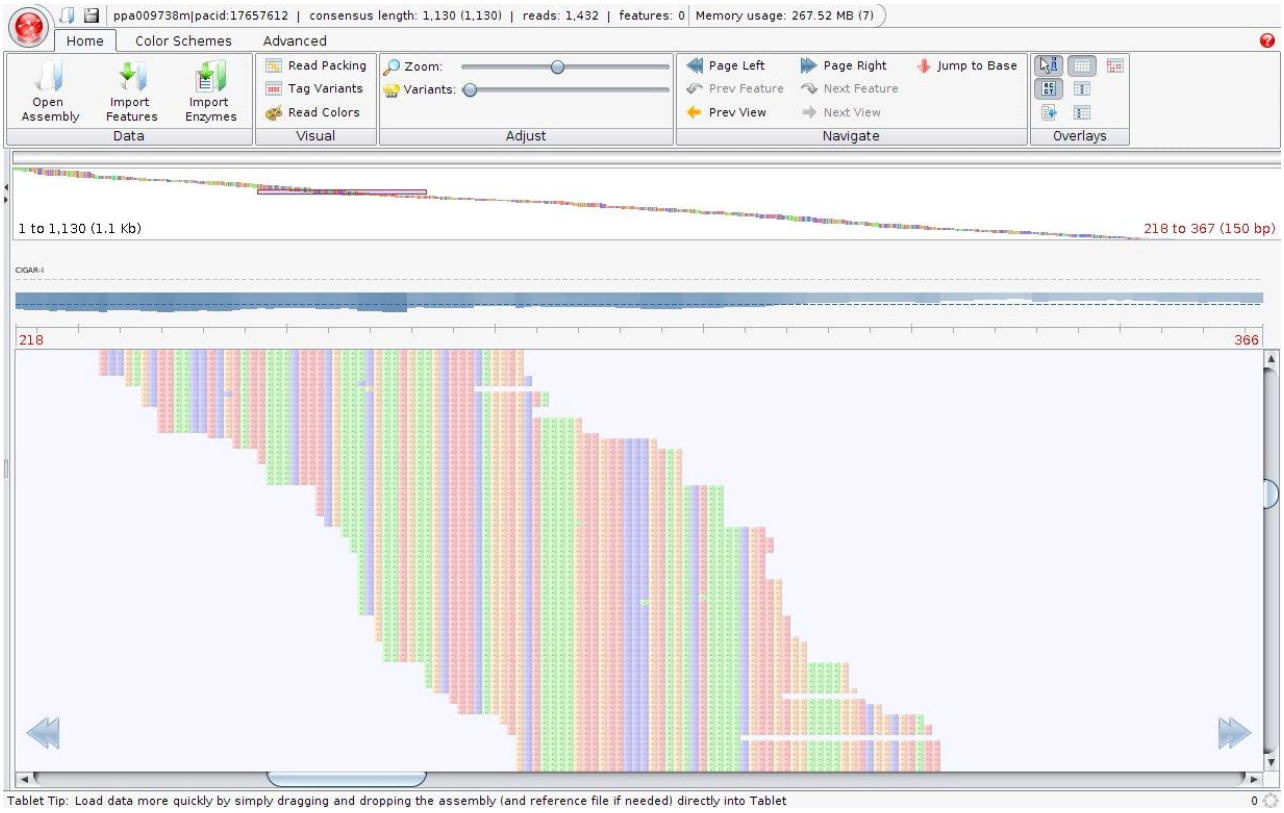


Fig.21 A screenshot of the stacked alignment using the tablet software

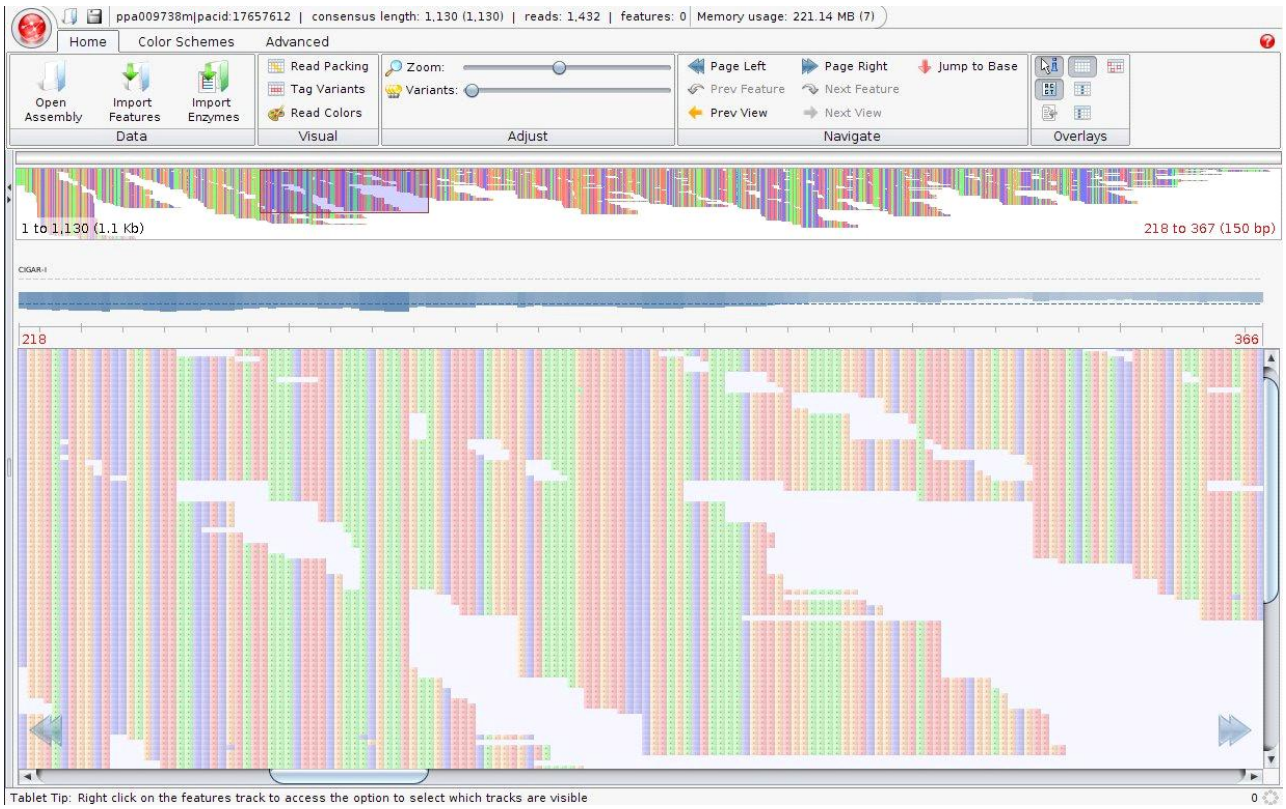


Fig.22 A screenshot of the packed alignment using the tablet software

5.3.3. Analysis of differential expression

Analysis was performed as described in materials and methods.

5.3.3.1. Normalization

The normalization step calculates coefficients which are used to convert read counts to Log2-cpm (count per million). The resulting values are summarized in the tab 62.

Tab 62. Normalization factor

Sample	norm.factor
typeA_time1_rep1	1.0694413
typeA_time1_rep2	0.9921793
typeB_time1_rep1	1.2112593
typeB_time1_rep2	0.9375753
typeC_time1_rep1	1.045361
typeC_time1_rep2	1.0249415
typeD_time1_rep1	1.0350526
typeD_time1_rep2	0.9375819
typeA_time2_rep1	0.9689879
typeA_time2_rep2	0.9179312
typeB_time2_rep1	1.1882614
typeB_time2_rep2	0.8519185
typeC_time2_rep1	0.9861486
typeC_time2_rep2	0.9558749
typeD_time2_rep1	1.0221422
typeD_time2_rep2	0.919982

The normalization factors shown here are very close to one, indicating that the libraries are very similar in composition.

After converting the reads count into Log2-cpm, the following plots were generated in order to visually confirm that the mean-variance relationship behave as expected. In fig. 23 the result is shown (left) in comparison with a token plot identifying a bad sample set (right).

Count data almost show non-trivial mean-variance relationships. Raw counts show increasing variance with increasing count size, while log-counts typically show a decreasing mean-variance trend.

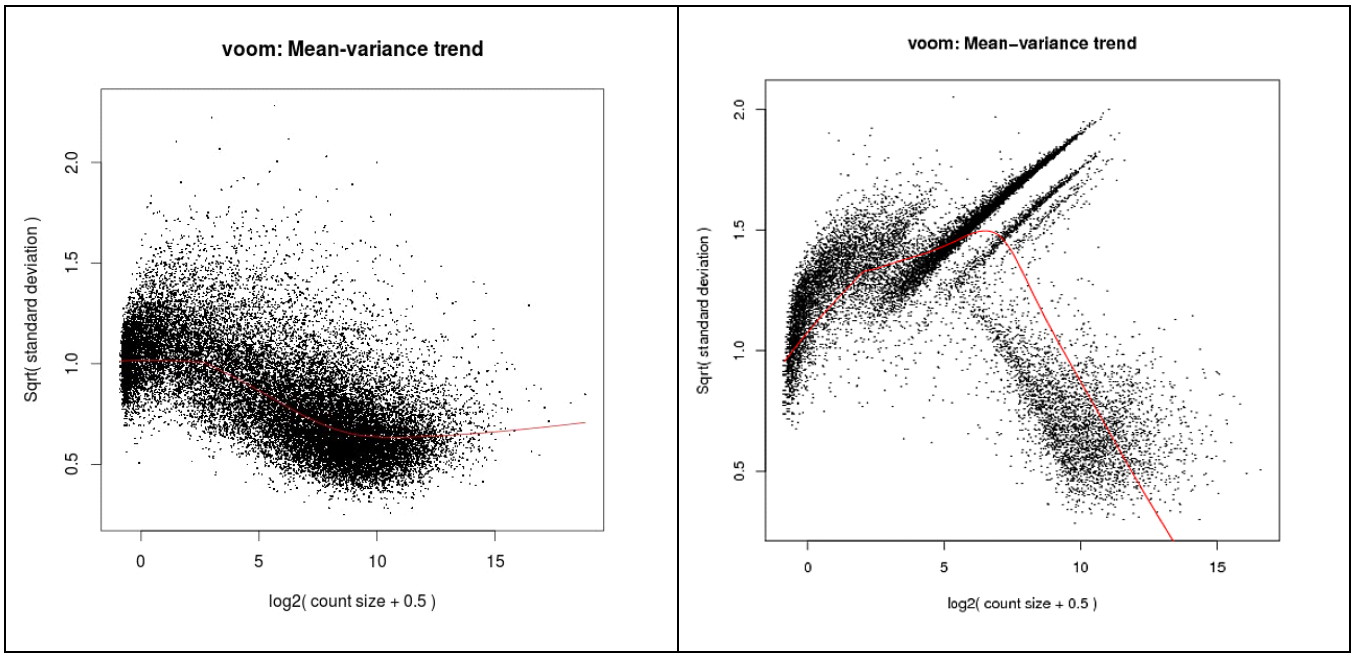


Fig. 23 Plot of mean variance trend.

5.3.3.2. Multidimensional scaling plot

The plot in fig. 24 is a variation on the usual multidimensional scaling (or PCA). By calculating the distance between samples for the top 500 genes, it gives visualization of whether the samples group together, as expected, or not. It can be highlighted that only SH flesh type group tightly together, while the other flesh phenotypes superimpose each other. It can be pointed out that for each type, the two time points group together.

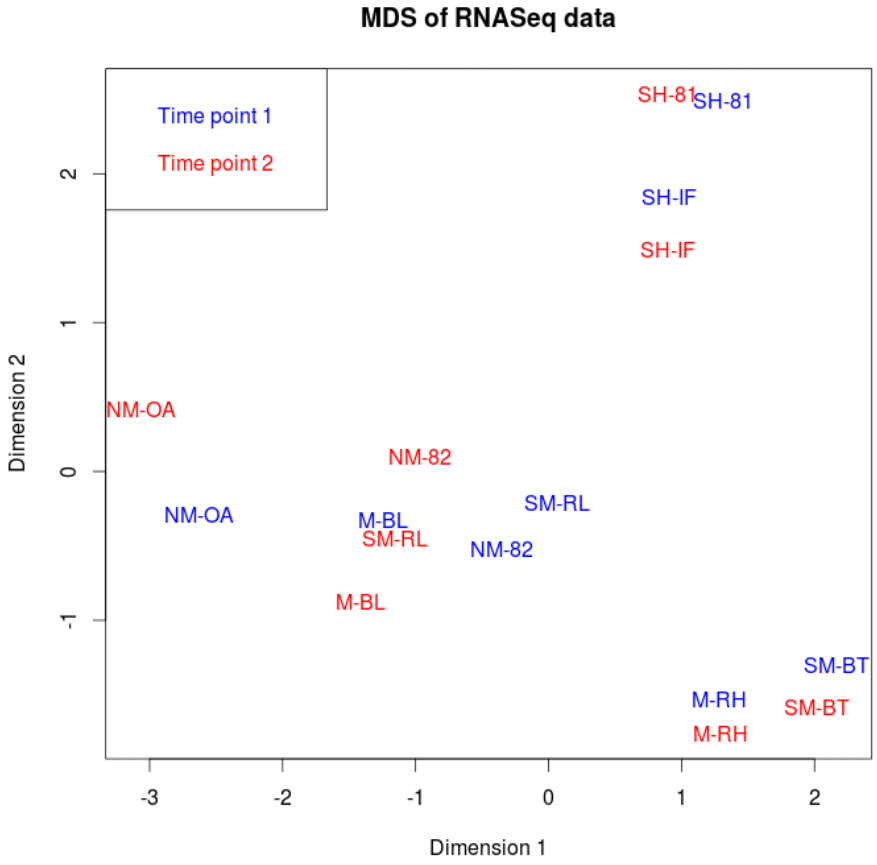


Fig. 24 Multidimensional Scaling (MDS) of RNA-Seq Data

5.3.3.3. Estimation of differential expression

Differential expression evaluation generates as result a table containing the gene ID, its expression value and some statistic useful to understand the reliability of the results. After this point there are many ways to further analyze or visualize such output. One example is MapMan (http://mapman.gabipd.org/web/guest), a user-driven software tool that displays large datasets onto diagrams of metabolic pathways. Because peach genome annotation it's still far from being "good", the more reliable Arabidopsis thaliana's annotation was used. For that reason a BLAST of the peach genome against the Arabidopsis genome (TAIR 10) was performed. Thanks to this step the peach genes with a good correspondence in Arabidopsis genome could have been visualized in MapMan (fig. 25).

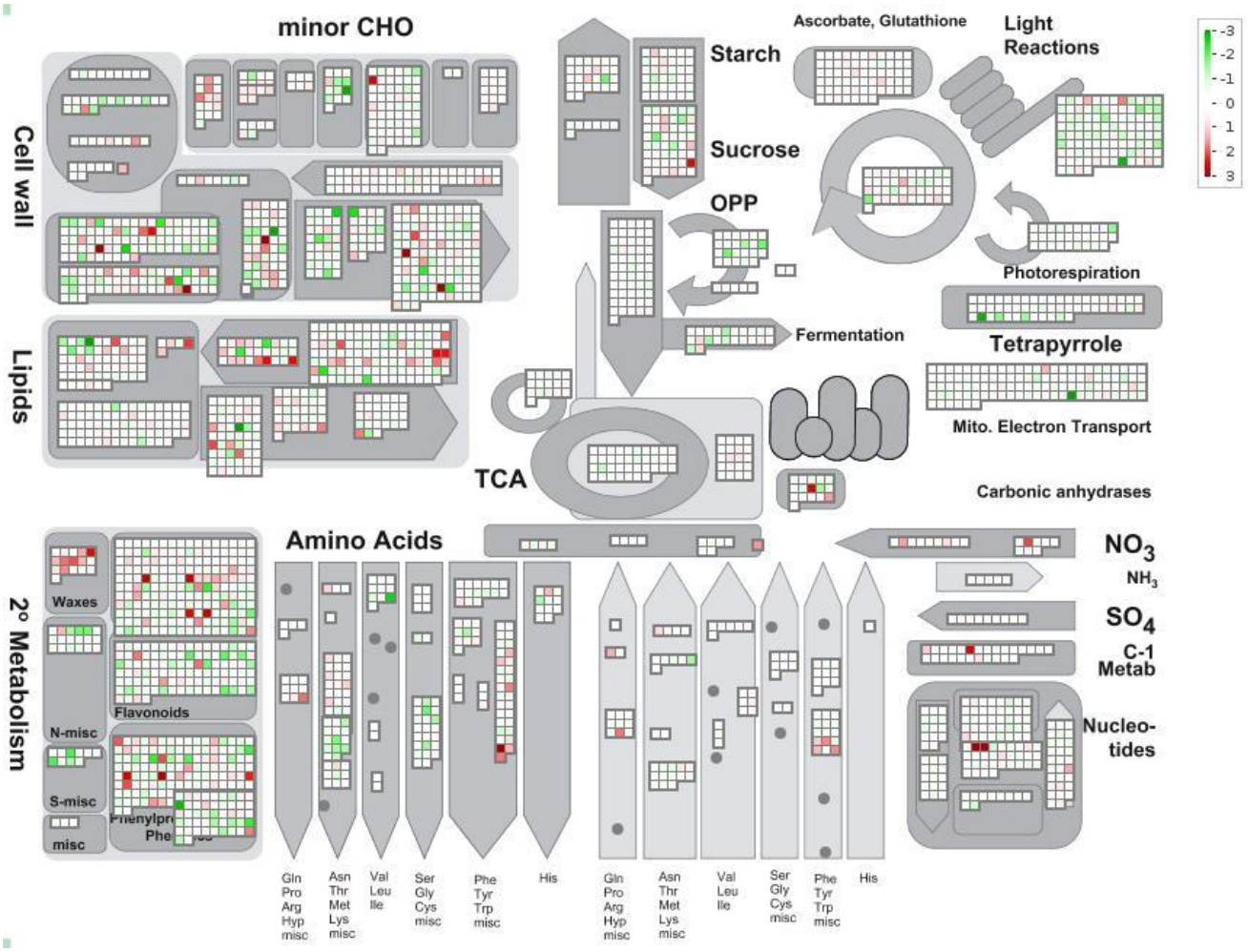


Fig. 25 General metabolism overview for the pairwise comparison M-T1 vs. SM-T1. In red the genes overexpressed in M-T1 and in green the genes downregulated in M-T1 (or otherwise overexpressed in SM-T1). Color scale correspond to Log2FC.

In the original annotation of the peach genome, hits against the human genome were present too. In order to clean from these hits, alongside the original annotation and the Arabidopsis derived, a third annotation was created using the Blast2GO software (http://www.blast2go.com/b2ghome), an all in one tool for automatic functional annotation. During the analysis all the three annotation were taken into account, and, in case of discordance, the most reliable was manually selected (using different criteria such as e-value, hit species and BLAST database).

The results were filtered using adjusted p-value of 0.05 as cutoff that corresponds to an expected proportion of false discoveries of 5%. Figure 26 reports two Venn diagram showing the differentially expressed genes for the two time point (veraison = left; commercial ripening = right) divided for classes that correspond to the initial hypothesis and subsequent contrasts. Only the contrast showing differentially expressed genes are here

reported. In table 63 the differentially expressed genes with Log2FC values and best annotation are reported for all the significant contrasts.

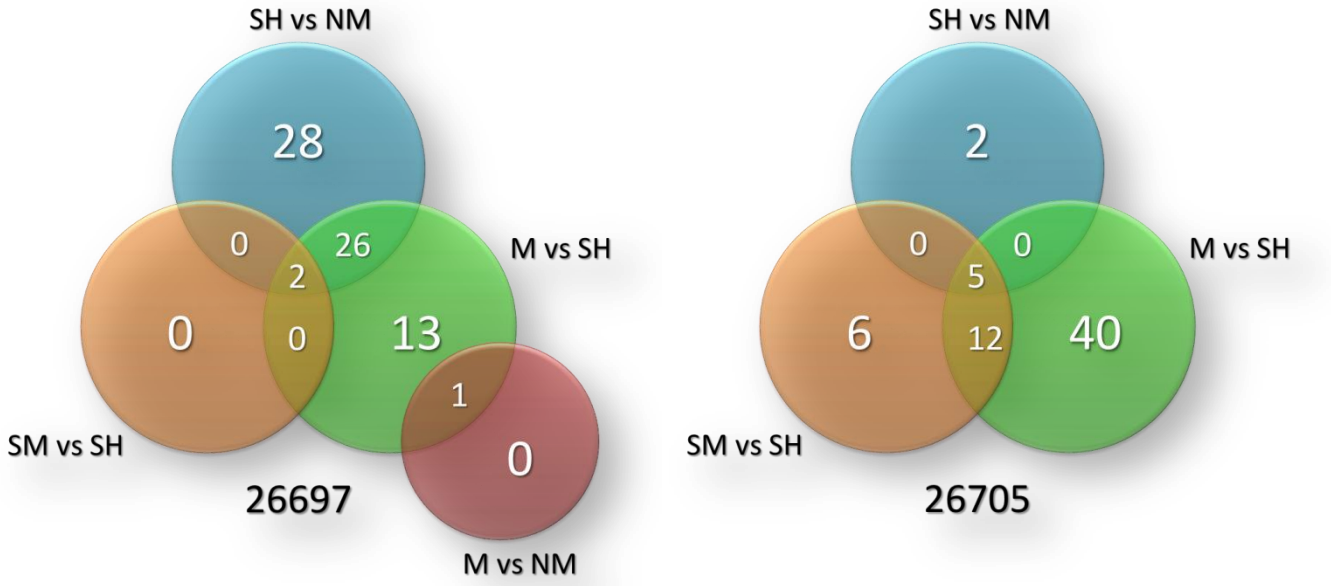


Fig. 26 Venn diagram showing the differentially expressed genes in T1 (veraison) = left and T2 (commercial ripening = right). Only the contrast showing differentially expressed genes are shown.

Tab. 63 Differentially expressed genes from the significant contrasts.

Gene ID	Veraison (Log2FC)				Comm. Ripening (Log2FC)			Description	BLAST evalue	BLAST hit	Hit species
	M-SH	M-NM	SM-SH	SH-NM	M-SH	SM-SH	SH-NM				
ppa013010m	4.67				5.77			14 kda proline-rich protein	1.12217E-25	gi|224137832	[Populus trichocarpa]
ppa014606m				4.54	-5.95			abc transporter g family member 26-like	0	gi|255563590	[Ricinus communis]
ppa011192m	3.38			-3.00	3.08			af446868_1 at3g14560 mie1_6	7.73617E-05	gi|297830000	[Arabidopsis lyrata]
ppa012202m	3.35			-2.95	3.08			af446868_1 at3g14560 mie1_6	9.45941E-05	gi|297830000	[Arabidopsis lyrata]
ppa024588m					-2.34			aldo-keto reductase yakc	1.8399E-178	gi|356526627	[Glycine max]
ppa000117m	2.02			-2.70			-2.36	alpha beta-hydrolases-like protein	0	gi|359484251	[Vitis vinifera]
ppa003717m					-3.25	-4.24		ankyrin repeat-containing protein at5g02620-like	0	gi|225439832	[Vitis vinifera]
ppa004428m	2.76							aspartic proteinase-like protein 1-like	0	gi|255545620	[Ricinus communis]
ppa000479m				-2.94	2.03			auxin response factor 25-like	0	gi|359484941	[Vitis vinifera]
ppa000946m				-2.21				auxin response factor 5	0	gi|27450533	[Prunus persica]
ppa011843m	4.72		4.14	-5.28	5.77	5.04	-5.18	auxin-induced protein	1.06285E-87	gi|302398587	[Malus x domestica]
ppa011935m	4.50		3.75	-4.95	5.99	4.75	-5.45	auxin-induced protein	6.46799E-87	gi|351725121	[Glycine max]
ppa022319m					4.50			auxin-induced protein	5.88935E-41	gi|2924327	[Malus x domestica]
ppa024497m	3.36				4.16			auxin-induced protein 15a	1.11886E-20	gi|2924327	[Malus x domestica]
ppa015904m				4.24	-4.02	-4.77		auxin-induced protein 5ng4	2.7941E-135	gi|255585897	[Ricinus communis]
ppa013765m	4.58			-4.48	5.02	4.19		auxin-induced protein 6b-like	1.25931E-41	gi|2924327	[Malus x domestica]
ppa026172m					7.08			auxin-induced protein 6b-like	1.02327E-47	gi|2924327	[Malus x domestica]
ppa010342m	3.32			-3.17	4.40	3.57		auxin-induced protein aux28	2.3715E-99	gi|359807079	[Glycine max]
ppa010501m	3.29			-3.16	4.42	3.57		auxin-induced protein aux28	1.05479E-92	gi|359807079	[Glycine max]
ppa012269m	3.33			-3.19	4.39	3.54		auxin-induced protein aux28	3.54199E-78	gi|359807079	[Glycine max]
ppa008953m	3.07			-3.30	3.21	2.37	-2.94	auxin-responsive protein	1.4845E-104	gi|302398583	[Malus x domestica]
ppa008961m	3.12			-3.39	3.28	2.38	-2.99	auxin-responsive protein	2.3249E-106	gi|302398583	[Malus x domestica]
ppa017497m						5.11		b3 domain-containing protein	9.68595E-30	gi|147789397	[Vitis vinifera]
ppa002707m					3.41			bzip transcription factor bzip133	0	gi|356528485	[Glycine max]
ppa001365m						-3.53		cation h(+) antiporter 20-like	0	gi|225440302	[Vitis vinifera]
ppa004768m	-3.58			3.78				cytochrome p450	0	gi|225431255	[Vitis vinifera]
ppa004095m						-2.53		cytochrome p450	0	gi|255580537	[Ricinus communis]
ppa025045m					-5.05			cytochrome p450	0	gi|33300600	[Prunus dulcis]
ppa024213m	-6.81			8.20				cytochrome p450 78a4-like	0	gi|224057703	[Populus trichocarpa]
ppa003346m	-1.76			1.98				dna binding	0	gi|356499663	[Glycine max]
ppa011235m	2.17							dna binding protein	2.1894E-108	gi|225458920	[Vitis vinifera]
ppa002229m					-1.40			dnaj heat shock n-terminal domain-containing protein	0	gi|359491697	[Vitis vinifera]
ppa007271m				-6.16				endopolygalacturonase	0	gi|51507375	[Pyrus communis]
ppa022313m					-4.69			fasciclin-like arabinogalactan protein 9	2.43631E-66	gi|255553599	[Ricinus communis]
ppa007792m	-3.15			3.15				f-box kelch-repeat protein	1.1985E-168	gi|255552951	[Ricinus communis]
ppa018277m	6.71	6.03			5.69			f-box protein	2.74908E-20	gi|224115754	[Populus trichocarpa]
ppa006765m	4.75			-5.06	5.95			f-box protein at2g32560-like	1.2829E-170	gi|224055285	[Populus trichocarpa]
ppa006985m	6.65							gibberellin 20-oxidase	0	gi|333440997	[Pyrus communis]
ppa008404m				-2.22				homocysteine s-methyltransferase	2.9098E-171	gi|255552095	[Ricinus communis]
ppa008421m				-2.33				homocysteine s-methyltransferase	7.2907E-169	gi|255552095	[Ricinus communis]
ppa010310m				-2.29				homocysteine s-methyltransferase	8.5711E-135	gi|255552095	[Ricinus communis]
ppa010689m				-2.31				homocysteine s-methyltransferase	2.42576E-81	gi|255552095	[Ricinus communis]
ppa006544m				-1.75				homogentisate phytyltransferase	0	gi|254596582	[Malus x domestica]
ppa012701m					-5.28			hypothetical protein	3.59317E-70	gi|341833958	[Pyrus x bretschneideri]
ppa010239m					3.62			hypothetical protein RCOM_1180720	1.1987E-50	gi|255585863	[Ricinus communis]
ppa022459m				5.77				hypothetical protein VITISV_007322	4.7508E-167	gi|147853623	[Vitis vinifera]
ppa003134m	5.60			-6.28	5.37	4.48	-5.19	indole-3-acetic acid-amido synthetase	0	gi|300680024	[Dimocarpus longan]
ppa002656m	4.33			-3.99	3.34			l-aspartate oxidase	0	gi|225452458	[Vitis vinifera]
ppa024091m					-3.65			leucine-rich repeat receptor-like kinase	2.6829E-170	gi|359482434	[Vitis vinifera]
ppa001082m				-4.90	4.48	4.50		lipoxygenase	0	gi|195957709	[Prunus persica]
ppa002308m				-4.92	4.52	4.54		lipoxygenase	0	gi|195957709	[Prunus persica]
ppa001112m				-4.97	4.46			lipoxygenase	0	gi|195957709	[Prunus persica]
ppa003030m					-2.21			microtubule-associated protein 70-2-like	0	gi|224086823	[Populus trichocarpa]
ppa010931m				1.95				mrna turnover protein 4	4.5562E-130	gi|225448095	[Vitis vinifera]
ppa003583m					-3.06			multicopper oxidase	0	gi|209420826	[Castanea mollissima]
ppa026318m	4.10			-4.15	3.98	3.72		nbs-containing resistance-like protein	0	gi|47059739	[Prunus persica]
ppa024268m				-4.29				nitrilase 3	4.425E-126	gi|224138410	[Populus trichocarpa]
ppa011900m	-3.04			3.49				oxygen-evolving enhancer protein 3-1	1.04907E-85	gi|255563649	[Ricinus communis]

Gene ID	Veraison (Log2FC)				Comm. Ripening (Log2FC)			Description	BLAST evalue	BLAST hit	Hit species
	M-SH	M-NM	SM-SH	SH-NM	M-SH	SM-SH	SH-NM				
ppa008550m							4.45	peroxidase 16	4.6943E-160	gi|211906542	[Gossypium hirsutum]
ppa000643m				-2.21				phytochrome a	0	gi|224122788	[Populus trichocarpa]
ppa006746m	-3.22							plastidic aldolase	0	gi|356538694	[Glycine max]
ppa002155m				-2.29				p-loop containing nucleoside triphosphate hydrolase family protein	0	gi|359486767	[Vitis vinifera]
ppa013300m					4.76			pop3 peptide	2.98467E-45	gi|217075526	[Medicago truncatula]
ppa022818m				-7.04				potassium transporter	0	gi|359491532	[Vitis vinifera]
ppa013026m				-4.51				predicted protein	5.1944E-33	gi|224092406	[Populus trichocarpa]
ppa013911m					-2.77			PREDICTED: uncharacterized protein LOC100803743	3.01208E-23	gi|356496422	[Glycine max]
ppa007279m						-3.03		probable carboxylesterase 2-like	0	gi|82697951	[Malus pumila]
ppa007155m	3.98			-3.08	2.86			protein	4.4936E-47	gi|224062537	[Populus trichocarpa]
ppa011755m	2.56			-2.32	2.43			protein	3.79E-113	gi|302398579	[Malus x domestica]
ppa000610m				5.50				protein	0	gi|225456989	[Vitis vinifera]
ppa004017m					-2.89			protein	0	gi|147861793	[Vitis vinifera]
ppa022354m				3.28				protein	3.8945E-107	gi|359478461	[Vitis vinifera]
ppa025482m				2.97				protein	0	gi|224140589	[Populus trichocarpa]
ppa004740m	3.41				3.89			protein kinase-like protein	0	gi|224087605	[Populus trichocarpa]
ppa008330m					-8.52			protein trichome birefringence-like 19	0	gi|356545636	[Glycine max]
ppa001576m					4.45			receptor protein kinase 1-like	0	gi|359492570	[Vitis vinifera]
ppb025587m				-5.24				receptor-like protein 12-like	2.47952E-67	gi|359493541	[Vitis vinifera]
ppa000282m				-2.46				regulatory-associated protein of tor 1-like	0	gi|359481183	[Vitis vinifera]
ppa013865m	4.54			-4.61	5.99			saur family protein	3.09621E-43	gi|2924327	[Malus x domestica]
ppa021647m	5.61			-6.94	6.01			saur family protein	5.05129E-40	gi|356517873	[Glycine max]
ppa024057m	6.24			-5.44	6.33			saur family protein	2.76762E-42	gi|356517873	[Glycine max]
ppa017462m	5.98							saur family protein	2.21965E-49	gi|225427870	[Vitis vinifera]
ppa026059m				-7.02	7.35			saur-like auxin-responsive protein	9.75879E-33	gi|297801964	[Arabidopsis lyrata]
ppa009553m	2.06							short chain alcohol	1.8039E-143	gi|356561253	[Glycine max]
ppa009906m				2.67				short-chain type	2.9728E-108	gi|255568942	[Ricinus communis]
ppa025818m	5.92							sob five-like 2 protein	6.65232E-10	gi|357477793	[Medicago truncatula]
ppa010287m					3.81			stem-specific protein tsjt1-like	1.2389E-135	gi|224125458	[Populus trichocarpa]
ppa001537m					2.89			sucrose synthase	0	gi|6683114	[Citrus unshiu]
ppa000096m				-3.55				tetratricopeptide repeat-containing protein	0	gi|359489732	[Vitis vinifera]
ppa1027179m	4.14			-3.00	5.17	3.68		tir-nbs-lrr resistance protein	0	gi|359493273	[Vitis vinifera]
ppa022091m	3.90				5.29			tmv resistance protein n-like	0	gi|359493273	[Vitis vinifera]
ppa005254m	2.09							transcription factor bhlh66	3.4719E-110	gi|225445889	[Vitis vinifera]
ppa023258m	-7.06			7.48	-7.86	-6.68		tyrosine dopa decarboxylase	7.86284E-52	gi|224059382	[Populus trichocarpa]
ppa015004m	-4.51			4.41	-4.83	-5.27		tyrosine-sulfated glycopeptide receptor 1-like	6.6249E-179	gi|359482434	[Vitis vinifera]
ppa019687m						-5.52		tyrosine-sulfated glycopeptide receptor 1-like	3.0795E-136	gi|359482434	[Vitis vinifera]
ppa018557m						-3.83		udp-glucose flavonoid 3-o-glucosyltransferase 6-like	0	gi|75288885	[Fragaria x ananassa]
ppa023851m					-5.07			udp-glycosyltransferase 85a2-like	0	gi|225459251	[Vitis vinifera]
ppa021673m					-4.31			vacuolar amino acid transporter 1-like	0	gi|225465048	[Vitis vinifera]
ppa010261m	1.94				2.11			zinc finger	1.0889E-107	gi|302398655	[Malus x domestica]

5.4. Conclusions

Looking at the scientific data bases, as far as we know, this is the first research that considers the approach of transcriptome analysis as a means to characterize peach flesh typology.

Fruit softening is determining fruit quality and post-harvest life and it involves several processes including disassembly of polysaccharide networks in the primary cell wall and middle lamella, and transpirational water/turgor loss (Ghiani et al., 2011). The melting phase of M peaches is also associated with marked increases in gene expression and enzymatic activity of endo-PG; contrariwise, NM fruit have a lower level of ripening-related endo-PG expression and activity (Ghiani et al., 2011). So far none of the different research groups working on this subject has been able to explain the SM phenotype, which shares some similarities with the M type, but with a different time course.

Trascriptome approach gives a broad spectrum of the comparison between two conditions, so we decided to proceed with a transcriptome analysis of the first survey regarding the difference between M and SM peaches, which is now becoming a trait of wide commercial interest. SM, in fact, is the most difficult phenotype to characterize in field and there is no genetic information like candidate genes responsible for the character or associated genetic markers.

Because of the complexity of this approach, it was decided to use more cultivars, each one bearing a single flesh character in order to reduce the variability due to the genetic background, supposing that cultivars belonging to the same flesh type should have, at a variable level, a common expression profile in the pathway involved in texture determination. Indeed in the data analysis the cultivars were treated in the same way in which biological replicates are usually treated.

The main differences highlighted with this approach were in M vs. SH and SH vs. NM at veraison, and again in M vs. SH at commercial ripening. It is interesting to note that, in the contrast M vs. SH, there are 28 genes which are differentially expressed in both the time points. 26 of the 28 are overexpressed in M type, while only 2 genes are downregulated. The expression values are consistent in the two time points showing a wide change in expression. Among these genes there are some ARS transcription factors (auxin response factor). ARS in Arabidopsis promotes flowering, stamen development and floral organ abscission and fruit dehiscence. It works independently of ethylene and cytokinin response pathways and may act as a cell division repressor and organ growth. Which are the pathways interested by this TF is still uncertain. One gene involved in defense (ppa026318m: disease-resistance relating genes) is also present, while the others remain so far uncharacterized.

The same ARS transcription factors are overexpressed in NM type in the comparison SH vs. NM at veraison (or downregulated in SH) in a magnitude similar to that seen in the M type (more than 10 times higher). However

too few genes showed differential expression, making it difficult to associate changes in metabolic pathways referring to different flesh types.

It can be highlighted, against our initial hypothesis, that no difference was evaluable in the contrast M vs. SM either in fruit veraison or in commercial ripening. This result was completely unexpected as it was supposed that cultivars belonging to the same flesh type should have a common expression profile. As it has been shown previously, in a PCA analysis taking into account the top 500 expressed genes, M and SM samples group separately in both dimensions, while NM group together at least in one axis and SH in both. From these results it seems that 'Big Top' (SM) is more similar to 'Red Haven' (M) instead of 'Rich Lady', the other SM cultivar. Once again 'Rich Lady' (SM) seems closer to 'Bolero' (M). This fact could generate a lot of variability in the data distribution, interfering with the differential expression estimation. This might explain why so few genes seem to be differentially expressed in all the considered contrasts.

In the light of these results we are going to review our experimental plan, by treating the cultivars singularly and proceeding with a series of pairwise comparisons, in order to evaluate the changes in expression for each combination. Only after this step will it be possible to compare the data on the basis of the flesh type in order to find similarities.

Because the experimental design to be used for a pairwise comparison analysis is less complex, a change of analytical software is now possible and probably advisable. In particular the RNA-seq reads can now be aligned with programs like TopHat, introducing the *de-novo* transcript discovery, increasing the sensitivity of the technique and hopefully allowing to dispel some of the fog covering the flesh type determination.

6. General conclusions

The main purpose of this research was to correlate physical or chemical differences to different flesh phenotypes, in order to develop tools capable of characterizing flesh textures. Accordingly, at the same time, the expression profiles of the different flesh textures were investigated in order to improve our understanding of which genes and pathways are involved in flesh types.

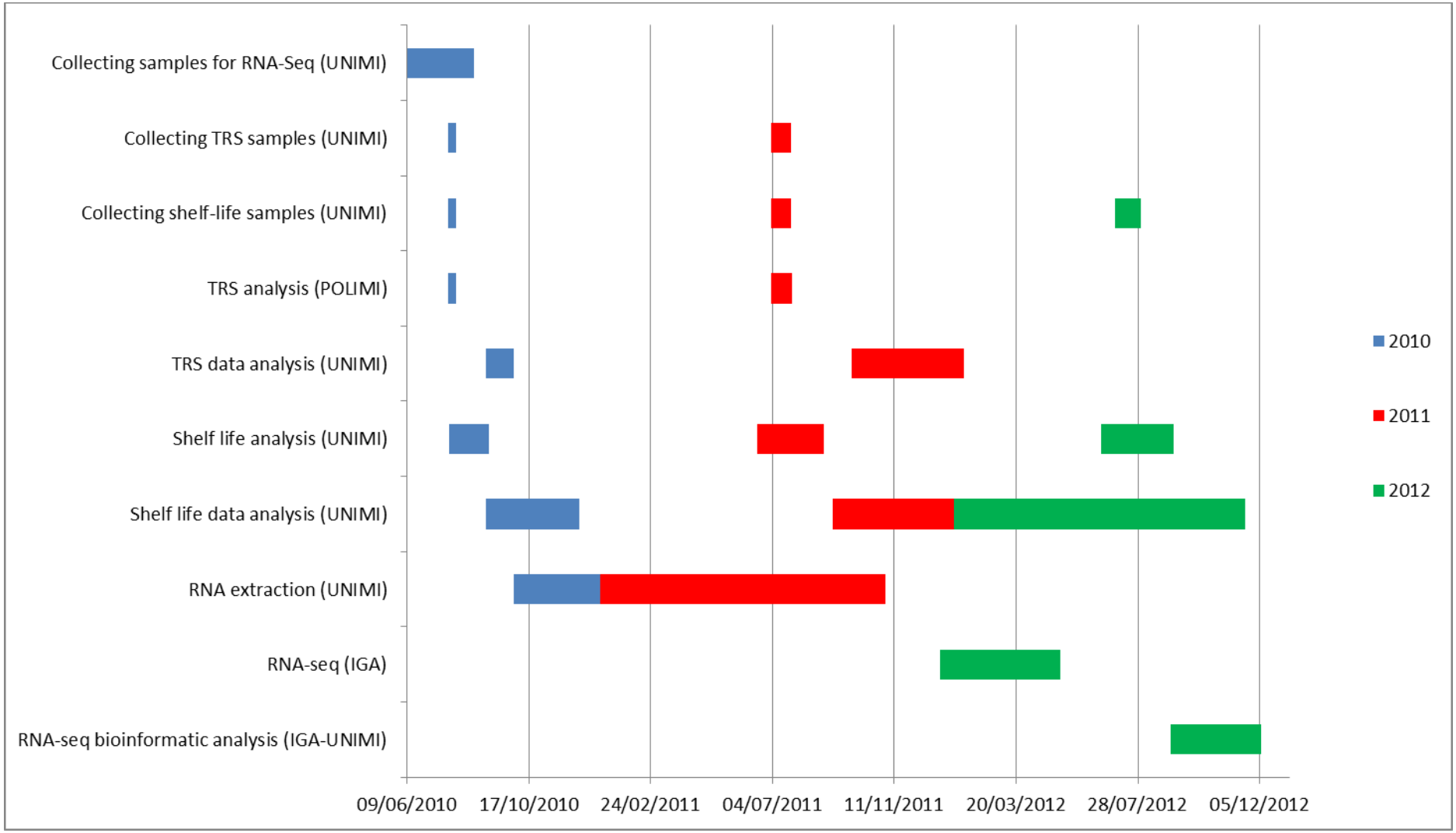
The possibility of applying the TRS technique to assess the texture phenotype of peach fruit was tested. This technique, allowing measurements of the scattering properties of the pulp independently of those of the absorption spectrum, was considered interesting because potentially capable of discriminating between the different flesh phenotypes in peaches. Nevertheless, despite these premises, the results showed that this technique has limited capabilities to discriminate between the different flesh phenotypes, as it can discriminate only three flesh phenotypes (Melting, Slow Melting and Stony hard) out of the four that were tested.

The chemical and physical characterization, allowed an evaluation of different parameters (weight loss, TA, SSC, firmness and expressible juice). From the results that have been obtained, it is possible to assert that, among all the parameters that were analyzed, expressible juice may be the key factor discriminating between the different flesh phenotypes. In fact it discriminated between all the flesh phenotypes in samples at physiological ripening in two consecutive years (2011 and 2012). Further studies are needed to determine whether this trait can be used as phenotyping method to be applied in QTL analysis to the end of finding molecular markers associated with this character. Moreover firmness discriminated between the different flesh phenotypes in samples at physiological ripening, but only after three weeks at 4°C.

The expression profile analysis gave partially unexpected results. In one of the most important contrasts of the experiment, M vs. SM, no differentially expressed genes were found, either in fruit veraison or in commercial ripening. This could be explained by the fact that in a PCA analysis taking into account the top 500 expressed genes, M and SM cultivars don't group separately, increasing the variability. On the contrary, but as expected, differences were found in M vs SH and SH vs NM at varaison and again in M vs SH at commercial ripening. All SH cultivars grouped together.

7. Work organization

This project involved different research facilities (Università degli Studi di Milano, Istituto di Genomica Applicata and Politecnico di Milano) and collaborators. Below, a brief outline of the work organization is reported. The institution to which the work is credited is indicated in brackets (the PhD candidate did the tasks named as UNIMI).


Collecting samples for RNA-Seq (UNIMI)
Collecting TRS samples (UNIMI)
Collecting shelf-life samples (UNIMI)
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TRS data analysis (UNIMI)
Shelf life analysis (UNIMI)
Shelf life data analysis (UNIMI)
RNA extraction (UNIMI)
RNA-seq (IGA)
RNA-seq bioinformatic analysis (IGA-UNIMI)
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