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Fruit Proteomics

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1. Introduction

Obesity has been recognized as a major threat to human health in the 21st century [Yun, 2010]. One of the central causes to such nutritional disturbance relies in the consumption boost of the so called "fast food", which is characterized by high levels of fat, salt and sugars [Rosenheck, 2008]. On the opposite side of the spectrum are the plant fruits, which are characterized by high levels of relevant nutrients such as phenolic compounds, vitamins and essential minerals [Prasanna et al., 2007]. Besides its direct positive effects on human health, fruit intake has also been associated with the prevention of age-related neurodegeneration and cognitive decline [Spencer, 2010]. Some relevant aspects that may help fruit become an alternative to the ingestion of "fast food" are its easiness of consumption, which helps in its fast intake, and attractive organoleptic characteristics, aspects that have driven the "fast food" adoption by the society. However, fruit are very perishable and most of the species have a distinct seasonal producing pattern, making access throughout the year a difficult task and increasing their costs. Therefore, a great effort has been placed to understand the molecular mechanisms that could affect the pre- and post-harvest life of fruit, based on the hypothesis that this knowledge could improve the quality and accessibility of these goods to the society [Palma et al., 2011].

In the present chapter, the main proteomic approaches used to assess fruit development and ripening are described. Examples that will help the reader understand and recognize the advantages and drawbacks of each method, in order to decide the one that best suits their own objectives, are provided.

2. Fruit ontogeny

The ovule, being the female structure that develops into seed, is central for seed-bearing plant reproduction. During evolution, a specialized structure was generated to protect it, giving rise to the angiosperms, as opposed to the more ancient gymnosperms. This organ, termed carpel, encloses the seeds, being the fruit precursor [Scutt et al., 2006]. Carpels usually are located at the innermost whorl of the angiosperm flower, the so called gynoecium. Either individual carpels or syncarpic gynoecia (where both organs are fused together) are divided into tissues which perform distinct roles in reproduction, such as the ovary, which accommodates the ovules and in which fertilisation takes place [Ferrandiz et

al., 2010]. Upon ovule fertilisation, the carpel tissues undergo a series of developmental changes that leads to the formation of the fruit, which not only protects and supports the developing seeds, but also contributes to its later dissemination [Scutt et al., 2006].
When an ovary develops into a fruit, the ovary wall becomes the pericarp, the fruit wall which is composed of three layers with characteristics that are species dependent: the exocarp, mesocarp and endocarp. The first one is the outermost protective layer, also known as peel or skin. The mesocarp, located at the middle, holds the succulent edible part of fruits such as peaches and mangoes, among others. The third inner layer is the endocarp [Levetin & McMahon, 2008].
Fruit can be broadly classified as dry or fleshy. In the former, the pericarp may be hard and woody or thin and papery [Levetin & McMahon, 2008]. Regarding the latter, virtually all parts of the total inflorescence structure could be, depending on the species, developed into fruit flesh, a bulky, succulent parenchymatous tissue that accumulates water and many organic compounds [Coombe, 1976]. Any of these diverse tissues could be the subject of study, making a universal protocol for its evaluation a difficult task to fulfil. Additionally, a variety of fruits are characterized by having large variations in interfering metabolites that occur during their development, mainly during the process of ripening [Martínez-Esteso et al., 2011; Palma et al., 2011]. This situation imposes further hurdles to the analysis of the samples, with one protocol suited for a particular developmental stage not necessarily the most appropriate for another.
Summary: Fruit ontogeny is quite complex, which entails difficulties in establishing a unique protocol for the proteomic analysis of their derived tissues. An empirical evaluation is almost certainly necessary for fruit that has not been tested before, even though certain guidelines can be followed on the basis of the previous work in the field.

3. Two dimensional gel electrophoresis

The process that leads to the successful completion of the two dimensional gel electrophoresis (2-DE), meaning a SDS-PAGE gel derived image with well resolved spots representing a proteome fraction of the fruit tissue under evaluation is comprised by five main steps: Protein extraction, isoelectrical focusing, equilibration, SDS-PAGE and protein visualization [Rabilloud & Lelong, 2011]. Since the nature of the fruit tissues is so diverse and particular, each of the above mentioned steps may have to be improved in order to achieve a proper final result, often through an empiric evaluation. However some general guidelines can be given as well as a rational basis to refine these steps.

3.1 Protein extraction from fruit tissues

Plant cells are characterized by the presence of extensive amounts of water, a crucial feature to maintain cell turgor, which helps the cell to accomplish several physiological processes. In terms of fruit post-harvest life, the turgor is directly involved in the organ integrity. However, this characteristic represents an important drawback for the protein extraction, since the amount of protein present per cell mass is very low due to this massive presence of water inside the fruit cells [Saravanan & Rose, 2004]. The presence of a cell wall also poses a difficulty for protein recovery, due to the nonspecific sticking of proteins to this polysaccharide matrix [Rose et al., 2004]. In addition, unlike other plant tissues, fruit tissues display a high content of proteases and metabolites such as phenolics, organic acids, lipids, pigments and polysaccharides, which interfere with protein extraction and gel image

analysis [Carpentier et al., 2005; Wang et al., 2008]. The presence of such contaminants may result in horizontal and vertical streaking as well as smearing, with the consequent reduction in the number of distinctly resolved protein spots on 2-DE gels [Saravanan & Rose, 2004]. Therefore, for a fruit-based proteomics analysis, the protein extraction method is a critical issue to address.

Of foremost importance is the avoidance of protein modifications during the extraction steps in order to diminish the probability of generating artefacts, such as false spots unrepresentative of the sample under analysis, which can lead to misleading conclusions. These modifications may be generated by chemical alterations of the proteins [Righetti, 2006] or by biological compounds such as proteases [Rabilloud & Lelong, 2011].

3.1.1 Tissue disruption

Even though tissue disruption could be considered the simplest step, the efficiency of the entire process relies heavily on this step [Giavalisco et al., 2003]. Based on our extensive experience on this topic and in the literature, by far the most used and efficient method to render proteins available for extraction is the liquid nitrogen assisted mortar/pestle method of tissue grinding (Table 1). Most of the times the finer the powder the higher the protein yield, therefore the use of auxiliary materials to improve the final grinding result, such as quartz sand, or equipment such as stainless steel blenders, may be advisable when dealing with hard, fibrous tissues such as non-ripe firm fruit [Giavalisco et al., 2003; Vincent et al., 2006]. Few authors report the use of sonication or homogenizers to assist fruit tissue disruption/sample homogenization [Lee et al., 2006; Di Carli et al., 2011]. Other methods have been proposed to accomplish similar and more reproducible plant tissue disintegration techniques such as acoustic related technologies [Giavalisco et al., 2003; Toorchi et al., 2008]. However, these methods require access to specialized equipment.

3.1.2 Sample homogenization

Upon proper cell disruption, the recovered tissue is homogenized. The main objectives of this step are to capture and separate the proteins from other metabolites that may interfere with the subsequent proteome characterization. The direct recovery of the proteins from the disrupted tissues by solubilizing the samples in an IEF lysis buffer has proved to be inadequate for these kinds of samples [Wang et al., 2003; Carpentier et al., 2005]. Therefore, alternative and more labour intensive procedures must be used. At least two methods have been widely used to perform this task and are extensively described in the literature. Tissue homogenization in an aqueous buffer followed by protein extraction with phenol or protein precipitation with trichloroacetic acid [Wang et al., 2008]. Importantly, both render proteins amenable for mass spectrometric analysis [Sheoran et. al, 2009].

In the phenol based method, an aqueous buffer is added to the pulverized tissue, followed by protein extraction with this solvent [Hurkman & Tanaka, 1986]. The nature of this buffer may differ greatly among protocols, but is usually composed of reducing and chelating agents which helps cope with polyphenols, metalloproteases and polyphenol oxidases, salts that promote protein extraction, and protease inhibitors dissolved in high pH buffer (Table 1). Polyvinylpolypyrrolidone (PVPP) has also been used to adsorb polyphenols, even though its action is restricted to those molecules in non-ionized states, such as in low pH environments [Carpentier et al., 2005]. The use of SDS and sample heating has been reported [Hurkman & Tanaka, 1986; Hu et al., 2011], albeit the surfactant should be removed prior

isoelectric focusing (IEF) in order to avoid its interference on this step [Molloy, 2000; Görg et al., 2004]. A recent report, where mesocarp proteins from *Prunus persica* fruit were evaluated, suggests that the direct phenol extraction of freeze-dried tissue, followed by the addition of an aqueous buffer, could improve both the protein yield as well as the number of detectable spots on 2-DE gels [Prinsi et al., 2011]. Thus, variations of the method have been performed, although the most used version is the one described by Hurkman and Tanaka (Table 1) [Hurkman & Tanaka, 1986].

Regarding the second method, several versions have been generated, most employing the addition of trichloroacetic acid (TCA) and acetone to a sample extracts to achieve protein precipitation. This step is followed by resolubilization in an appropriate IEF buffer (see below). Variations are mainly focused in the solubilisation of the pulverized tissue in an aqueous buffer, similar to the one used in the phenol based method, prior to the addition of TCA/acetone [Saravanan & Rose, 2004]. A combination of TCA/acetone washes followed by phenol-based protein extraction proved to be successful in dealing with plant samples rich in lipids and pigments, such as mature grape berry clusters [Wang et al., 2003; Vincent et al., 2006]. These interfering compounds are the main contaminants of the phenol-based protocol, since they do not partition in the buffer phase during the first steps of this procedure [Carpentier et al., 2005].

Direct comparisons of these methods, using tissues such as tomato pericarp and grape berry, indicates that the phenol based procedure outperforms the TCA/acetone precipitation method both in terms of protein yield and qualitative characteristics of the 2-DE gels (Table 1)[Saravanan & Rose, 2004; Carpentier et al., 2005]. These differences may arise from dissimilar capacities of both protocols to nullify the proteases activity, and in difficulties in resolubilizing the proteins precipitated by the TCA/acetone protocol [Carpentier et al., 2005]. Since the latter is still the method of choice for many researchers, alternative methodologies to overcome this problem have been evaluated (see below).

More elaborated pre-treatments have been used for extraction of proteins from highly recalcitrant tissues, such as grape berry pericarp [Martínez-Esteso et al., 2011]. Mesocarp were homogenized at 4°C in extraction buffer containing 50 mM Na_2HPO_2 pH 7.0, 1 mM EDTA, 0.1 M PVPP, 1 mM $Na_2O_5S_2$, 10 mM ascorbic acid, and a cocktail of protease inhibitors. The homogenate was filtered through eight layers of cotton gauze and the filtrate was centrifuged. The resulting pellet was washed once in a buffer containing 50 mM Na_2HPO_2 pH 7.0, 1 mM EDTA, 0.1 M NaCl, 10 mM ascorbic acid, and recovered by centrifugation. Afterwards the pellet was cleaned with ethyl acetate:ethanol 1:2 (v/v), followed by TCA and acetone, as described by Wang and others [Wang et al., 2003; Martínez-Esteso et al., 2011]. Another alternative cited in the literature, with a similar performance to TCA/acetone, was used to extract protein from coffee seeds, tissues rich in polyphenols. Samples were milled with liquid nitrogen and extracted in a solution containing 0.1 M acetic acid, 3 M urea and 0.01% CTAB. Extracts were then centrifuged and supernatants were precipitated in an anhydrous solution of acetone and methanol. The samples were stored at low temperature and then centrifuged. The resulting pellet was resuspended in an appropriate IEF buffer [Gil-Agusti et al., 2005].

Summary: An efficient tissue disruption using liquid nitrogen assisted mortar and pestle followed by phenol-based extraction of the proteins has proven to be the best option to achieve a reproducible and adequate amount of proteins that can be used in the subsequent electrophoretic separation. If the samples are especially rich in lipids and pigments, an initial wash with organic solvents, such as TCA and acetone, prior to protein extraction with phenol, is recommended.

Table 1. Sample preparation

Species	Tissue	Tissue disruption			Homogenization buffer		Protein extraction		Resuspension/solubilization buffer										References
		N_2 assisted grinding		Other	Tris pH 8, reducing agent, protease inhibitor, EDTA, salt	Tris pH 8, SDS, salt, incubated at high °T	Aqueous/phenol two phase[1]	TCA/acetone, reducing agent	Chaotrope			Detergent				Reducing agent		Ampholytes		
			PVPP						Urea		Thiourea	CHAPS	ASB-14	SB3-10	OTHER	DTT	OTHER	pH 3-10	pH 4/5-7/8	
									7M	5M	2M									
Capsicum annuum	Placental tissue	X		X[2]					>[3]			X								Lee et al., 2006
Citrus reticulata	Juice sacs	X					?		>			X				X			X	Yun et al., 2010
Elaeagnus umbellata	Mesocarp	X			X		X		>		X	X			X[4]	X		X		Wu et al., 2011
Fragaria x ananassa	Whole fruit	X			X		X		>			X				X		X		Hjernø et al., 2006
Fragaria x ananassa	Whole fruit[5]	X		X[6]		X		X	X		X	X	X			X		X		Zheng et al., 2007
Fragaria x ananassa	Whole fruit	X	X	X[6]	X		X		X		X	X				X		X		Zheng et al., 2007
Fragaria x ananassa	Accrescent receptacle	X			X		X		>			X				X[7]		?[7]	?[7]	Bianco et al., 2009
Malus domestica	Pseudocarp	X			X		X		>			X				X		X		Guarino et al., 2007
Malus domestica	Peel	X		X[6]		X		X	X		X	X	X			X		X		Zheng et al., 2007
Malus domestica	Peel	X	X	X[6]	X		X		X		X	X				X		X		Zheng et al., 2007
Malus domestica	Pericarp[8]	X		X[6]	X			X		X	X	X		X		X	X[9]	X	X	Song et al., 2006
Malus domestica	Pericarp[8]	X		X[6]		X		X	X		X	X	X			X		X	X	Song et al., 2006
Musa spp	Meristem cultures	X						X	X		X	X				X		?	?	Carpentier et al., 2005
Musa spp	Meristem cultures	X			X		X		X		X	X				X		?	?	Carpentier et al., 2005
Musa spp	Mesocarp	X		X[6]	X			X		X	X	X		X		X	X[9]	X	X	Song et al., 2006
Musa spp	Mesocarp	X		X[6]		X		X	X		X	X	X			X		X	X	Song et al., 2006
Musa spp	Meristematic tissue	X			X		X		X		X	X				X		?	?	Carpentier et al., 2007
Persea americana	Exocarp	X	X					X	X		X	X				X				Barraclough et al., 2004
Prunus avium	Mesocarp	X			X[10]		X		X		X	X				X		X	X	Chan et al., 2008
Prunus persica	Mesocarp	X	X		X[11]		X[12]		X		X	X								Borsani et al., 2009
Prunus persica	Mesocarp	X			X[10]			X	X		X	X			X[13]	X		X	X	Chan et al., 2007
Prunus persica	Mesocarp	X			X		X		X		X	X	X			X	X[9]	X	X	Nilo et al., 2010
Prunus persica	Mesocarp	X			X		X[14]		X		X	X			X[13]	X		X		Prinsi et al., 2011
Prunus persica	Mesocarp				X		X[14]		X		X	X			X[13]	X		X		Prinsi et al., 2011
Prunus persica	Mesocarp	X	X			X	X		X		X	X				X			X	Hu et al., 2011
Prunus persica	Endocarp	X	X			X	X		X		X	X				X			X	Hu et al., 2011
Pyrus communis	Flesh[15]	X			X[16]		X		X		X	X				X				Pedreschi et al., 2007
Pyrus communis	Flesh	X			X		X		X		X	X								Pedreschi et al., 2009
Solanum lycopersicum	Pericarp	X	X	X[17]	X		X		X		X	X				X			X	Saravanan & Rose, 2004
Solanum lycopersicum	Pericarp	X	X	X[17]				X	X		X	X				X			X	Saravanan & Rose, 2004
Solanum lycopersicum	Pericarp	X	X	X[17]	X			X	X		X	X				X			X	Saravanan & Rose, 2004
Solanum lycopersicum	Fruit	X			X		X		>			X			X[4]	X		X		Rocco et al., 2006
Solanum lycopersicum	Pericarp	X			X		X		>			X			X[4]	X		X		Faurobert et al., 2007
Vitis vinifera	Berries[18]	X	X	X				X[19]	X		X	X			X[4]	X		X		Giribaldi et al., 2007
Vitis vinifera	Berries and stem	X		X[20]				X	>			X	X				X[21]	X	X	Vincent et al., 2006
Vitis vinifera	Berries and stem			X[20]				X[22]	X		X	X			X[4]	X		X	X	Vincent et al., 2006
Vitis vinifera	Berries and stem			X[20]	X		X			X	X	X				X		X	X	Vincent et al., 2006
Vitis vinifera	Pericarp	X	X	X[23]	X		X	X	X		X	X								Martínez-Esteso et al., 2011
Vitis vinifera	Mesocarp	X	X	X[24]	X		X	X	X		X	X								Martínez-Esteso et al., 2011
Vitis vinifera	Mesocarp			X[25]				X[22]	X		X	X			X[4]	X		X	X	Sarry et al., 2004
Vitis vinifera	Exocarp	X	X		X		X		X		X	X				X		?	?	Deytieux et al., 2007
Arachis hypogaea	Peanut pegs	X					X[26]	X	X[26]											Zhang et al., 2011

1. Aqueous/phenol two phase protein recovery followed by cold NH4-acetate dissolved in methanol precipitation and washes with the same solution and acetone.
2. Sonication.
3. > - higher values than the annotated were used.
4. Triton X-100.
5. Protein extracts obtained from this protocol were further purified using a 2-D Clean-Up Kit.
6. Stainless steel blender was used prior to mortar and pestle.
7. According to the manufacturer, samples cannot be solubilised in a buffer with any primary amines, such as ampholites and DTT, if they are to be labelled with DIGE CyDyes (Chakravarti et al., 2005).
8. Pericarp was reported, even though the succulent tissue from this fruit is denominated pseudocarp.
9. TCEP.
10. Triton X-100 was added.
11. SDS was added.
12. Final wash used cold ethanol.
13. NP-40.
14. The mixing sequence was inverted, see text.
15. Tissue samples were taken from the equatorial region excluding the skin and core.
16. Variant II is reported.
17. The mixture was homogenized at low temperature using a polytron PT 10/35 with an SM standard generator.
18. Berries were cut, deseeded and pulverized with a steel roll-on mechanical grinder half filled with liquid nitrogen.
19. Frozen powder was vortexed in Tris-HCl (pH 7.5) containing 2 M thiourea, 7 M urea, 2% Triton X-100, 1% DTT and 2% PVPP previous to TCA/acetone wash.
20. Stainless steel blender plus dry ice was used prior to mortar and pestle under liquid nitrogen.
21. HED.
22. Washed twice with ethanol.
23. Frozen pericarp were directly washed with ethyl acetate:ethanol at –20 °C with periodic vortexing, and the pellet recovered by centrifugation.
24. Mesocarp were homogenized in 50 mM Na2HPO2 pH 7.0, 1 mM EDTA, 0.1 M NaCl, PVPP, 1 mM Na2O5S2, 10 mM ascorbic acid, and a cocktail of protease inhibitors, filtered, centrifuged, and washed in 50 mM Na2HPO2 pH 7.0, 1 mM EDTA, 0.1 M NaCl, 10 mM ascorbic acid, and recovered by centrifugation.
25. Raw material was crushed in TCA/acetone.
26. Samples were washed with TCA/acetone, precipitated and finally with phenol plus DTT. For more details refer to Zhang et al., 2011.

3.2 Isoelectrical focusing

The initial step in the process of two dimensional gel electrophoresis first described by O'Farrell [O'Farrell, 1975] is based on the protein separation due to their intrinsic charge, in a process called isoelectric focusing. Even though this procedure is of foremost importance for the correct completion of the two-dimensional gel electrophoresis, many publications that deal with fruit tissues rely on protocols developed for animal tissues. Therefore, the

results are far from optimal and reflected in gels of poor quality and a low number of spots displayed, greatly undermining the capacity of this approach. A more exhaustive approach requires the appropriate selection of isoelectric focusing buffer used for the resuspension of samples. This result in a consistent way to improve the protein profiles detected in 2-DE gels.

Prior to IEF, proteins should be completely solubilised, disaggregated, denatured and reduced in order to resolve as many of the molecules as possible [Shaw and Riederer, 2003]. Under these conditions proteins are loaded onto an immobilized pH gradient strip and subject to increasingly higher field strengths, until they reach their isoelectric point (pI). However, at this moment, when their net charge is closest to zero, they have a tendency to aggregate and precipitate [Rabilloud & Lelong, 2011]. In order to overcome these constrains, methodological procedures have been optimized and a series of chemical reagents tested, leading to continuous improvements in IEF.

3.2.1 IEF solubilisation buffer

One of the main focuses to improve IEF has been the evaluation and introduction of novel chaotropes, detergents and reducing agents that could help in sample solubilisation. The presence of chaotropes, compounds that disrupt non-covalent interactions between the molecules present in the sample, are essential to render proteins disaggregated and denatured [Rabilloud et al., 1997; England & Haran, 2011]. However, the exposition of the hydrophobic patches, normally buried inside these molecules, to a hydrophilic environment increases the already strong tendency of proteins to precipitate [Molloy, 2000; Rabilloud & Lelong, 2011]. In order to avoid this phenomenon, surfactants are added to the solubilisation buffer. Due to their amphipathic nature, these molecules help in the protein dispersion both through the stabilization of the proteins hydrophobic patches as well as by interacting with ionic and hydrogen bonds of the molecules in solution. The disruption of intramolecular and intermolecular disulfide bonds for complete protein unfolding and linearity is also mandatory, not only at this stage, but also for proper molecular weight based separation in the SDS-PAGE gels [Molloy, 2000]. This can be accomplished with the use of reducing agents.

Two different chaotropes, both of which do not display a net electric charge in solution over the pH range used for IEF, are the most used in at this stage: urea and thiourea [Shaw and Riederer, 2003; Rabilloud, 2009]. The capacity of the latter to improve the protein solubilisation has prompted its wide use (Table 1). However, certain constraints to the composition of the IEF buffer have been imposed by its presence, since thiourea is only soluble in a water-based buffer when high concentrations of urea are added. In turn, the most efficient surfactants already tested are not compatible with these urea concentrations, limiting therefore the amount of thiourea that can be used to solubilize proteins [Rabilloud et al., 1997].

Among the detergents, the most frequently used is the 3-[(3-cholamidopropyl) dimethylammonio] propane sulfonate (CHAPS), a sulfobetaine-type switterionic surfactant. Its compatibility with high urea concentrations commonly used in 2-DE and superior efficiency compared to nonionic detergents have driven its use [Rabilloud et al., 1997; Molloy, 2000]. Other alternatives include amidosulfobetaine-14 (ASB14), Sulfobetaine 3-10 (SB 3-10), 4-n-Octylbenzoylamido-propyl-dimethylammoniosulfobetaine (C8Φ) and 3-(4-heptyl) phenyl 3-hydroxypropyl dimethylammonio propane sulfonate (C7BzO) [Molloy, 2000; Maserti et al., 2007]. In terms of disulfide reducing agents, thiol-reducing agents and

phosphines have gained widespread use in 2-DE, being dithiothreitol (DTT) the most often used. Since DTT is charged, especially at alkaline pH, during IEF it will migrate out of the gel, with a concomitant loss of solubility for some proteins and 2-DE horizontal streaking [Herbert, 1999; Molloy, 2000]. Therefore, its use in combination with other reducers or its substitution by compounds such as tributyl phosphine (TBP), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCP) and hydroxyethyl disulfide (HED) is advisable [Méchin et al., 2003; Sarma et al., 2008; Acín et al., 2009; Zhang et al., 2011]. Another advantage of using phosphines is the possibility of shortening the length of the equilibration step, therefore diminishing the loss of proteins at this point [Zuo & Speicher, 2000]. This can be accomplished by performing the reducing and alkylating procedures at the same time, since the phosphines such as TBP do not react with alkylating agents such as acrylamide and 2-vinylpyridine [Molloy, 2000].

Salt ions help stabilize proteins; therefore their absence may lead to protein precipitation. One way to overcome this situation is to add ampholytes to the IEF solution. These molecules enhance solubility of individual proteins as they approach their pI. They also buffer changes in conductivity, scavenge cyanate derived from urea, prevent interactions between hydrophobic proteins and IEF matrix and assist nucleic acids precipitation during centrifugation [Shaw and Riederer, 2003; Khoudoli et al., 2004; Gorg et al., 2009; Rabilloud & Lelong, 2011].

As expected, improvements in the composition of the IEF solubilisation buffer should help overcome some of the problems mentioned earlier in this chapter. For instance, the use of a reducing and an alkylating agent, TBP and 2-vinylpyridine, dissolved in a strong chaotrope such as guanidine hydrochloride to resuspend a dry fruit (e.g. peanut pegs) protein pellet obtained after TCA/acetone washes and phenol-based precipitation, have improved the spot number and resolution on 2-DE gels [Zhang et al., 2011]. Advances in solubilisation of acetone precipitated plant proteins have also been achieved by incremental changes in the concentration of Tris-base in the resuspension buffer, with a maximum effect obtained at 200 mM Tris-base. This result was probably due to the reduction in the protein-protein associations existing at this salt concentration, enhancing their release into the solution [Cho et al. 2010]. It is important to mention that a final dilution of the high salt IEF buffer was performed, in order to avoid a possible Joule heating during the focusing process [Wu et al., 2010; Rabilloud & Lelong, 2011].

Also interesting is the powerful result achieved with maize endosperms proteins when 2% of the surfactant SB 3-10, which is not compatible with high concentrations of urea, was combined with urea 5M, thiourea 2M, CHAPS 2%, DTT 20 mM, TCEP 5 mM, and two carrier ampholites (designated R2D2 by the authors). Compared to the more classical mixture of urea 7M, thiourea 2M, CHAPS 4%, DTT 25 mM and ampholytes, protein solubilisation and spot resolution were clearly enhanced [Méchin et al., 2003]. A similar improvement was observed when mesocarp derived *P. persica* 2-DE protein patterns were compared among samples resuspended in the R2D2 buffer and the T8 buffer evaluated by Méchin and co-authors (Nilo et al., 2011 – submitted).

3.2.2 Sample application

The sample application protocol has also demonstrated its relevance in improving the final 2-DE protein pattern. The now widely used immobilized pH gradients are supplied as a dehydrated gel matrix with plastic backing. Therefore, they have to be rehydrated before the IEF run, by "sample in-gel rehydration" or without the protein samples present in the rehydration solution by cup-loading or by paper-bridge loading. There are advantages and

disadvantages for each technique, mainly when working with hydrophobic or very high molecular weight proteins. Nonetheless, in some cases the use of one or the other method may be crucial, e.g. very alkaline proteins should be loaded by cup-loading, even though paper-bridge has been reported as a good alternative especially when samples are scarce and a broad pI range is to be assessed [Kane et al., 2006; Gorg et al., 2009]. The sample in-gel rehydration can be performed by a passive or active IPG strip rehydration. The latter option improves the entry of higher molecular weight proteins into the gel matrix [Gorg et al., 2009].
Recently, a novel strategy for sample loading, called G-electrode-loading method (GELM), has been introduced [Koga, 2008; Koga and Minohata, 2011]. This method allows a higher amount of protein to be loaded and therefore available for IEF. However, its performance has not been tested thus far with fruit derived proteins.

3.2.3 IEF running program improvement

The quality of the IEF is fundamental to achieve high quality 2-DE gels. However, most of the times an empirical assessment of the IEF program is performed until satisfactory results are achieved. This process can be time consuming and even be detrimental to the equipment being used, since the high heat generated by a sample that has not been properly desalted can burn the IEF machine plastic support where the samples are applied. For instance, salt interference is highly detrimental for 2-DE reproducibility, with concentrations lower than 10 mM recommended [Heppelmann et al., 2007]. Salt ions may affect IEF by slowing down its progression due to increased conductivity; producing artefacts and inducing protein modifications. Unfortunately, mandatory salt removal procedures will lead to sample loss and can result in the generation of a technical bias [Wu et al., 2010]. Therefore careful and reproducible procedures have to be implemented to deal with this kind of contamination.
One of the symptoms of salt contamination is the generation of a low voltage during the initial focusing of the IPG strip, which leads to suboptimal focusing. The presence of protein gaps and of streaking at the end of second dimension gels are also indicators of this problem [Heppelmann et al., 2007]. An estimation of salt conductivity, through the use of instruments such as portable conductivity meters, could help to confirm the presence or absence of salts as the source of these problems [Wu et al., 2010]. Additionally, it has been reported that IPGs washes, even when the focusing process has already commenced, could help to get remove salts and help to achieve adequate 2-DE results [Heppelmann et al., 2007].
In order to evaluate and compare results from different IEF runs the Volt hour (Vh) values should be recorded. The Vh reflects the total supplied energy to the system and should be optimized to produce the lowest value. The amount should be sufficient to reach a steady-state IEF, appropriate for protein focusing. This will depend on the sample, but also on the pH gradient, the IEF gel size and the amount of protein loaded (Table 2) [Gorg et al., 2009]. One way to avoid the cumbersome empirical evaluation of the IEF program for each new sample would be the use of a recently published algorithm, designed to predict the total Vh required for proper protein focusing during IEF [Wu et al., 2010].
Summary: IEF quality is fundamental in achieving high quality 2-DE gels. Besides the appropriate selection of IEF resuspension buffer components, some of which are almost standard nowadays (e.g. urea, thiourea, CHAPS, DTT), a careful evaluation of the sample application procedures and program settings required for reproducible IEF are crucial. High salt concentrations in the sample must be avoided. It is highly advisable that all of these points have been evaluated and optimized prior to running highly expensive experiments with scarce samples.

Species	Tissue	IEF Program			pI range	IEF gel size (cm)	# spots analyzed [1]	Image analysis	Spot visualization	References
		Pre-focusing step		Final kVh						
		Passive	Active							
Capsicum annuum	Placental tissue	?[2]	?	?	4-7; 4.5-5.5; 5.5-6.7; 6-9 L	?	1200; 600; 550; 200	Melanie IV	Coomassie	Lee et al., 2006
Citrus reticulata	Juice sacs	X		80	4-7 L	17	489	PDQuest	Coomassie	Yun et al., 2010
Elaeagnus umbellata	Mesocarp	?	?	?	4-7 L	?	1030	PDQuest	Silver	Wu et al., 2011
Fragaria x ananassa	Whole fruit	X		73	3-10 NL	24	1000	DeCyder	DIGE	Hjernø et al., 2006
Fragaria x ananassa	Whole fruit	X		30	3-11 L	18	956	PDQuest	SYPRO Ruby	Zheng et al., 2007
Fragaria x ananassa	Whole fruit	X		30	3-11 L	18	1368	PDQuest	SYPRO Ruby	Zheng et al., 2007
Fragaria x ananassa	Accrescent receptacle	X		27	3-10 (?)	18	622	Image Master 2D Platinum	DIGE	Bianco et al., 2009
Malus domestica	Pseudocarp	X		52	4-7 L	18	470	PDQuest	Coomassie	Guarino et al., 2007
Malus domestica	Peel	X		30	3-11 L	18	849	PDQuest	SYPRO Ruby	Zheng et al., 2007
Malus domestica	Peel	X		30	3-11 L	18	1422	PDQuest	SYPRO Ruby	Zheng et al., 2007
Malus domestica	Pericarp	X		30	3-11 NL	11	500	PDQuest	Silver	Song et al., 2006
Malus domestica	Pericarp	X		30	3-11 NL	11	500	PDQuest	Silver	Song et al., 2006
Musa spp	Meristem cultures	X		60	3-10 (?)	24	1348	Image Master 2D Platinum	Silver	Carpentier et al., 2005
Musa spp	Meristem cultures	X		60	3-10 (?)	24	1500	Image Master 2D Platinum	Silver	Carpentier et al., 2005
Musa spp	Mesocarp	X		30	3-11 NL	11	394	PDQuest	Silver	Song et al., 2006
Musa spp	Mesocarp	X		30	3-11 NL	11	394	PDQuest	Silver	Song et al., 2006
Musa spp	Meristematic tissue	X		55	4-7 L	24	1657	Image Master 2D Platinum	Coomassie	Carpentier et al., 2007
Persea americana	Exocarp			140	3-10 NL	18	?	ImageMaster 2D Elite software	SYPRO Ruby	Barraclough et al., 2004
Prunus avium	Mesocarp			9	3-10 (?)[3]	13	600	Image Master 2D Elite software	Coomassie	Chan et al., 2008
Prunus persica	Mesocarp		X	68	4-7 L	17	600	Image Master 2D Platinum	DIGE	Borsani et al., 2009
Prunus persica	Mesocarp			7	3-10 (?)[3]	13	?	Image Master 2D Elite software	Coomassie	Chan et al., 2007
Prunus persica	Mesocarp		X	70	3-10 NL	17	242	Delta 2D	DIGE	Nilo et al., 2010
Prunus persica	Mesocarp	X		90	3-10 NL	24	1128	Image Master 2D Platinum	Coomassie	Prinsi et al., 2011
Prunus persica	Mesocarp	X		90	3-10 NL	24	516	Image Master 2D Platinum	Coomassie	Prinsi et al., 2011
Prunus persica	Mesocarp		X	65	5-8 L	24	601	PDQuest	Coomassie	Hu et al., 2011
Prunus persica	Endocarp		X	65	5-8 L	24	714	PDQuest	Coomassie	Hu et al., 2011
Pyrus communis	Flesh	X		24	5-8 L[4]	24	800	Image Master 2D Platinum	Silver	Pedreschi et al., 2007
Pyrus communis	Flesh		X	91	4-7 L	24	?	Progenesis	DIGE	Pedreschi et al., 2009

Species	Tissue	IEF Program			pI range	IEF gel size (cm)	# spots analyzed [1]	Image analysis	Spot visualization	References
		Pre-focusing step		Final kVh						
		Passive	Active							
Solanum lycopersicum	Pericarp	X		100	4-7 L	17	679[5]	Progenesis	Coomassie	Saravanan & Rose, 2004
Solanum lycopersicum	Pericarp	X		100	4-7 L	17	679[5]	Progenesis	Coomassie	Saravanan & Rose, 2004
Solanum lycopersicum	Pericarp	X		100	4-7 L	17	679[5]	Progenesis	Coomassie	Saravanan & Rose, 2004
Solanum lycopersicum	Fruit	X		72	4-7 L	24	638	PDQuest	Coomassie	Rocco et al., 2006
Solanum lycopersicum	Pericarp	X		>64	4-7 L	24	1730	Image Master 2D Platinum	Silver	Faurobert et al., 2007
Vitis vinifera	Berries		X	105	3-10 NL	18	792	Image Master 2D Platinum	Coomassie	Giribaldi et al., 2007
Vitis vinifera	Berries and stem		X	85	3-10 NL	17	326	PDQuest	Coomassie	Vincent et al., 2006
Vitis vinifera	Berries and stem		X	85	3-10 NL	17	844	PDQuest	Coomassie	Vincent et al., 2006
Vitis vinifera	Berries and stem		X	85	3-10 NL	17	942	PDQuest	Coomassie	Vincent et al., 2006
Vitis vinifera	Pericarp	X		56	3-10 NL	18	921	Progenesis	DIGE	Martínez-Esteso et al., 2011
Vitis vinifera	Mesocarp	X		56	3-10 NL	18	804	Progenesis	DIGE	Martínez-Esteso et al., 2011
Vitis vinifera	Mesocarp		X	120	3-10 NL	?	270	PDQuest	Coomassie	Sarry et al., 2004
Vitis vinifera	Exocarp		X	64	3-10 NL	?	700	Image Master 2D Platinum	Coomassie	Deytieux et al., 2007
Arachis hypogaea	Peanut pegs	X		>80	3-10 ?	11	?	Dymension III	Silver	Zhang et al., 2011

Table 2. 2-DE Conditions

1. Maximum number evaluated.
2. Not determined.
3. Gels were polymerized in glass tubes: The IEF gel solution contained 10% NP-40, 30% w/v acrylamide, 9.5 M urea, 10% ammonium persulfate, and an equal mixture of 2% carrier ampholytes pH 3.5–10 and 5–8.
4. Other pI ranges were also reported.
5. A clear indication of the differences in the number of spots detected is not delivered.

3.3 Equilibration and SDS-page

After IEF, focused protein samples must be negatively charged with SDS to ensure exclusive molecular weight based separation during the second dimension. In parallel, proteins must be reduced and alkylated, a pre-requisite for keeping proteins unfolded during the SDS-PAGE step. This objective is accomplished in two main steps. First, the proteins are reduced by the action of DTT, and subsequently they are alkylated in the presence of iodoacetamide. Even though Gorg and colleagues have set the proper conditions for IPGs equilibration [Gorg et al., 2009], improvements can be achieved by speeding-up the process. These would allow a reduction in the levels of proteins lost during this step [Zuo & Speicher, 2000]. One way to achieve this task is to use vast excess of a high specific low molecular mass disulphide, which blocks the cysteines thiols [Olsson et al., 2002; Rabilloud, 2010]. Another option is to reduce and alkylate cysteine residue thiol groups prior to the IEF step, by using reagents such as TBP and 2-vinylpyridine [Zhang et al., 2011].

Regarding SDS-PAGE, some alternatives that may allow the strengthening of the fragile acrylamide-bis-acrylamide based matrix have been identified. However, their use has been

restricted due to problems associated with mass spectrometry (MS) incompatibility or by negatively affecting the electrophoresis itself. This has precluded the generation of large gels, which would have a much better resolution since this parameter is dependent on the surface area of the gel [Rabilloud, 2010]. An alternative to these large gels is to improve the area occupied by the spots during the second dimension in the conventional gels. This can be achieved through the generation of acrylamide gradients, which can encompass diverse ranges. Due to the difficulty in achieving reproducible gradient home-cast gels, their use is not widespread, with adoption by few authors whose work is summarized in Table 1 and 2 [Lee et al., 2006; Song et al., 2006; Nilo et al., 2010]. Finally, the 2-DE reproducibility heavily relies on this part of the process, with most of the noise and technical bias being generated at this stage [Choe and Lee, 2003; Lilley and Dupree, 2006]. Therefore, extreme care must be taken in order to avoid technical derived artefacts.

Summary: Equilibration is a well-defined and very important step of 2-DE, even though some improvements in the process can still be accomplished. Regarding SDS-PAGE, an increment in the gel resolution can be achieved through the use of acrylamide gradients. Due to the fact that SDS-PAGE is not a steady-state separation technique, an additional effort must be employed in order achieve highly standardized running conditions.

3.4 Protein visualization

After completion of SDS-PAGE, several alternatives are available for the detection of the protein spots present in this matrix. Some of them, such as Coomassie Brillant Blue (CBB) and its variant, colloidal Coomassie Brilliant Blue, as well as silver staining, are readily accessible and cost effective. Their use enables the detection of proteins in the sub-microgram range. However, silver has quite a poor linear dynamic range and proteins excised from gels stained by this means can be problematic to identify by MS [Patton, 2000].

Alternatives to these methods which are more sensitive (detection limit in the picogram range) as well as more reliable for protein quantitation, due to their linear dynamic range of at least three orders of magnitude, are the fluorescent dyes. Among the most sensitive are the Deep Purple (DP) and SYPRO Ruby (SR). Additionally, some of these fluorescent stains allow the detection of post-translational protein modifications on 2-DE gels, such as glycoproteins and phosphoproteins [Patton, 2000; Rieder 2008, Gauci et al., 2011].

Other factors that must be considered when choosing the visualization method are the inter-protein variability, ease of use, compatibility with subsequent MS analyses, among others [Gauci et al., 2011].

3.4.1 Difference gel electrophoresis – DIGE

Difference gel electrophoresis (DIGE) is a powerful tool for proteomics analysis. It provides the user with an internal standard control on each gel run, therefore strengthening the process of image comparison, which leads to more statistically robust results [Unlü et al., 1997; Lilley and Dupree, 2006]. Additionally, in the same way as other fluorophores, like DP and SR, allow the detection of protein amounts below the nanogram threshold [Patton, 2000; Gauci et al., 2011].

However, the use of this technology imposes several restrictions that may hamper its use when working with fruit samples. First, the protein concentration recommended by the manufacturers is of 5-10 mg/ml, which is not easy to achieve from fruit samples. Second, the sample pH needs to be adjusted to between 8.0 and 9.0. Fruit are characterized by

having highly acid components, which makes this adjustment a difficult task. In fact, since pH is adjusted with NaOH or Tris-HCl, it may lead to an increase of salts present in the sample, and therefore poor IEF [Tannu & Hemby, 2006; Wu et al., 2010]. Third, since the cye-dye labelling process must be performed at low temperatures, the solubility of compounds present at high molarity, such as thiourea and urea [Wahl et al., 2006], and also of detergents such as SB 3-10, will decrease. Since these molecules have proved very important to keep the proteins soluble for IEF, this situation may be detrimental in obtaining consistent 2-DE gels.

Summary: The use of fluorescent dyes to detect the presence of protein spots on 2-DE gels is advantageous both in terms of sensitivity, specificity and linearity. However, the requirement of high-cost equipment to excite and detect the fluorescence emitted by these molecules imposes some restrictions to their broad use. Some hurdles must also be addressed, concerning the use of the DIGE technology, to fully exploit its advantages for fruit proteome characterizations.

4. Literature evaluation – Fruit proteomics

A comprehensive search of the literature lead to the identification of 30 publications, produced in the last seven years, where 2-DE gels with fruit protein samples had been evaluated. Over 40% of the studies were performed by using well established plant fruit models such as tomato (*Solanum lycopersicum*), grape (*Vitis vinifera*) and peach (*Prunus persica*) (Figure 1A). The economical relevance of these species is also clear, with grapes been the most cultivated fruit plant throughout the world [Alexander & Grierson, 2002; Shulaev et al., 2008; Giribaldi & Giuffrida, 2010].

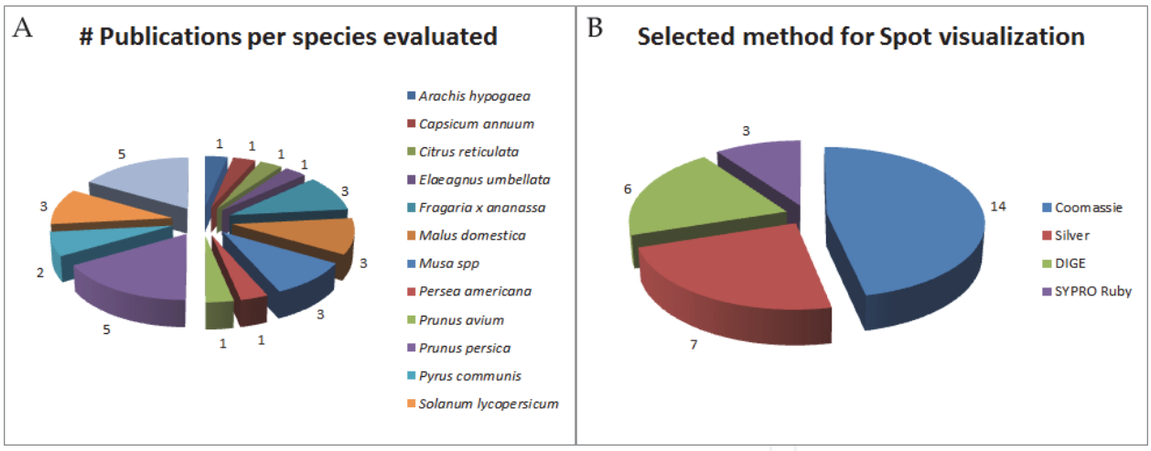

Fig. 1. General evaluation of the data displayed on Tables 1 and 2. The pie charge on **A** illustrates the species that have been assessed through a proteomic 2-DE gel based approach. Most of these evaluations have been performed using Coomassie based procedures (**B**).

One of the main goals of 2-DE is to maximise the numbers of detectable spots [Khoudoli et al., 2004]. Therefore, this criterion could be used to evaluate some of the parameters collected from the fruit proteomic literature (Tables 1 and 2), and used to discriminate which method would be the most relevant in order to achieve high quality 2-DE gels. However, there are a series of variables that may influence this parameter, as mentioned earlier. For

instance, it has been reported that the number of spots detected in a gel are largely dependent on the software package used [Stessl et al., 2009; Dowsey et al., 2010]. The progress in these programs as well as the report in the literature of other quality parameters associated with spot resolution, such as intensity and circularity, could help to improve this kind of evaluations.

Contrary to what one would expect from the previous statement, by far the most used procedure to detect spots on 2-DE gels is the least sensitive, that being coomassie staining (Table 2 and Figure 1B). This is probably due to the simplicity of the protocol and associated the low costs. However, Carpentier et al. was able to reach the highest level of spot detection in the literature (Table 2) by using the most sensitive version of this staining protocol, colloidal coomassie, with 24 cm gels and loading as much as 400 micrograms of protein per gel [Carpentier et al., 2007].

Another striking point is the broad inclusion of thiourea, CHAPS and also of Triton X-100 in the IEF buffer. The use of more powerful surfactants is less popular, possibly due to lack of information regarding the benefits of their use. A similar phenomenon can be observed regarding the gel size, which is still mainly limited to 17-18 cm (Table 2). Regarding the method of IPG sample in-gel rehydration, the passive mode was preferred over the active for most of the researchers.

5. Concluding remarks

Despite the enormous relevance of fruit for human nutrition and its usefulness as a powerful biological model to understand processes of great scientific interest, to date fruit from very few species have been assessed through the use of 2-DE technology. As described in this chapter, this may be due to the intrinsic complexity of the fruit samples, which hampers the adequate development of the 2-DE generating process if a minimal set of precautions are not followed. Fortunately, several of the cited publications have reached outstanding results, which foster the use of this powerful proteomic tool to dissect the fruit associated phenomena under evaluation. Regarding the protein extraction method, the use of phenol-based approaches has proved to be superior compared to the other alternatives published. The development of alternative non-toxic compounds, with similar efficiency to extract proteins, but less prone to solubilize phenols and lipids, would be of great importance.

It is interesting to stress that there are no discernable trends in the use of protein solubilisation cocktails (Table 1). Few publications have addressed this point using a systematic assessment, probably due to the enormous number of factors that would have to be confronted. In other systems, the Taguchi method, a statistical tool that allows the evaluation of a limited number of experiments that generates the most information, has been used to achieve this goal [Khoudoli et al., 2004; Rao et al., 2008]. Using solely animal tissues, Khoudoli and colleagues were able to improve 2-DE gel aspects such as resolution and reproducibility [Khoudoli et al., 2004]. To date no similar studies have been performed with fruit tissues, even though similar enhancements were achieved by others with maize endosperms when similar guidelines were followed, namely combinations of zwitterionic detergents and optimization of the concentration of carrier ampholytes [Méchin et al., 2003]. In parallel, the development of an algorithm to improve the IEF running protocol by estimating the optimal amount of Vh required for protein focusing [Wu et al., 2010], will also be of invaluable interest for those that are beginning to work with scarce, complex fruit derived samples.

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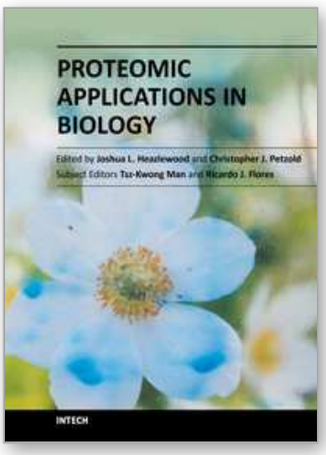

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The past decade has seen the field of proteomics expand from a highly technical endeavor to a widely utilized technique. The objective of this book is to highlight the ways in which proteomics is currently being employed to address issues in the biological sciences. Although there have been significant advances in techniques involving the utilization of proteomics in biology, fundamental approaches involving basic sample visualization and protein identification still represent the principle techniques used by the vast majority of researchers to solve problems in biology. The work presented in this book extends from overviews of proteomics in specific biological subject areas to novel studies that have employed a proteomics-based approach. Collectively they demonstrate the power of established and developing proteomic techniques to characterize complex biological systems.

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