



María Jesús Lerma-García¹
Maria Nicoletti²
Ernesto Francisco
Simó-Alfonso¹
Pier Giorgio Righetti²
Elisa Fasoli²

¹Department of Analytical
Chemistry, University of
Valencia, Valencia, Spain

²Department of Chemistry,
Materials and Chemical
Engineering “Giulio Natta”,
Politecnico di Milano, Milan,
Italy

Received July 25, 2018

Revised September 27, 2018

Accepted September 27, 2018

Research Article

Proteomic fingerprinting of apple fruit, juice, and cider via combinatorial peptide ligand libraries and MS analysis

Combinatorial peptide ligand libraries coupled to MS was applied to extensively map the proteome of apple fruit, and to detect its presence in commercial apple juice and cider to evaluate their authenticity and genuineness. Using the Uniprot.*Malus* database, 96 proteins were detected in apples, among which 30 proteins were specifically captured via combinatorial peptide ligand libraries. Next, three proteins, previously recognized in fruits, were found in apple juice, which were involved in cellular metabolism of fruit maturation and in allergenic reactions. On the other hand, only one *Malus* allergen was identified in cider beads eluate, demonstrating that the industrial processes did not prevent any negative effects in sensitive subjects. Thus, the present study not only increases the knowledge of the apple proteome but also offers a reliable analytical method to assess quality and genuineness of commercial products, which could be also used to inform consumers about the presence of allergens.

Keywords:

Apple allergens / Apple fruit juice and cider / Combinatorial peptide ligand library / Mass spectrometry / Proteomic fingerprinting

DOI 10.1002/elps.201800320



Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Apple (*Malus domestica* L. Borkh) is a pome fruit of the Rosacea family and one of the most widely planted tree fruits in the temperate zones [1]. The health benefits associated to apple consumption are due to nutritional components stored in the vacuole [2], defined as a multifunctional organelle occupying up to 90% of total mature cell volume, and storing nutrients and metabolites contributing to fruit quality [3]. In addition, the vacuole is very important during fruit ripening because both metabolism and accumulation of carbohydrates, organic acids and amino acids in apple are developmental stage-dependent in this organelle [4]. Also the maturation processes are due to biochemical dysfunction of vacuole which causes a dramatic effect on plant metabolism,

including ethylene biosynthesis, pigmentation, chlorophyll degradation, cell wall degradation, organic acid accumulation and production of volatiles resulting in changes in fruit traits such as color, texture, flavor and aroma [5].

Apples are considered as one of the healthiest foods: they are high in fiber, antioxidants, flavonoids and vitamin C, on the contrary they are low in calories with only a trace of sodium and no fat or cholesterol. The phytonutrients and antioxidants in apples may help to reduce the risk of developing cancer [6], neurological diseases [7], hypertension, diabetes, and cerebrovascular pathologies [8].

While previous studies on apple components were focused on polysaccharides able to affect cancer cell growth [9], many recent researches have reported their phytochemicals function as antioxidants with antiproliferative effects on cancer cells [10]. So, the increased consumption of apple could also be favored by demonstration of important health benefits such as cancer prevention [11].

Recent mapping of apple genome [12] has made possible the application of genome-wide transcriptomic and proteomic analysis techniques, able to explore the expression profiling in apple in order to understand fruit development and ripening [13]. In particular, proteomics is a useful tool to investigate the changes on protein composition during fruit development and maturation, improving the

Correspondence: Dr. Elisa Fasoli, Department of Chemistry, Materials and Chemical Engineering “Giulio Natta”, Politecnico di Milano, 20131 Milan, Italy.

Fax: +39-02-23993080

E-mail: elisa.fasoli@polimi.it

Abbreviations: CPLL, combinatorial peptide ligand library; **Fru**, fructose; **Sor**, sorbitol; **Suc**, sucrose; **TCA**, tricarboxylic acid cycle

knowledge on ripening process [14] and on defensive processes against pathogens [15]. Quantitative proteomics, such as MS-based stable isotopic labeling, should provide information on changes of protein levels with high accuracy: it is applied to understand the relationship between metabolites or proteins and apple fruit quality [16].

In this manuscript, a systemic proteomic study of *Malus domestica* L. Borkh tissues using combinatorial peptide ligand libraries (CPLLs) for reduction of dynamic range of the apple proteome, increasing the possibility to detect also trace species, is reported. After sample incubation with CPLLs, proteins were separated by SDS-PAGE and identified by nLC-MS/MS. This proteomic analysis could elucidate the specific roles of apple's proteins within cellular processes. Moreover, the recognition of apple's proteins in some commercial products, like apple juice and apple cider, should demonstrate the genuineness and the traceability of commercialized products, protecting consumers against any possible food adulteration or alteration. Finally, considering that Rosacea fruits are most often responsible for allergenic reactions [17], the last aim of this research was to detect well known allergens or correlated isoallergens both in fruit and in commercialized products for a deep evaluation of allergenicity in food and major information of consumers about possible negative reactions.

2 Materials and methods

2.1 Chemicals and samples

ProteoMiner™, Laemmli buffer, 40% acrylamide/bis solution, TEMED, molecular mass standards (Bio-Rad Laboratories, Hercules CA), β -mercaptoethanol, ammonium persulphate, CHAPS, DTT, iodoacetamide, ammonium bicarbonate (AmBic), ACN, TFA, SDS, formic acid (FA) and all other chemicals used were from pure analytical grade (Sigma-Aldrich, St Louis, MO). Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Roche Diagnostics, (Basel, CH).

The apples used in this work, which came from five different cultivars (Ambrosia; Fuji, Gala, Golden, and Stark), were purchased in a local supermarket. One biologic apple juice (NaturaSi, Verona, Italy) and one biologic apple cider (Baule Volante, Castel Maggiore, Italy) were also analyzed.

2.2 Protein extraction and CPLL treatment

Before apple protein extraction, the surface of apple fruits was washed with lukewarm 1% SDS solution, to eliminate bacterial and surface contamination from human hands. Next, apples were sliced and reduced to very minute fragments via a treatment in a fruit blender for 10 min at maximum power. To study the whole proteome of apple fruits, 75 g of minced fruit were mixed with 40 mL of an extraction buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM NaCl and 2% (m/v) CHAPS and gently shaken for 3 h at room

temperature. Protease inhibitor cocktails were also added to this buffer to prevent any proteolytic attack. Prior to capture with CPLLs, the homogenate was centrifuged at 18 000 rpm for 10 min to isolate the clarified protein solution from the insoluble residues. The obtained homogenate was next divided into two fractions, which were titrated at pH 7.2 using 1 M NaOH and at pH 2.2 by addition of FA and 0.1% TFA. Next, both aliquots were incubated with the CPLLs via gentle shaking overnight at room temperature. The adsorbed proteins were then desorbed with a solution containing 4% SDS and 20 mM DTT for 15 min, under boiling conditions [18]. Three technical replicas on two different biological samples were performed.

For detection of proteinaceous material in the apple juice and cider, two aliquots of 300 mL of juice/cider were added to 30 mL of an extraction buffer after centrifugation (18 000 rpm for 10 min), in order to eliminate insoluble materials. One of the aliquots was adjusted to pH 7.2 and the other one was titrated to pH 2.2 as previously described. The CPLL treatment was performed as previously indicated.

As control samples, 50 μ L of apple fruit aliquot and 2.4 mL of apple juice/cider aliquots were precipitated with MeOH/chloroform [18]. The obtained pellets were dissolved in 30 μ L of Laemmli buffer for SDS-PAGE [19].

2.3 SDS-PAGE, MS and data analysis

SDS-PAGE was performed in a Bio-Rad Mini-Protean system (Hercules, CA) according to previous works [18], with the only modification of the staining of the cider control sample, which was stained following the silver protocol [20], while all the other gels were stained with the Coomassie Blue.

SDS-PAGE samples bands were cut into thin slices and digested with trypsin [18] to be injected on a nano chromatographic system (UltiMate 3000 RSLCnano System, Thermo Scientific) coupled to a LTQ XL mass spectrometer (Thermo Scientific) using the same experimental conditions than in previous works [18]. The MS data were analyzed by Mascot search engine (Version 2.3.01), using Proteome Discoverer software (v. 1.2.0 Thermo) and consulting Uniprot *Viridiplantae* (937204 sequences, 321079394 residues) and *Malus* databases (3420 sequences, 1051115 residues). Oxidation of methionine residues was set as variable modifications; one missed cleavages were allowed to trypsin; peptide mass tolerance was set to 1 Da, fragment mass tolerance to 0.8 Da, ion source cut-off of 20 and significance threshold of $p < 0.01$. MS analysis was performed in technical duplicates.

3 Results

The SDS-PAGE profiles of apple extracts, characterized by control (untreated sample) and two eluates from CPLL beads after capture at pH 2.2 and 7.2 are depicted in Fig. 1. As shown in this figure, the CPLLs incubations at different pH values have contributed to detect higher protein content than

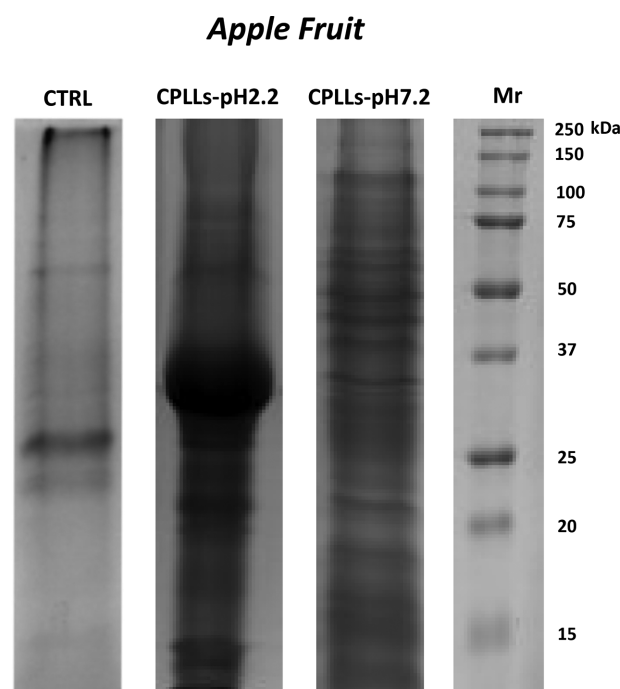


Figure 1. Coomassie Blue stained SDS–PAGE gels corresponding to protein extracts from apple fruit obtained for the control and the CPLL eluates at pH 2.2 and 7.2.

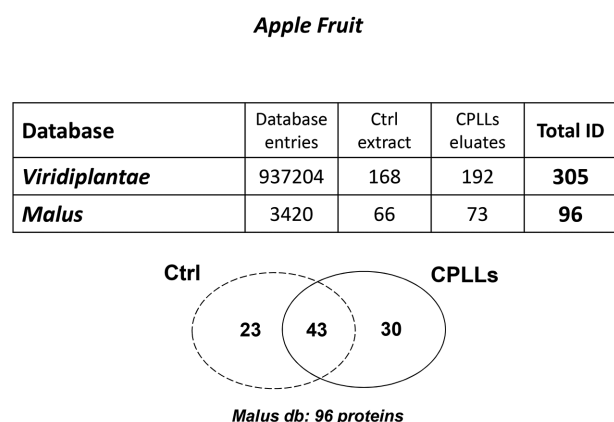


Figure 2. Total number of proteins identified in control and CPLL eluates of apple fruit using both Uniprot.*Viridiplantae* and Uniprot.*Malus* databases. Venn diagram of total proteins identified in apple fruit using *Malus* database: untreated sample [Ctrl] and in the two combined CPLL eluates at pH 2.2 and 7.2.

the untreated extract, thus increasing the number of resolved bands. In addition, the two pH values were characterized by different profiles, concerning both number and intensity of proteins bands.

Although the SDS–PAGE profiles were different, the numbers of identified proteins in both control and CPLLs samples were quite similar as reported in table of Fig. 2. The explanation was the different experimental conditions. In fact, while proteins in control were extracted in native buffers, CPLLs eluates contained SDS and DTT. Consequently, in

initial extracts proteins maintained their native structures with presence of physiological dimers/trimers, showing a simple profile in electrophoresis. On the contrary, in CPLLs eluates, proteins were completely denatured, destroying any aggregates and losing their 3D structure. For this reason, the corresponding SDS–PAGE profiles were enriched in protein bands referred to released monomers and denatured aminoacid sequence. Notwithstanding the increase of protein bands, the number of identified proteins was quite similar in the control extract and in CPLLs eluates because while in denatured conditions one protein could be resolved in more than one band, in native conditions the same is characterized by a specific band. Despite this, CPLLs treatment has contributed to identify unique gene products undetectable in the control, probably for their low abundance.

The number of total identifications are reported in table of Fig. 2, divided into two different databases, Uniprot.*Viridiplantae* and Uniprot.*Malus*. In detail, a total of 305 non-redundant proteins were identified in the apple extracts using Uniprot.*Viridiplantae* db, while 96 were identified using Uniprot.*Malus* db. Considering the interest in exploitation of apple's proteins, a Venn diagram (reported in Fig. 2) was constructed to compare *Malus* unique gene products, identified in control and in the CPLL eluates. In this latter, 23 proteins were only found in untreated sample and 30 in CPLL eluates, while 43 were commonly recognized in both untreated sample and CPLL eluates. Finally, the biological role of *Malus* identified proteins (96) was evaluated by a gene ontology analysis, performed by using the web available software QuickGO (www.ebi.ac.uk/QuickGO) (see Supporting Information Fig. 1). The three main molecular functions were represented by catalytic (22.94%) and hydrolase (18.82%) activities, and metabolic processes (17.65%).

In commercialized products derived from apples, such as juice and cider, the conservation of proteome and the presence of allergens were next investigated. As shown in the SDS–PAGE profiles of apple juice extracts in Fig. 3A, the CPLL eluates were characterized by a higher number of bands than untreated sample. The qualitative data of electrophoresis was confirmed by MS analysis (see Supporting Information Table 2): a total of 32 proteins were identified in apple juice using Uniprot.*Viridiplantae* database. Only eight proteins were found in untreated sample and 22 in CPLL eluates, while two proteins were commonly recognized in both untreated sample and CPLL eluates (see Venn diagram of Fig. 3A). Finally, the SDS–PAGE profiles of apple cider extracts are shown in Fig. 3B: despite the high sensitivity of silver staining, no protein band has been detected in the untreated cider. On the contrary, after CPLLs treatment, faint and smeared protein bands were stained by Coomassie Blue solution and the presence of trace proteins after industrial process was confirmed by MS. In fact, MS analysis has identified 2 and 1 proteins belonging to Uniprot.*Viridiplantae* and Uniprot.*Malus* databases, respectively. In the case of commercialized products the CPLLs treatment has been shown to be effective in the detection of proteins derived from natural ingredients.

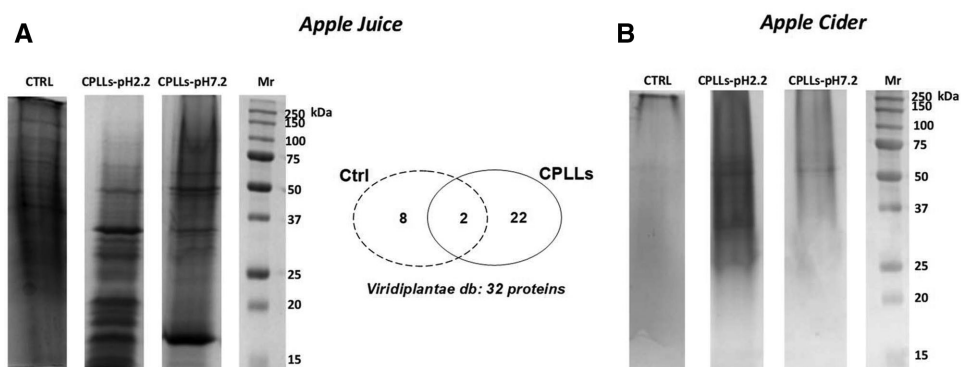


Figure 3. Coomassie Blue stained SDS-PAGE gels corresponding to protein extracts from apple juice (A) and cider (B) obtained for the control (Ctrl) and the CPLL eluates at pH 2.2 and 7.2. Control cider eluate was silver stained. Venn diagram corresponding to the total proteins identified in apple juice using Uniprot_Malus database.

4 Discussion

The increasing interest in fruit proteomics is to improve the knowledge on biological functions of nutritional and health-related proteins, such as a more detailed and comprehensive characterization of allergens [21]. For a deep investigation of apple proteome, the first requirement was to apply an analytical technology able to normalize samples by reducing the signals of high abundance species, which normally hide the trace ones, thus preventing their detection. Considering previous proteomic researches on fruits [22], apple's extracts have been treated with CPLs at two different pH values (2.2 and 7.2) in order to see if the CPLs treatment was able to decrease the concentration range of proteins. The SDS-PAGE profile obtained at pH 2.2 (see Fig. 1) was mainly characterized by an intense band at 37 kDa, which could be attributed to high abundance proteins, while the eluate at pH 7.2 has largely reduced the concentration of these proteins, enhancing additional bands around the same molecular mass value. The CPLs reduction of dynamic range has made possible to identify a higher number of proteins exceeding the detection limit of MS analysis. In fact, more than 45% and 31% were specifically recognized in beads eluates using Uniprot_Viridiplatae and Uniprot_Malus databases, respectively. Although the number of total proteins found by using Uniprot_Malus database was threefold lower than the corresponding one obtained with Uniprot_Viridiplatae database (96 vs. 305) (see Fig. 2 and Supporting Information Table 1), the Uniprot_Malus database is more specific, allowing a deep exploitation of apple proteome and a larger investigation of molecular functions, required to understand the role of proteins in cellular processes. While many proteomic researches have recently provided information on biological processes, such as fruit maturation, ripening and senescence [5], the relationship between proteome and molecular pathways is not completely clear. For this reason, this investigation has aimed to understand the role of apple proteins in complex biochemical mechanisms present during physiological maturation/growing. Therefore, based on the MS identifications made by the Uniprot_Malus database, protein networks were studied using gene ontology annotation terms and a first molecular function was investigated (see Supporting Information Fig. 1). This study has revealed high percentages of

catalytic and hydrolase activities, demonstrating the involvement in metabolic processes like carbohydrates metabolism via glycolysis and tricarboxylic acid cycle (TCA). In fact, at early stages of apple development, carbohydrates are rapidly metabolized for fruit growth: the sugar quantity is very low while organic acid and amino acid levels are high. During apple maturation, carbohydrate metabolism slows down because fructose (Fru) and sucrose (Suc) are accumulated and the excess is converted in starch for storage. At late stage of apple development, starch breaks down, Suc continues to be accumulated and the levels of organic acids decrease, elevating apple sweetness to a maximum at maturity. Apple, like other fruits of *Rosaceae*, is able to synthesize both sorbitol (Sor) and Suc in leaves and to convert them into Fru in fruit. Sixty to eighty percent of Sor is produced in apple leaves [23] and almost all Sor and half of Suc are converted to Fru, estimating that 80% of total carbon flux is due to Fru production in fruit [4]. Many MS identifications (listed in Supporting Information Table 1 using Uniprot_Malus database) are proteins involved in sugar cycle and transport: Sorbitol-6-phosphate dehydrogenase (A0A0S1TQH9.MALDO) converts Sor after being taken up into the cytosol of parenchyma cells, into Fru imported in vacuole for accumulation [24]. The balance between sugar and organic acids, due to both glycolysis and TCA cycle, is largely responsible for apple flavor: the most predominant organic acids are malic acid and citric acid, which decrease their abundance during maturation process [25]. Despite the low abundance, CPLs were able to capture Glyceraldehyde-3-phosphate dehydrogenase (X5D281.MALBA) and Aldehyde dehydrogenase (AL7A1.MALDO), involved in glycolysis, and Malate dehydrogenase (A3DSX0.MALDO), an enzyme present in TCA cycle [26]. A malic enzyme (A0A023IRK2.MALDO), involved in both malate synthesis and degradation, was also identified in bead eluates. Like sugars, malate and citrate are stored in vacuole via vacuolar malate transporters [27]. Despite any absence of ripening effects on treated apples, MS analysis has identified some proteins involved in oxidation-reduction processes and expressed as response to oxidative stress: Superoxide dismutase (Q6DTU3.MALBX), Polyphenol oxidase (I1U4K3.MALDO) and Glutathione peroxidase (Q6A4W8.MALDO), demonstrating the presence of antioxidant-redox processes as a defense response during

apple maturity [28]. Finally, in both control and CPLLs samples, Ubiquitin (Q84XC3.MALDO) and Polyubiquitin (B2LUR5.MALDO) were recognized, indicating a possible protein degradation due to apple maturation or a possible beginning of ripening mechanism.

Another interesting consideration based on MS data was the investigation on allergens identification because apple, like *Rosacea* fruits, is most often responsible for allergic reactions in large parts of Europe and Northern America [17]. Apple allergy manifests various clinical symptoms mediated by an IgE-response, such as respiratory, cutaneous and gastrointestinal disturbances to systemic anaphylaxis [29]. The principal apple allergens, identified so far, are: Mal d 1, a pathogenesis-related protein (PR-10) [30–33], Mal d 2, a thaumatin-like protein (TLP) [33, 34], Mal d 3, a lipid transfer protein (LTP) [33, 35] and Mal d 4, a profiling protein [33, 36]. All mentioned allergic proteins were found in the apple samples used in this work, both before and after CPLLs treatment, confirming their moderate abundance directly detectable via MS analysis. Mal d 1, Bet v 1 family member, is defined the major apple allergen and its amount depends on storage conditions like Mal d 3 whose level, dependent on cultivar and tree position, decrease during storage [37]. Recent studies have demonstrated that food processing conditions and food preservatives may influence apple allergenicity [38]; for this reason, the conservation of proteome and the presence of allergens in commercialized products derived from apples like juice and cider has been investigated.

Considering apple juice, the CPLLs treatment has increased by 70% the protein discovery using Uniprot_Viridiplantae database as shown in Venn Diagram of Fig. 3A. Focusing on Uniprot_Malus database, the MS analysis has not identified any protein with more than one peptide in juice control, while eight proteins were identified after CPLL treatment (see Supplementary Table 2). These proteins were the same ones previously identified in apple fruit: Glyceraldehyde-3-phosphate dehydrogenase (X5D281.MALBA) and Non-specific lipid-transfer protein (Q5GLH0.MALDO). The first is an enzyme involved in TCA cycle up-regulated during fruit maturation for high degradation of organic acids. Ethylene receptor is overexpressed during fruit ripening due to a significant burst of respiration and ethylene production [39]. Furthermore, the other identified protein is one of major and well-known allergen present in fruits: the nsLTP or Mal d 3, which increases its level at the end of maturity when apples are suitable for consumption. Previous studies have identified two genes encoding mature nsLTP [40], whose expression has been demonstrated to be independent of each other [41]. Moreover, the expression of nsLTPs is mediated by a complex mixture of genetic and environmental stimuli, like for example ethylene the major ripening-hormone regulator in apple [37]. Although the levels of Mal d 3 are significantly lower in juice than in fresh fruits, this beverage could trigger an allergic reaction in sensitized individuals. Moreover the same protein was the only one captured by CPLLs and identified via MS analysis also in cider: our study has firstly identified this unique gene

product belonging to *Malus* taxonomy in cider, while previous researches have used capillary electrophoresis to separate and to purify cider proteins, finally confirmed through SDS-PAGE gel [42].

Finally the detection of proteins, specific of apple fruit, could be useful to assess the authenticity of commercial products, like apple juice and cider, ensuring beverages genuineness and protecting consumer's health from adulteration or negative effects.

5 Concluding remarks

In this work, it has been demonstrated that the CPLLs methodology is a reliable analytical tool to enhance signals of trace components, finally identified by MS analysis in apple fruits and in derived commercialized beverages, like juice and cider. The principal aim was to discover apple proteome profiling as a useful “fingerprinting” to evaluate apple's nutritional properties related to fruit's maturation and/or alteration. Ninety-six proteins belonging to Uniprot_Malus database were identified in apple fruits and 30 were specifically captured by CPLLs. After investigation of their biological functions, focused on understanding their role in fruit maturation, in nutritional benefits and in development of adverse reactions like allergies, the next purpose was to confirm the presence of such proteomic signature also in commercialized beverages to assess their authenticity and genuineness. In the chosen apple juice, three CPLLs captured proteins, identified with Uniprot_Malus database, were previously recognized in fruit and they were involved in cellular metabolism of fruit maturation and in allergenic reactions. Finally only one *Malus* allergen (mal d 3) was identified in cider beads eluate, demonstrating that industrial processes did not prevent any negative effects in sensitive subjects. In conclusion, we have tried to combine reliable analytical tools to assess food safety and traceability, preventing any adulteration or alteration during production and storage, and to defend consumers, informing them about the presence of allergens.

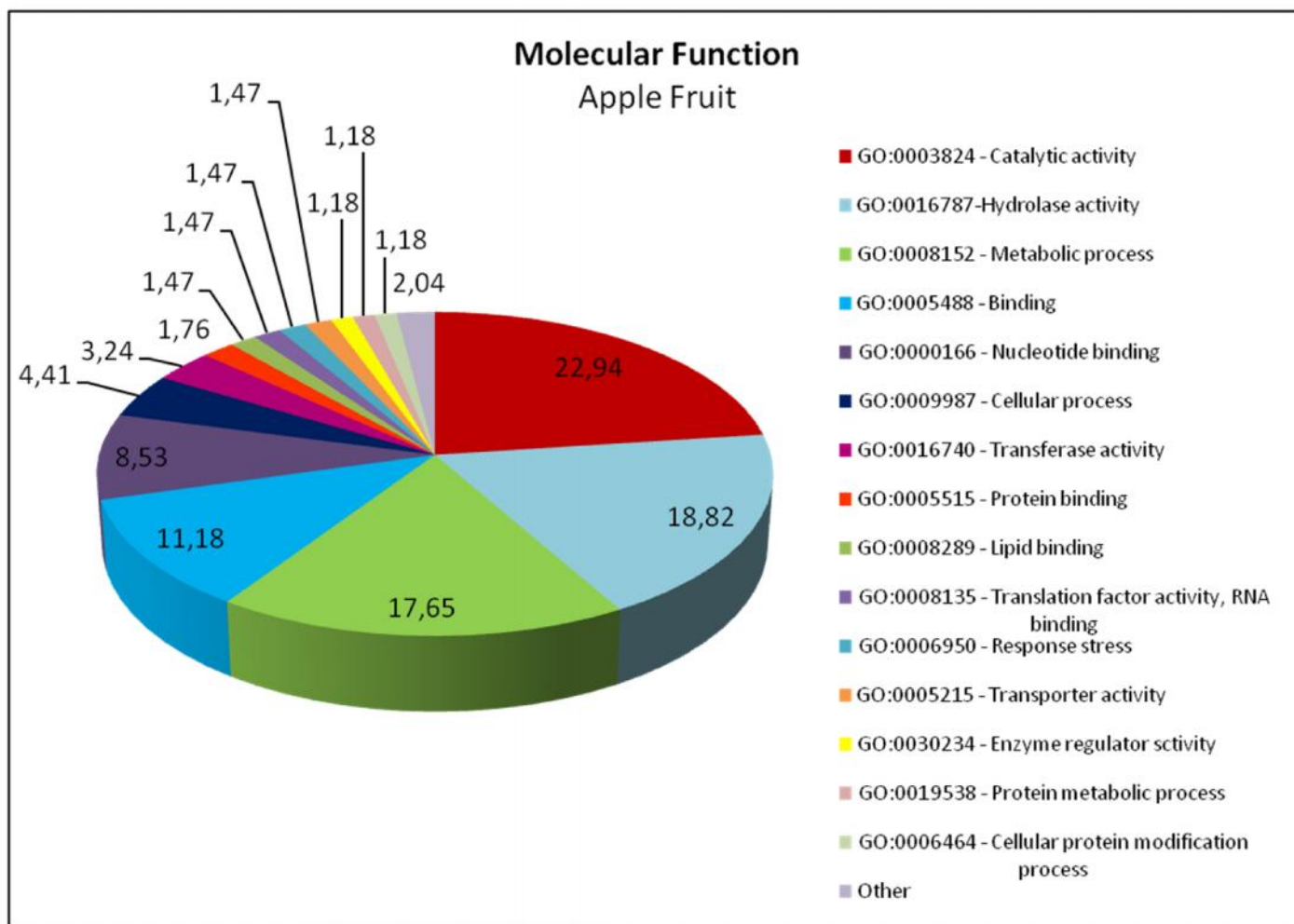
M.J. Lerma-García thanks the Generalitat Valenciana for a VALi+d postdoctoral contract.

The authors have declared no conflict of interest.

6 References

- [1] Eccher, G., Ferrero, S., Populin, F., Colombo, L., Botton, A., *Plant Biosyst.* 2014, 148, 157–168.
- [2] Khan, A. M., Olsen, K. M., Sovero, V., Kushad, M. M., Korban, S. S., *Plant Genome* 2014, 7, 1–18.
- [3] Zhang, C., Hicks, G. R., Raikhel, N. V., *Front. Plant Sci.* 2014, 5, 476.
- [4] Li, M., Feng, F., Cheng, L., *Plos One* 2012, 7, e33055.
- [5] Palma, J. M., Corpas, F. J., del Río, L. A., *J. Proteomics* 2011, 74, 1230–1243.

- [6] Adom, K. K., Liu, R. H., *J. Agric. Food Chem.* 2005, **53**, 6572–6580.
- [7] Heo, H. J., Choi, S. J., Choi, S. G., Shin, D. H., Lee, J. M., Lee, C. Y., *J. Food Sci.* 2008, **73**, H28–32.
- [8] Knekt, P., Isotupa, S., Rissanen, H., Heliövaara, M., Järvinen, R., Häkkinen, S., Aromaa, A., Reunanen, A., *Eur. J. Clin. Nutr.* 2000, **54**, 415–417.
- [9] Zhang, D., Li, Y. H., Mi, M., Jiang, F. L., Yue, Z. G., Sun, Y., Fan, L., Meng, J., Zhang, X., Liu, L., Mei, Q. B., *Nutr. Res.* 2013, **33**, 839–848.
- [10] Yang, S., Zhang, H., Yang, X., Zhu, Y., Zhang, M., *Food Funct.* 2015, **6**, 3789–3798.
- [11] Lin, S.-T., Tu, S.-H., Yang, P.-S., Hsu S.-P., Lee, W.-H., Ho, C.-T., Wu, C.-H., Lai, Y.-H., Chen, M.-Y., Chen L.-C., *J. Agric. Food Chem.* 2016, **64**, 6826–6837.
- [12] Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., Fontana, P., Bhatnagar, S. K., Troggio, M., Pruss D., Salvi, S., Pindo, M., Baldi, P., Castelletti, S., Cavauiolo, M., Coppola, G., Costa, F., Cova, V., Dal Ri, A., Goremykin, V., Komjanc, M., Longhi, S., Magnago, P., Malacarne, G., Malnoy, M., Micheletti, D., Moretto, M., Perazzolli, M., Si-Ammour, A., Vezzulli, S., Zini, E., Eldredge, G., Fitzgerald, L. M., Gutin, N., Lanchbury, J., Macalma, T., Mitchell, J. T., Reid, J., Wardell, B., Kodira, C., Chen, Z., Desany, B., Niazi, F., Palmer, M., Koepke, T., Jiwan, D., Schaeffer, S., Krishnan, V., Wu, C., Chu, V. T., King, S. T., Vick, J., Tao, Q., Mraz, A., Stormo, A., Stormo, K., Bogden, R., Ederle, D., Stella, A., Vecchietti, A., Kater, M. M., Masiero, S., Lasserre, P., Lespinasse, Y., Allan, A. C., Bus, V., Chagné, D., Crowhurst, R. N., Gleave, A. P., Lavezzo, E., Fawcett, J. A., Proost, S., Rouzé, P., Sterck, L., Toppo, S., Lazzari, B., Hellens, R. P., Durel, C. E., Gutin, A., Bumgarner, R. E., Gardiner, S. E., Skolnick, M., Egholm, M., Van de Peer, Y., Salamini, F., Viola, R., *Nat. Genet.* 2010, **42**, 833–839.
- [13] Segonne, S. M., Bruneau, M., Celton, J. M., Le Gall, S., Francin-Allami, M., Juchaux, M., Laurens, F., Orsel, M., Renou, J. P., *BMC Plant Biol.* 2014, **14**, 375–393.
- [14] Shi, Y., Jiang, L., Zhang, L., Kang, R., Yu, Z., *Hortic. Res.* 2014, **1**, 6.
- [15] Buron-Moles, G., Wisniewski, M., Viñas, I., Teixidó, N., Usall, J., Droby, S., Torres, R., *J. Proteomics* 2015, **114**, 136–151.
- [16] Li, M., Li, D., Feng, F., Zhang, S., Ma, F., Cheng, L., *J. Exp. Bot.* 2016, **67**, 5145–5157.
- [17] Cuesta-Herranz, J., Lázaro, M., Figueredo, E., Igea, J. M., Umpiérrez, A., De-Las-Heras, M., *Clin. Exp. Allergy* 2000, **30**, 1411–1416.
- [18] Vergara-Barberán, M., Lerma-García, M. J., Nicoletti, M., Simó-Alfonso, E. F., Herrero-Martínez, J. M., Fasoli, E., Righetti, P. G., *J. Proteomics* 2017, **164**, 52–58.
- [19] Candiano G., Dimuccio V., Bruschi M., Santucci L., Gusmano R., Boschetti E., Ghiggeri, G. M., *Electrophoresis* 2009, **30**, 2405–2411.
- [20] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., *Anal. Chem.* 1996, **68**, 850–858.
- [21] Li, L., Luo, Z., Huang, X., Zhang, L., Zhao, P., Ma, H., Li, X., Ban, Z., Liu, X., *J. Proteomics* 2015, **120**, 44–57.
- [22] Lerma-García, M. J., D'Amato, A., Simó-Alfonso, E. F., Righetti, P. G., Fasoli, E., *Food Chem.* 2016, **196**, 739–49.
- [23] Cheng, L., Zhou, R., Reidel, E. J., Sharkey, T. D., Dandekar, A. M., *Planta* 2005, **220**, 767–776.
- [24] Wei, X., Liu, F., Chen, C., Ma, F., Li, M., *Front. Plant Sci.* 2014, **5**, 569.
- [25] Pratelli, R., Pilot, G., *J. Exp. Bot.* 2014, **65**, 5535–5556.
- [26] Yao Y. X., Li, M., Zhai, H., You, C. X., Hao, Y. J., *J. Plant Physiol.* 2011, **168**, 474–480.
- [27] Khan, S. A., Beekwilder, J., Schaart, J. G., Mumm, R., Soriano, J. M., Jacobsen, E., Schouten, H. J., *Tree Genet. Genomes* 2013, **9**, 475–487.
- [28] Torres, R., Valentines, M. C., Usall, J., Viñas, I., Larigaudière, C., *Postharvest Biol. Technol.* 2003, **27**, 235–242.
- [29] Fernández-Rivas, M., Bolhaar, S., González-Mancebo, E., Asero, R., van Leeuwen, A., Bohle, B., Ma, Y., Ebner, C., Rigby, N., Sancho, A. I., Miles, S., Zuidmeer, L., Knulst, A., Breiteneder, H., Mills, C., Hoffmann-Sommergruber, K., van Ree, R., *J. Allergy Clin. Immunol.* 2006, **118**, 481–488.
- [30] Fernandes, H., Michalska, K., Sikorski, M., Jaskolski, M., *FEBS J.* 2013, **280**, 1169–1199.
- [31] Ahammer, L., Grutsch, S., Tollinger, M., *Biomol. NMR assign.* 2016, **10**, 287–290.
- [32] Ahammer, L., Grutsch, S., Kamenik, A. S., Liedl, K. R., Tollinger, M., *J. Agric. Food Chem.* 2017, **65**, 1606–1612.
- [33] Herndl, A., Marzban, G., Kolarich, D., Hahn, R., Boscia, D., Hemmer, W., Maghuly, F., Stoyanova, E., Katinger, H., Laimer, M., *Electrophoresis* 2007, **28**, 437–48.
- [34] Marzban, G., Herndl, A., Pietrozotto, S., Banerjee, S., Obinger, C., Maghuly, F., Hahn, R., Boscia, D., Katinger, H., Laimer, M., *Food Chem.* 2009, **112**, 803–811.
- [35] Gómez, F., Aranda, A., Campo, P., Díaz-Perales, A., Blanca-López, N., Perkins, J., Garrido, M., Blanca, M., Mayorga, C., Torres, M. J., *Plos One* 2014, **9**, e107304.
- [36] Ma, Y., Zuidmeer, L., Bohle, B., Bolhaar, S. T. H., Gadermaier, G., Gonzalez-Mancebo, E., Fernandez-Rivas, M., Knulst, A. C., Himly, M., Asero, R., Ebner, C., Van Ree, R., Ferreira, F., Breiteneder, H., Hoffmann-Sommergruber, K., *Clin. Exp. Allergy* 2006, **36**, 1087–1096.
- [37] Sancho, A. I., Foxall, R., Rigby N. M., Browne, T., Zuidmeer, L., Van Ree, R., Waldron, K. W., Clare Mills, E. N., *J. Agric. Food Chem.* 2006, **54**, 5098–5104.
- [38] Marzban, G., Kinaciyan, T., Maghuly, F., Brunner, R., Gruber, C., Hahn, R., Jensen-Jarolim, E., Laimer, M., *J. Agric. Food Chem.* 2014, **62**, 7622–7630.
- [39] Giovannoni, J. J., *Plant Cell* 2004, **16**, S170–S180.
- [40] Pastorello, E. A., D'Ambrosio, F. P., Pravettoni, V., Farioli, L., Giuffrida, G., Monza, M., Ansaloni, R., Fortunato, D., Scibola, E., Rivolta, F., Incorvaia, C., Bengtsson, A., Conti, A., Ortolani, C., *J. Allergy Clin. Immunol.* 2000, **105**, 371–377.
- [41] Gao, Z. S., van de Weg, W. E., Schaart, J. G., van der Meer, I. M., Kodde, L., Laimer, M., Breiteneder, H., Hoffmann-Sommergruber, K., Gilissen, L. J., *Theor. Appl. Genet.* 2005, **110**, 479–491.
- [42] Blanco-Gomis, D., Mangas-Alonso, J. J., Junco-Corujedo, S., Gutiérrez-Álvarez, M. D., *J. Agric. Food Chem.* 2007, **55**, 2526–2531.



Other: DNA binding; Kinase activity; Structural molecule activity; RNA binding; Nucleic acid binding

Jerma-García et al. - Supplementary Fig. 1