Develonutri Year 3 report

Work performed and main achievements in the period including contractors involved (summary)

The third reporting period has proved the most challenging of all the reporting periods with the demonstration activities coming to fruition and the advances and protocols developed as part of the 1st and 2nd periods being applied in a real food scenario. In addition, the translation of this research to a the wider community has been well served by publications, presentations and meetings with the stakeholder community

WP1 – The focus of the WP has been the application of the technologies tested and standardised during the reporting periods 1 and 2 to the outputs of WP4: real foods and raw crop materials. The crops potato, tomato, and durum and bread wheat were analysed using the standard and high throughput technologies for metabolite/(micro)nutrient biodiversity, and the implications for nutritional variation, the impact of growing location and the impact of agricultural regimes (conventional /organic). It was shown that even within the relatively limited germplasm analysed here there was nutrient diversity evident and that these growing location was a major influence on the nutritional content. In addition at the specific component level, e.g. carotenoids, as with respect to the metabolome per se the technologies were generally able to distinguish between the same germplasm grown in conventional and organic systems and the driving factors were identified. This analytical approach was extended to the associated production lines and food products and identified differences in specific and global nutritional changes and, in the case of potato processing, indentified stages where nutritional variation occurred.

WP2 - In WP2, the advances made in the second year with respect to AMDIS development were progressed very significantly. A new database (DEVELONUTRI REFERENCE BOOK; Annex 1 tomato) based on the Adobe Acrobat platform was designed and implemented to hold the libraries and tabular and graphical data from consortium members and facilitate data comparisons. The REFERENCE BOOK will also be web based. The naming system for known and unknown compounds was developed with contributions from all partners. Mass spectral libraries in AMDIS and NIST formats were curated and included in the REFERENCE BOOK. The suitability of multi-platform AMDIS software was evaluated for LC/MS analysis (quadrupole and ion trap mass spectrometers) and recommendations made to NIST concerning software modification. During demonstration activities carried out in WP4 the composition of the databases present in the prototype DEVELONUTRI were validated and components checked with authentic standards (P2). A modification to AMDIS (C++ code) to enable data array output for quantitative/metabolomic analysis has been provided to NIST for incorporation into a future release of AMDIS software. A publication is being prepared (with NIST) on quantitative aspects of AMDIS using Develonutri data sets

WP3 – The emergent technologies focussed upon in this final period were a mix of very high end technology (MALDI-ToF/ToF-MS, FT-MS etc) and the more establish technology of FT-IR, the latter of which, although in most analytical laboratories, is not used in a metabolomic nutritional assessment scenario. The trialling of the MS technologies identified that once beyond the analytical LC-MS systems, such as the Thermo Orbitrap, then the MALDI- and FT-MS systems suffered problems predominantly associated with quantification and that these technologies are best used in a qualitative metabolite identification approach. The LC-FT-MS system appeared on paper to be the best analytical system available for metabolite/nutrient analysis but in practise the technology

was let down by its poor MS scan rate which meant that, although it yielded give very detailed structural information via an impressible accurate mass resolution, it could not resolve closely eluting peaks and had limited scans across a singly eluting peak with a consequential poor quantitative robustness and reproducibility. Significant advances were made using FT-IR in a metabolomic/nutrient analytical capacity and this was shown to be applicable to the simultaneous quantification of a broad range of metabolites/nutrients in a real food systems. Furthermore when used in an unbiased (untargeted) metabolomic manner it was also able to distinguish the impact of growth location and agricultural systems. Its utility was shown to be best exploited in concert with the other metabolomic technologies, LC & GC-MS.

WP4 – The emphasis here was on providing biological material to prove the efficacy and utility of the technologies developed as part of WP1 and 3 during reporting period 1 and 2. Tomato and wheat material grown in several countries was generated and for the latter the comparison of conventional and organic systems was trialled. Potato growth was confined to P1. For all crops exemplar GM was made available via gift aided material from other (often EU) projects. The translation of this material through to the food processing chain and/or products was also tested. For wheat this was done via the generation of bread and pasta and for potato by a detailed analysis of the processing chain. SMEs (P14 and 15) provided support for the pasta and potato work whilst tomato product analysis was led by P6 an 11 via provision of a range of national products such as juice, puree, etc.

WP5 – A new website was established and populated during this period www.develonutri.info and this will be used to host the crop-specific metabolite/(micro)nutrient reference books developed as part of WP2. Furthermore, engagement with the EU stakeholders was advanced on several levels not least via the publication of two project overviews in journals used browsed by EU officials; Projects and Innovation International. Several presentations have been given and these have been used to disseminate the output to different stakeholder and end-users: International Symposium on Recent Advances in Food Analysis (Prague) and 4th International Biotechnology Symposium (Rimini). These addressed the planned outputs of the proposed (but cancelled) workshop objective.

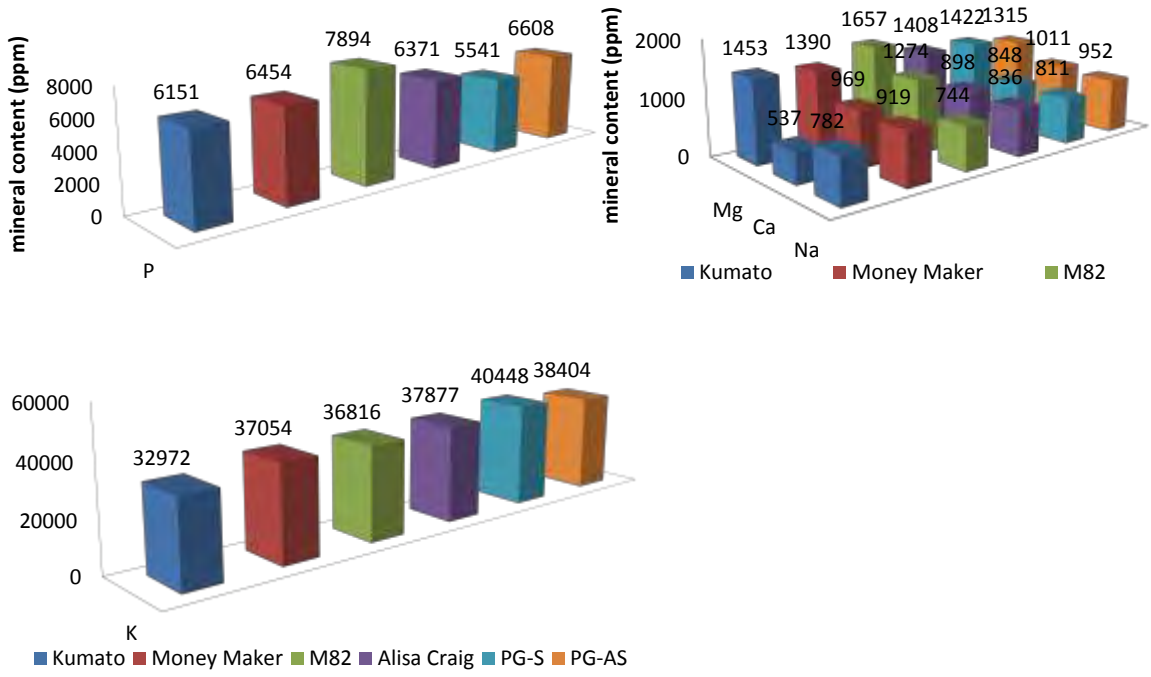
Section 2 – Workpackage progress of the period

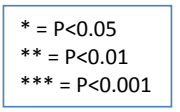
Workpackage 1: Chemical standards, inter-lab calibration, validation and ring testing of profiling approaches

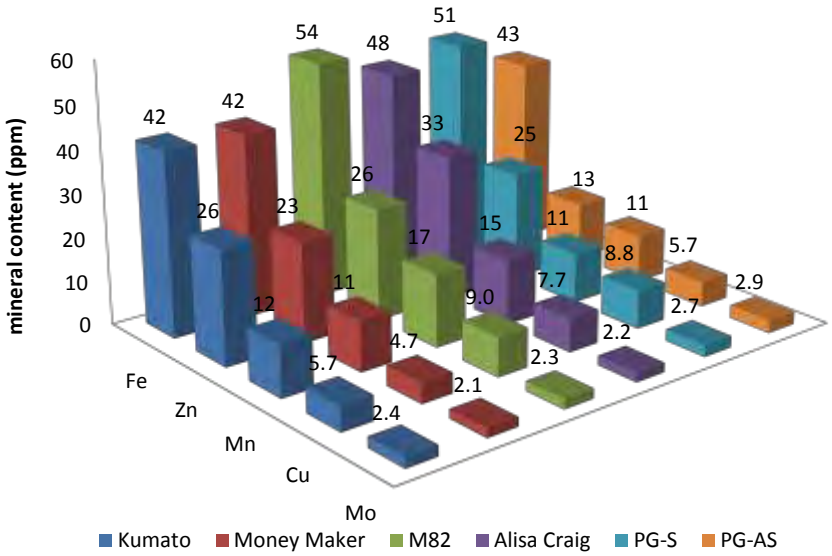
This work within this third and final period of the project largely focussed on the analysis of material generated from the latter deliverables of WP4; biological material and the demonstration activities (see the table of materiuals in Year 2)

Tomato

The range of material generated was broad encompassing the fresh and processed market. P4 showed that, at the level of micronutrient, changes, perhaps unsurprisingly, varied with the genotypes but not dramatically.



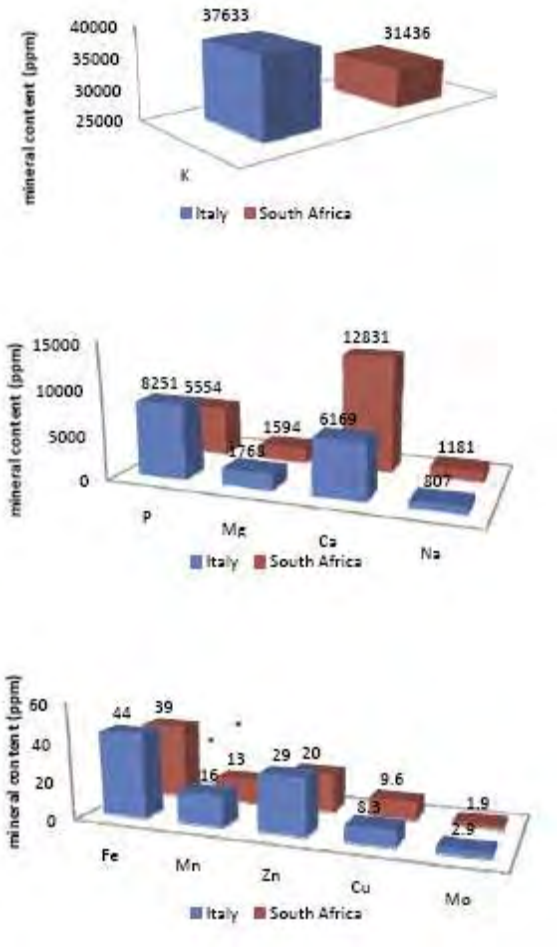




The micronutrient contents of select tomato lines.

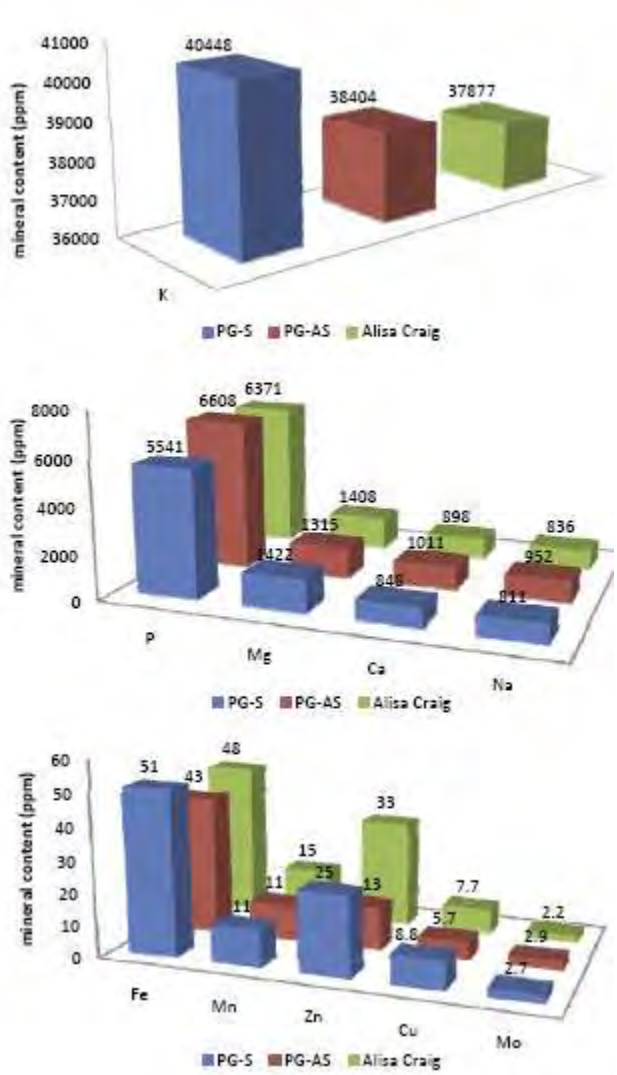
The variety M82 appeared to contain slight greater levels of micronutrients with the exception of sodium and potassium. However, none of these differences were statistically significant. The tomato varieties Roma and Rodade were also analysed for the effect of the location of growth. Although the measured differences between the locations in the variety Rodade are part wise consistent with the other variety - e.g. potassium (upper panel) or phosphorus (middle panel) [see below], none of these differences were

significant besides the small difference in Manganese content in the variety Rodade. Even this difference has a relatively high error probability. Genetically engineered tomato was grown only at one location in the greenhouse. .



Micronutrient differences in tomato cultivar Rodade grown in Italy and South Africa

The transgene seems to affect sodium and zinc content. However none of these differences were statistically significant

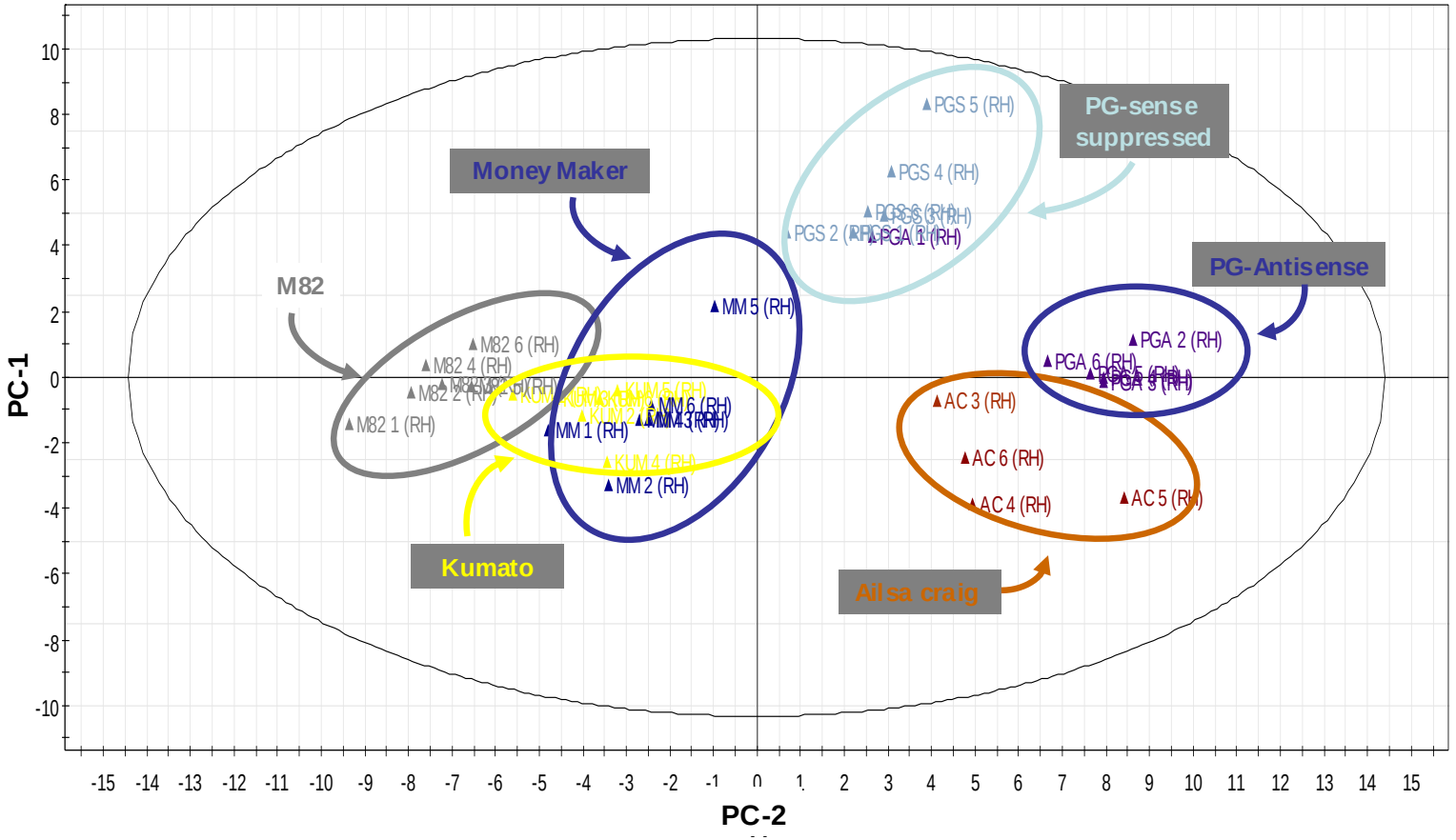


Micronutrient differences in GM and parent (Ailsa Craig) lines

Extending the profiling to the untargeted high throughput LC and GC technologies allowed all the biological materials to be clustered following PCA (see below)

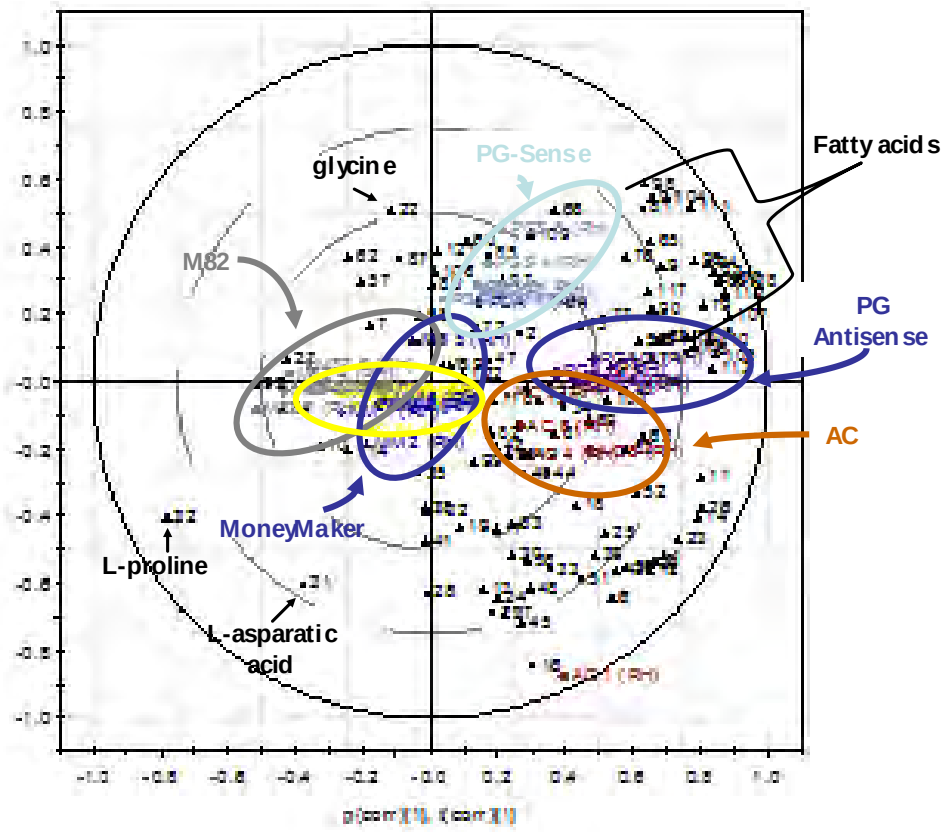
Principal component analysis (PCA) performed on GM and non-GM tomato varieties cultivated at RHUL

(GC-MS data 122 metabolites)



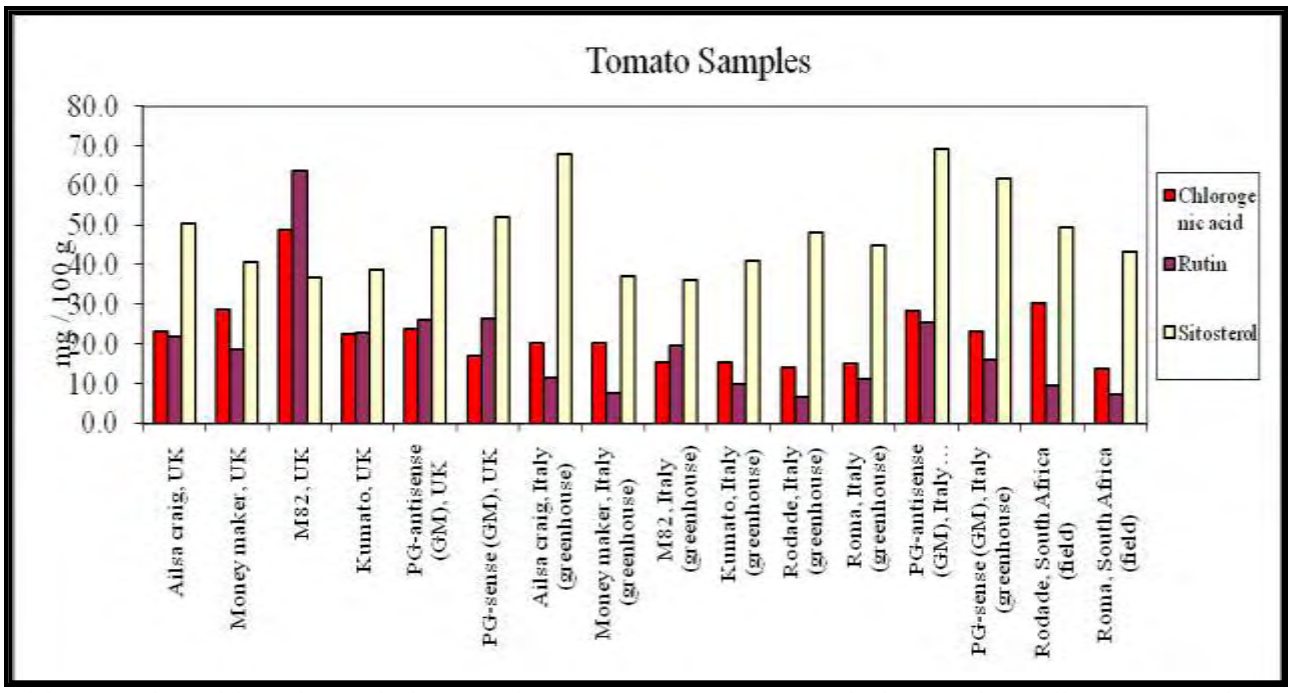
PCA of GC-MS data derived from analysis of selected tomato varieties and GM lines. (Ex P2)

Simultaneous score and loadings scatter plots on GM and non-GM tomato varieties cultivated at RHUL (GC-MS data 122 metabolites)



Metabolite mining of the GC-MS data derived from analysis of selected tomato varieties and GM lines (Fig. 6). (Ex P2)

A more compound-specific approach to tomato analysis was undertaken by P10 who analysed for chlorogenic acid, a common *solanaceae* polyphenol, quercetin-3-rutinoside and sitosterol the latter two being linked to dietary mediated reductions of risk of incidence of CVD. There were, as expect, no GM derived differences here.



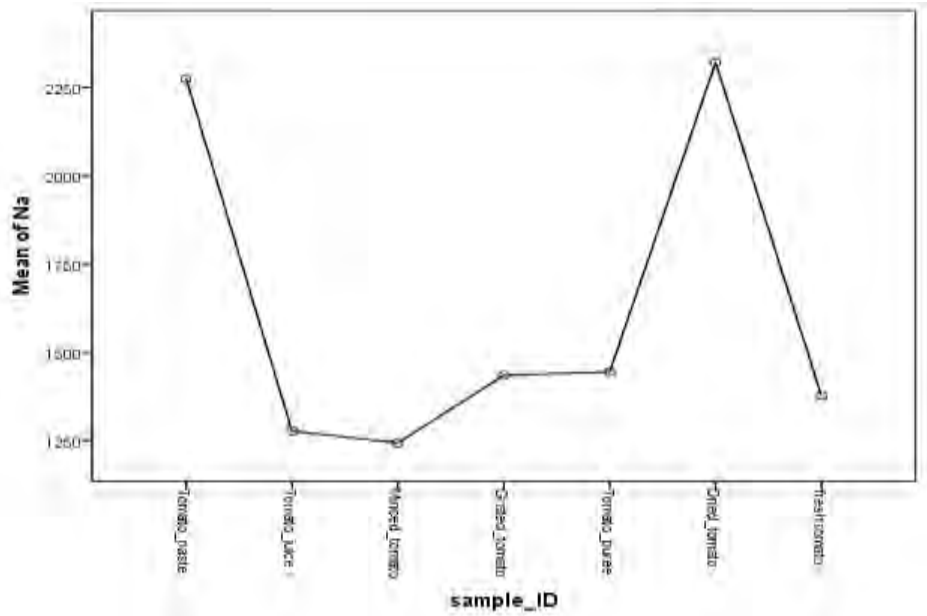
Chlorogenic acid, rutin and sitosterol contents of tomato varieties and GM lines grown in the UK, Italy and South Africa (P10).

. Interestingly there was however a significant variation in the polyphenols (chlorogenic acid and rutin) with levels varying by almost 6-fold (Roma & Rodade (Italy) v M82, UK). There was a shift in the ratio of chlorogenic acid: rutin in the PG sense and antisense lines when comparing growth in the UK with Italy. The UK derived GM material (±sense) had a chlorogenic acid: rutin ratio of <1 whilst the corresponding value for the Italian grown material was >1. The reasons for this are unclear. Sitosterol did vary but the level of variation was not so extensive (~80%). None of the compounds exhibited a specific geographic trend

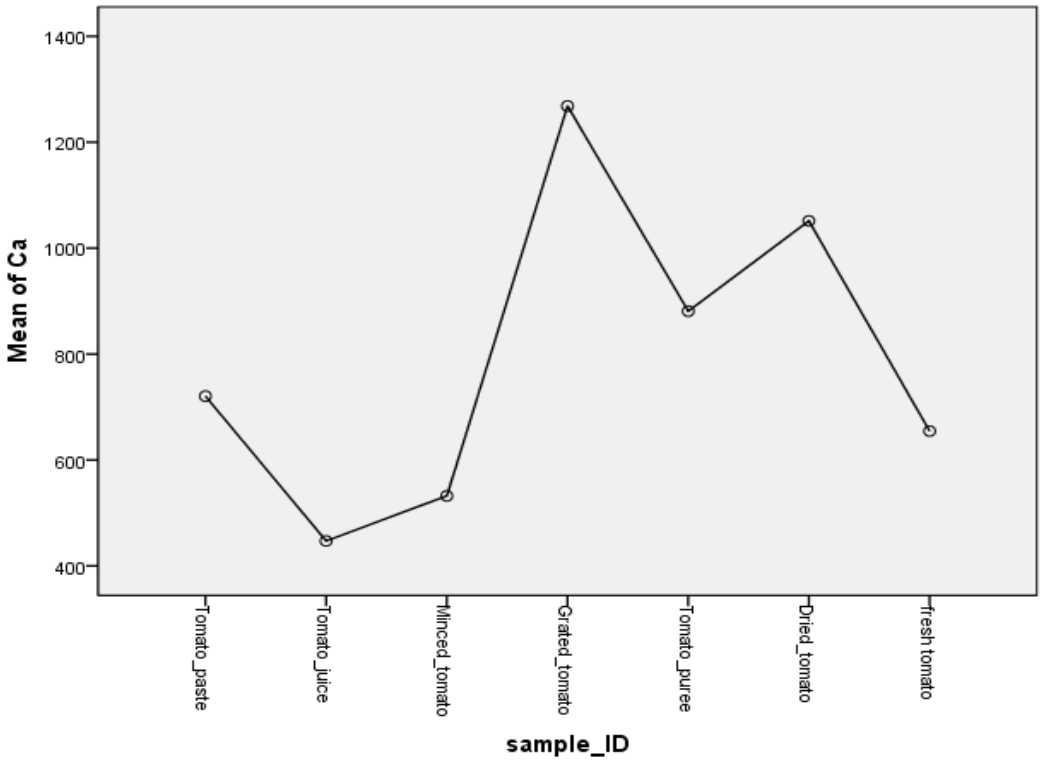
Progress to the processed material utilised several sources. As was previously outlined initial interest by tomato processing SMEs did not materialise and therefore commercially available tomato based products were utilised. For example, P6 was instrumental in sourcing a range of commercial pastes (x6), juices (x4), minced tomato products (x5), purees (x2), grated (x2) and dried (x5) tomatoes. These were compared against the appropriate fresh tomato. P11 added a further set of samples (paste, peeled and canned, juice and sundried). These samples were then extensively analysed for several specific (micro)nutrients such as carotenoids and fatty acids as well as with the SOPS developed for GC and LC-MS analysis (metabolomics)

Analysis at the micronutrient level showed that there was a significant (weight per weight) difference with respect to sodium (Na) with the concentrated products, paste and sun-dried, exhibiting levels almost 100% higher. However when calcium is considered this pattern was not replicated suggesting that there is a definite process effect evident. For calcium the grated tomato exhibited the greatest level with the dried and puree elevated

also. The associated juices were relatively depleted in calcium suggesting a skin/dry matter-related calcium loss.



Variation (ANOVA) of sodium content in the processed tomato samples supplied by P6



Variation (ANOVA) of calcium content in the processed tomato samples supplied by P6

The samples were also analysed for specific carotenoid contents by P11: lycopene, lutein, zeaxanthin, canthaxanthin and □-carotene. P11 found that that for the Tat brand (http://www.koc.com.tr) tomatoes contained the highest levels of lycopene and these were found in minced tomatoes followed by tomato puree and tomato paste. The highest level of β-carotene was also found in minced tomato followed by tomato juice and then tomato

puree. This supports the previous suggestion that the processing of the tomatoes influences the level of carotenoid found in the final product. One has to bear in mind however that there could be line and/or geographic differences at work here as was seen earlier with the fresh tomato GC and LC-MS analyses

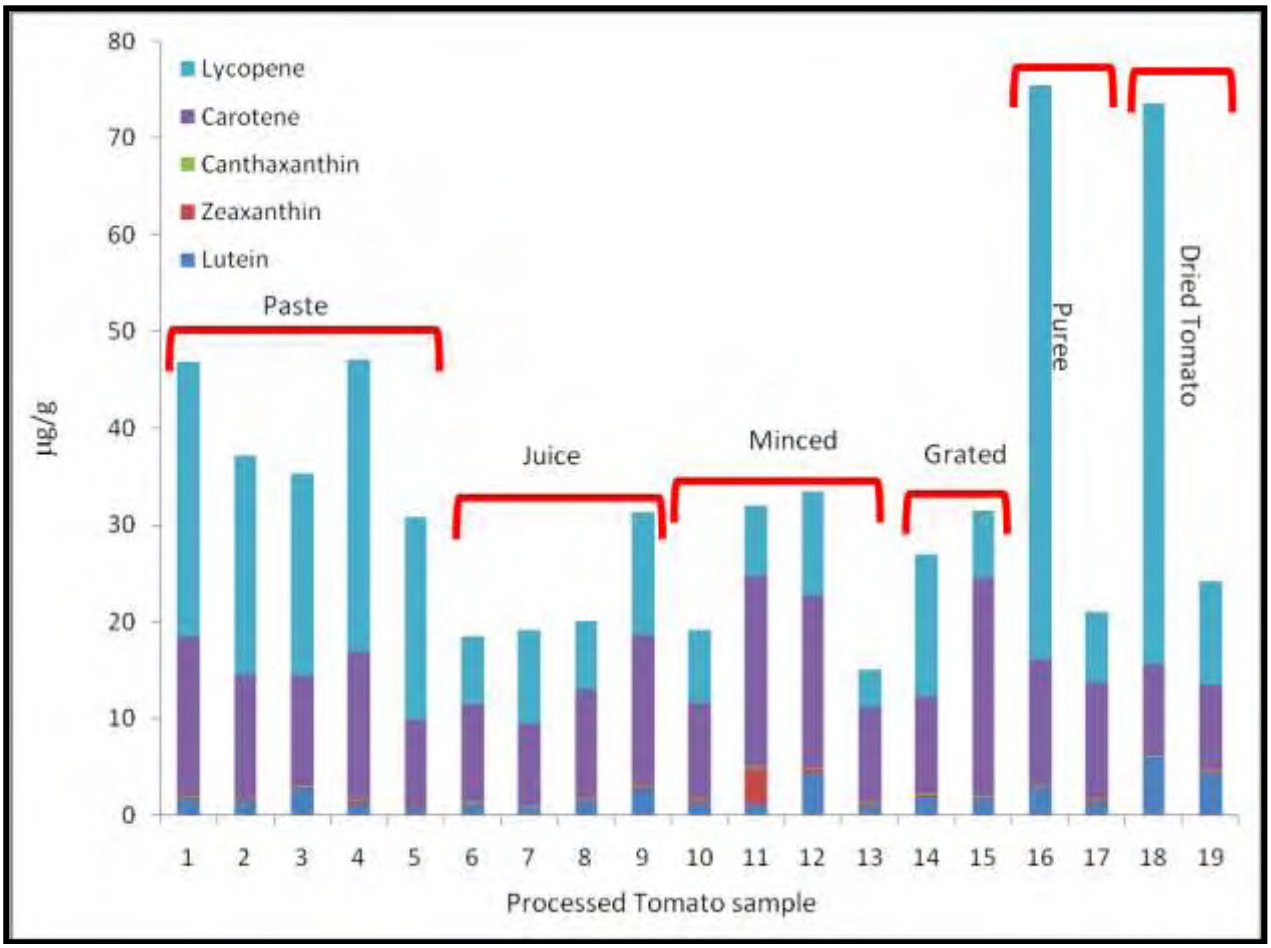
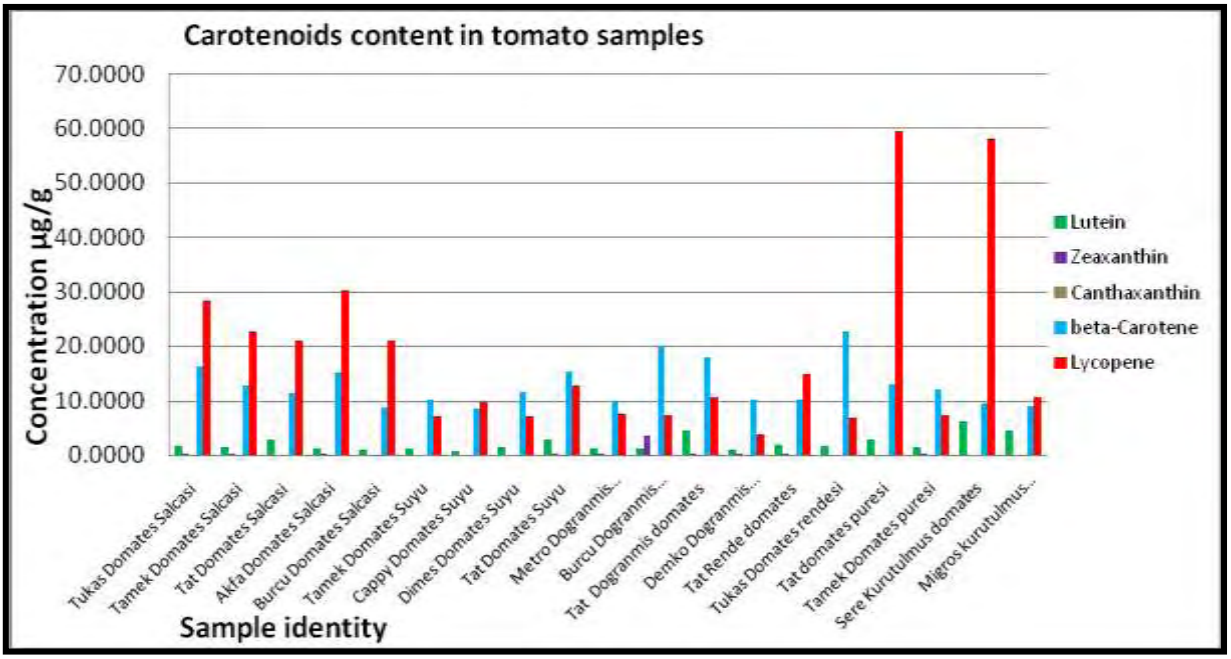
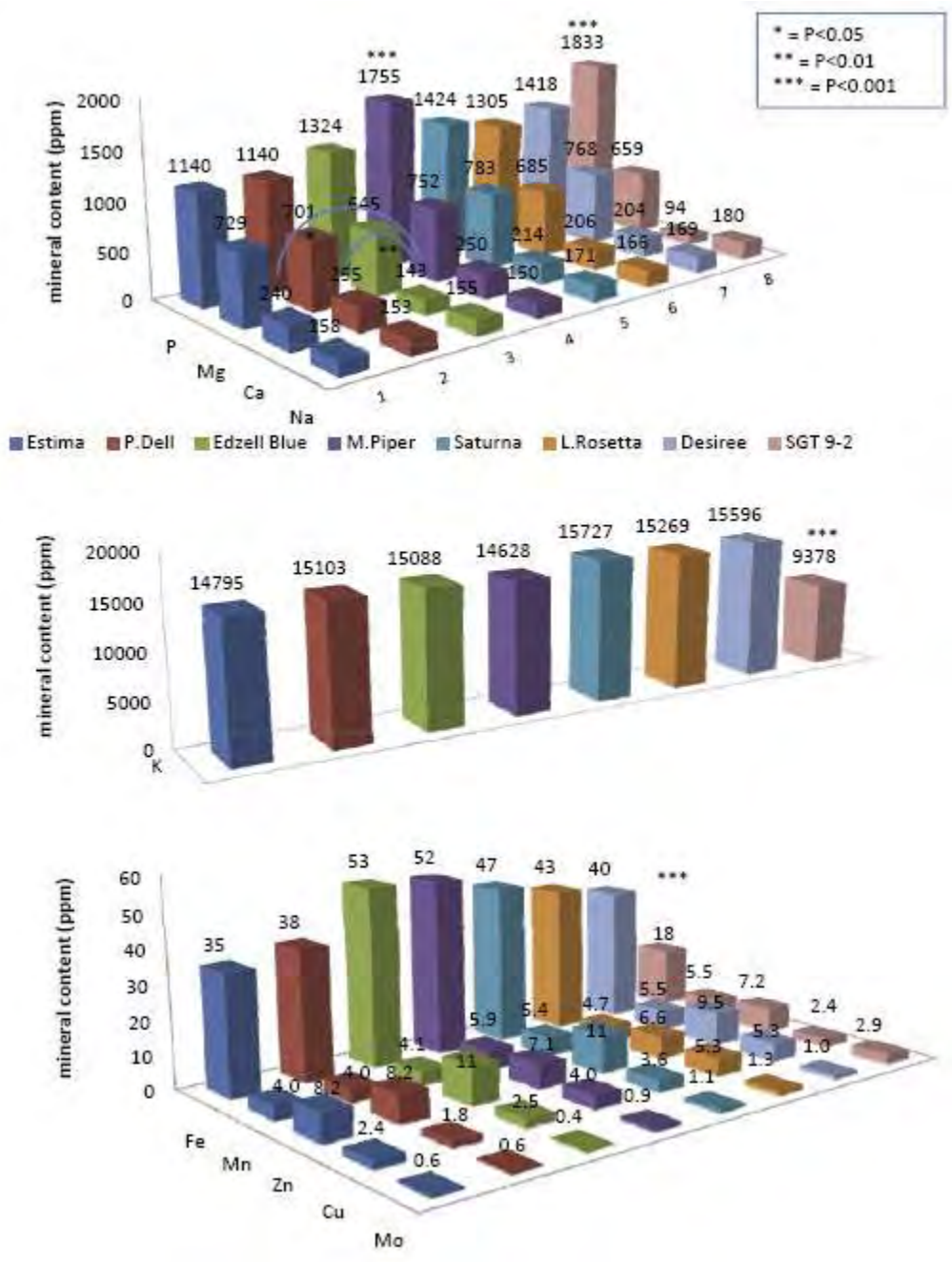


Figure 11. Variation in total and specific carotenoid content of P6 supplied processed tomato products.



Potato

As for tomato, potato was subject to a combine analytical approach. Only the GM line (SGT9-2, with a modification in the glycoalkaloid pathway) differed in several components, i.e. phosphorus, sodium and iron, otherwise only c.v. M. Piper contained more phosphorus (See below, upper panel).



Micronutrient content in selected potato varieties and a GM line (SGT 9-2).

In contrast to the genotype-derived variation, the production of the potato product appeared to exhibit effects on micronutrient content. The progression from first wash to peeled was accompanied by a dramatic reduction in Fe contents and this is corroborated by the recent study by Subramanian et al (2009; (http://www.scri.ac.uk/webfm_send/741) highlighting the peel localisation of Fe (See below, lowest panel). The phosphorus content was slightly influenced by process changes particularly the 24hr water soak suggesting an

osmotic efflux of P-compounds. The dramatic sodium peak in the processing step “sliced DIP” is due to the preservative (sodium metabisulphite) and disappears in the next steps of the processing again. This is important since there is concern in the potato industry with regard to the potential pressure to abolish the use of sodium metabisulphite but this shows that at least the sodium cation is rapidly reduced at the next stage (and even further following overnight storage). Selected ion chromatography identified that the counter anion metabisulphite performed similarly. Interestingly, the overnight step looks to be a good idea in this respect.

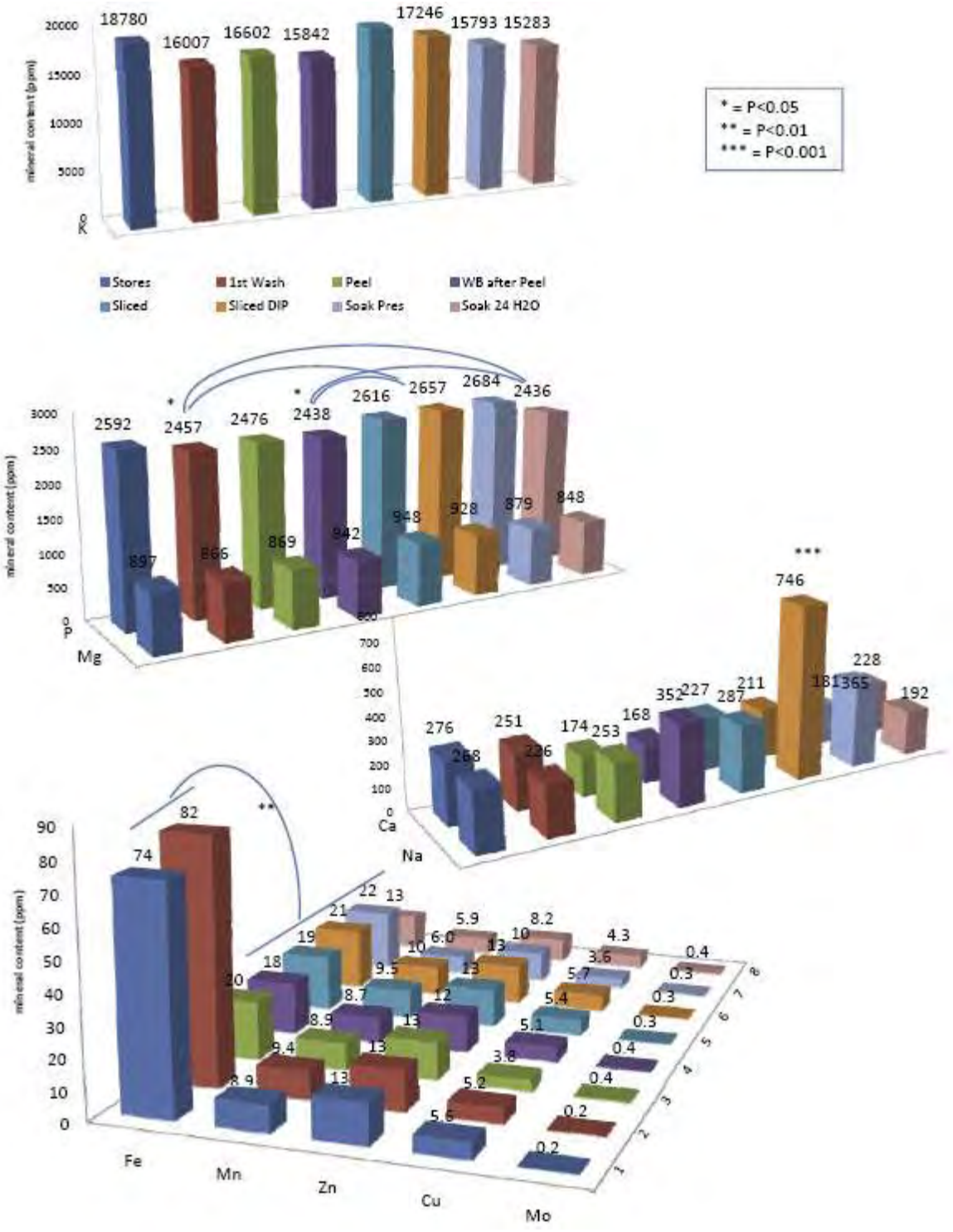
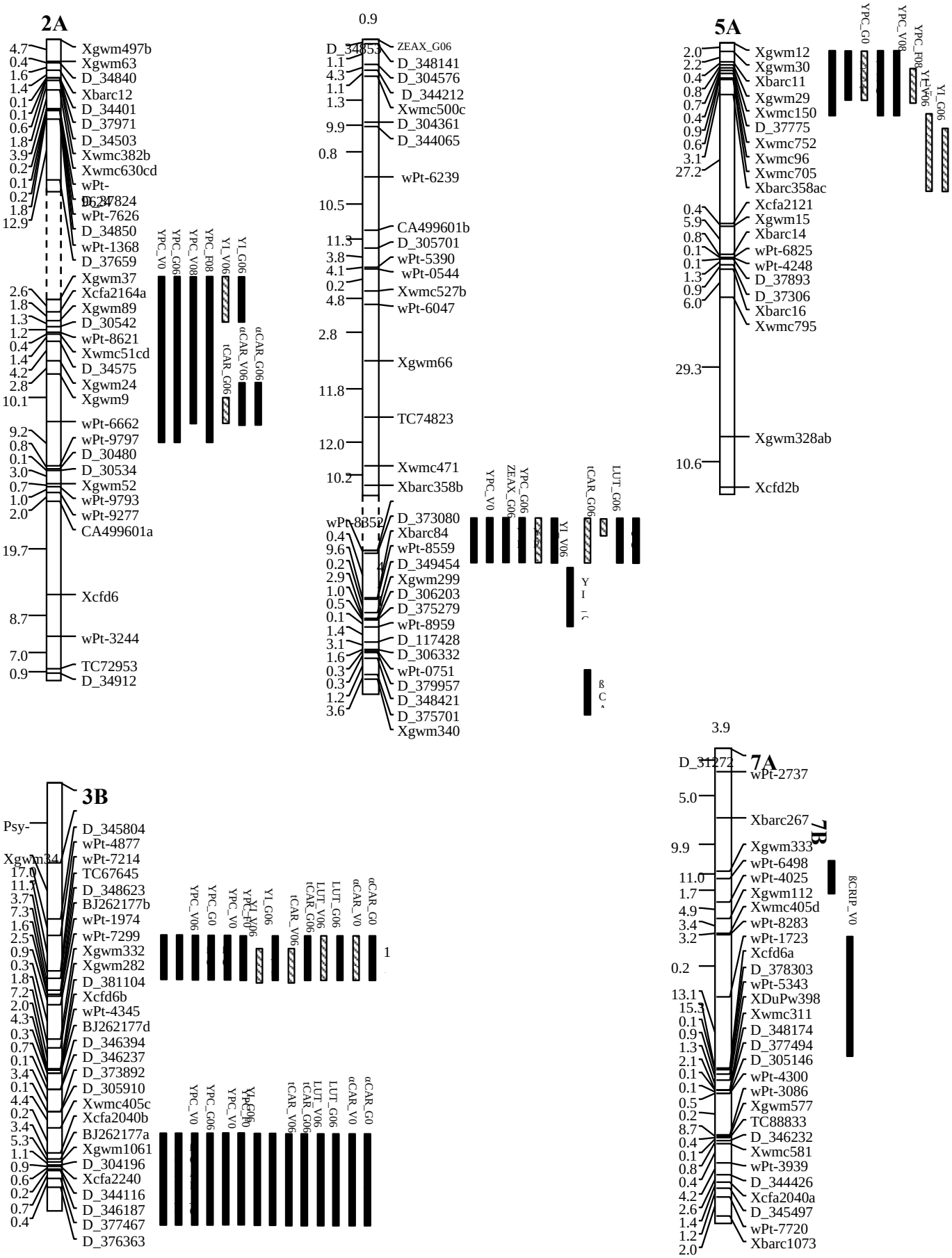


Figure 17. Micronutrient content in samples derived from the potato processing line of P15 (see 2nd year annual report Fig, 24).

Wheat

During the third year of the project **P5** completed demonstration activity based on HPLC target analysis of carorenoids in durum wheat. Quantitative trait loci (QTLs) for Yellow Pigment Content (YPC), yellow index (YI) and individual carotenoid compounds were generated using a segregating population of 121 F_2:F_{3-4} families, derived from the cross of cultivars Latino and Primadur, grown at four environments. Total carotenoids amounted to 37% of YP, indicating unknown colour-producing compounds in the durum extracts. Lutein was the most abundant carotenoid, followed by zeaxanthin, α-carotene and β-carotene, while β-cryptoxanthin was minor component. Phytoene synthase marker *Psy-A1*, 150 SSR and EST-SSR markers, and 345 DArT® markers, were used to construct the linkage map for subsequent QTL analysis (not as part of, but as a complement to, DEVELONUTRI). Composite interval mapping identified five QTLs on chromosomes 2A, 3B, 5A and 7A, accounting for a large proportion of the phenotypic variation for YPC and YI. Two major QTLs for YPC were detected in two separate intervals on 7AL, thus confirming that allelic differences at *Psy1*, and at least one additional gene in the distal region of homoeologous group 7L are associated with differences in YPC. Clusters of QTLs for total and/or one or more carotenoid compounds were detected on the same chromosome regions where QTLs for YPC and YI were identified (figure 24).

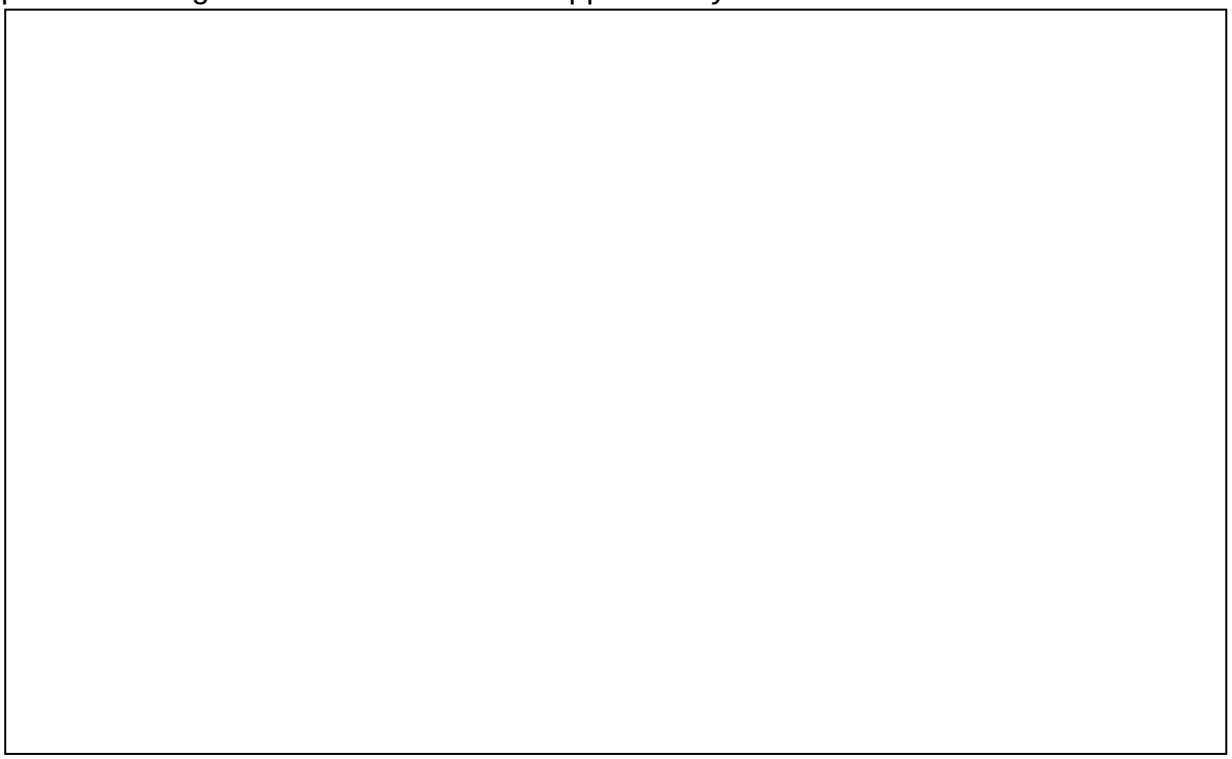


Genetic map and QTL summary for yellow pigment content, yellow index and carotenoid compounds detected in 121 F_2:F_3 families derived from a cross between the cv. Latino and Primadur of durum wheat. QTLs are represented by bars (2-LOD interval). Solid bars

represent QTLs significant at LOD≥3.0 and diagonal hatch bars represent suggestive QTLs at the sub-threshold 2.0<LOD<3.0 value. QTL names also indicate trait (YPC = yellow pigment content; YI = yellow index; tCAR = total carotenoids; LUT = lutein; αCAR = α-carotene; bCAR = β-carotene; bCRIP = β-cryptoxanthin; ZEAX = zeaxanthin) and environment (V06 = Valenzano 2006; G06=Gaudiano 2006; V08=Valenzano 2008; FG08=Foggia 2008).

However, the metabolomic analysis was the predominant focus for durum wheat, bread wheat and their end-products in the third period. The polar and non-polar metabolites detected using a GC-MS metabolomic approach by P5 are listed below

The polar and non-polar metabolites detected in durum wheat, bread wheat and their end-products using a GC-MS metabolomic approach by P5

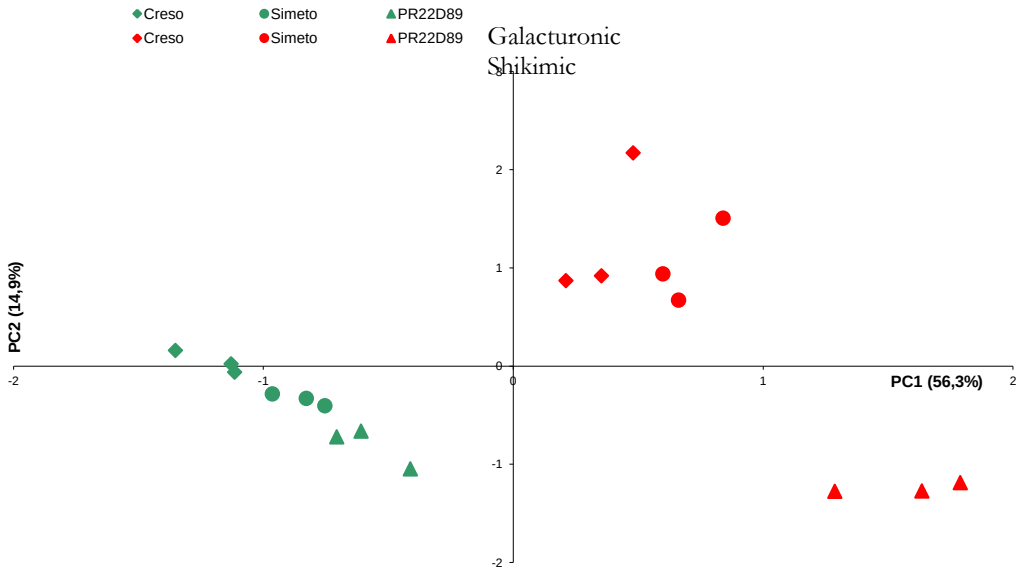


The core wheat used to test the SOPs generated as part of the years 1-2 research were as follows (Specifics are in Table 1);

Durum wheat: The analyses were design to assess i) the metabolite variations due to different growing locations (three cultivars grown in two environments representing typical durum wheat growing area in north and south Italy); ii) the effects of conventional vs organic farming on metabolites content and iii) the metabolic perturbations due to the industrial processing (from semolina to dried pasta).
Bread wheat: The analyses were design to assess i) the metabolite variations due to different growing locations (three cultivars of different origin were grown in three locations across Europe (Italy, Scotland and Swiss); ii) the metabolic differences between two GM lines and the corresponding wt; iii) the metabolic perturbations due to the industrial processing (from flour to bread) to highlight critical points in the production chain.

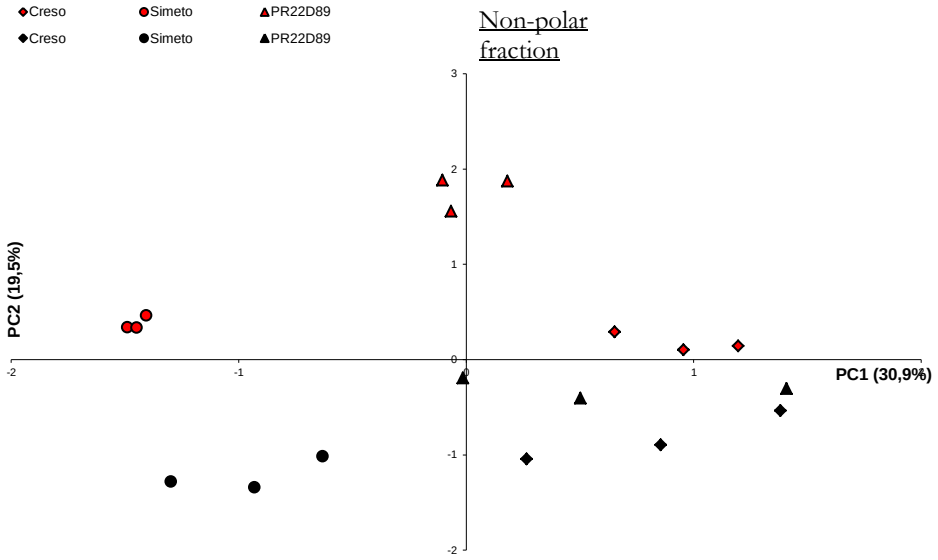
Figure 25 highlights an example of the PCA derived from the analysis of the durum variety derived GC-MS data. The first component accounts for 56% while the second for 15% of total variance. The compounds that had a significant effect on PC1 loading were amino

acids, maltose, hydroxy-fatty acids and saturated fatty acids. The other sugars, alanine and tocopherols impacted upon significant loading on PC2. The samples grown in North Italy (marked in red) differed significantly from those grown in the south reflecting the higher amounts of the outlined metabolites associated to this factor. Amongst the northern Italy grown samples there is also a greater spread between the varieties that was seen in the southern derived samples. This may reflect the more comparatively more varied weather patterns evident in the north compared to the south resulting in a greater metabolome diversity.



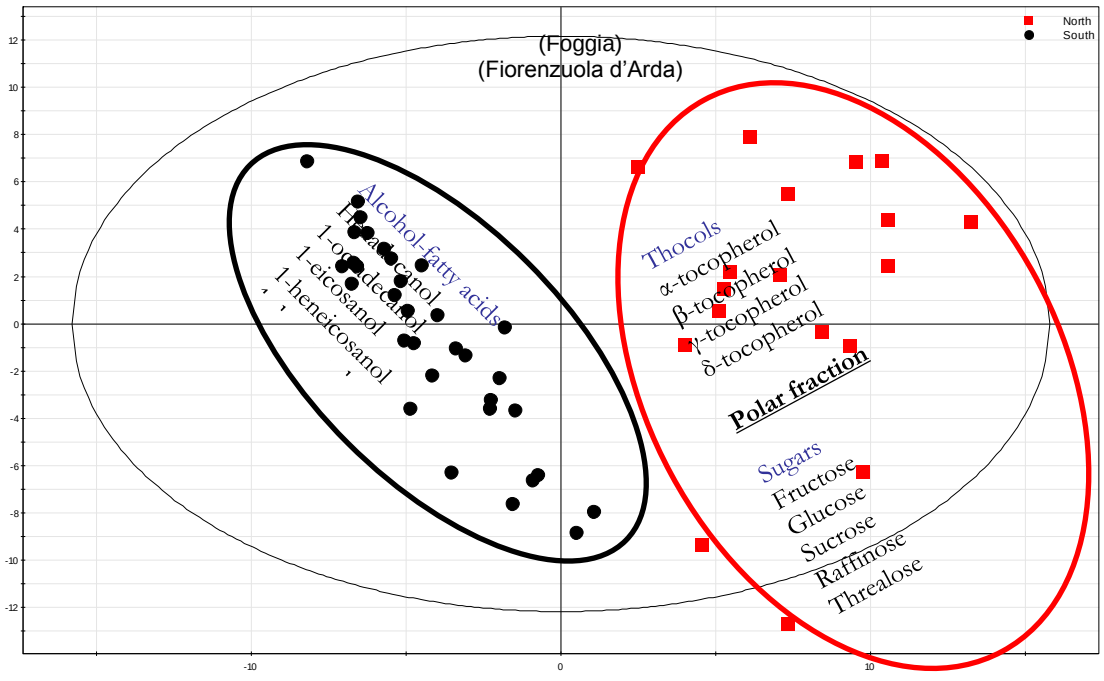
Durum wheat cultivars grown in North Italy (Fiorenzuola d'Arda: red annotation) and green marks: durum wheat cultivars grown in South Italy (Foggia: green annotation). Analysis by P5

The metabolomic-PCA approach was also used to attempt to distinguish between durum wheat samples grown in organic vs conventional farming systems. The two agronomic protocols differed with respect to fertilization and herbicide treatment. The consequences at the metabolome level were that the wheat samples were significantly different particularly with respect to fructose, glucose, myo-inositol and octacosanoic acid wherein they were present at a 2-fold greater level in the organically grown samples.

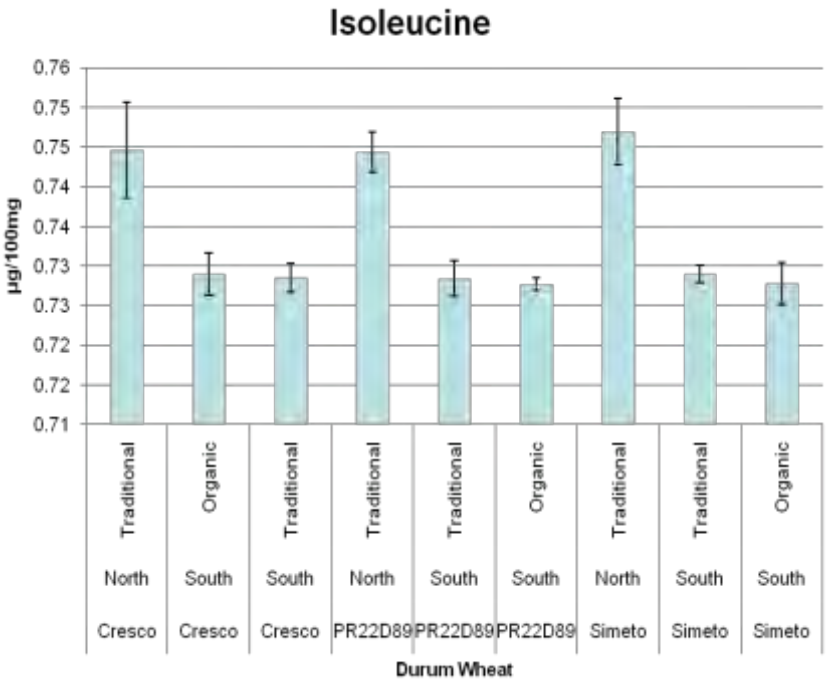


Durum wheat cultivars from organic farming (red annotation) and conventional farming (black annotation).

Analogous analysis undertaken by P1 corroborated this finding (see below) with the varieties segregated clearly by geographic location (North and South) with the wheat grown in northern Italy exhibiting relatively higher concentrations of e.g. alanine, methionine, oxo-proline phosphoric acid, glycerol, malic acid and sucrose.



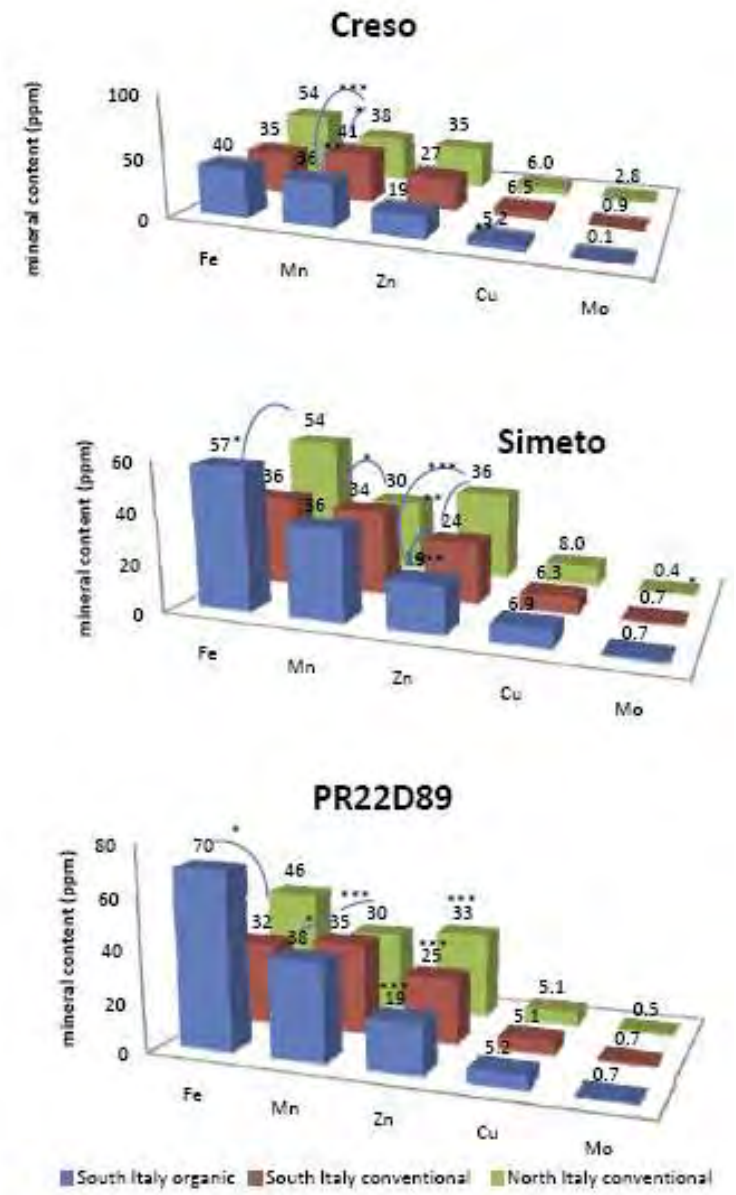
GC-MS(polar fraction)-PCA of Durum wheat cultivars grown in North Italy (Fiorenzuola d'Arda: red annotation) and green marks: durum wheat cultivars grown in South Italy (Foggia: black annotation). Analysis by P1.



An example of location and agricultural regime derived amino acid variation in durum wheat. Here isoleucine variation is highlighted

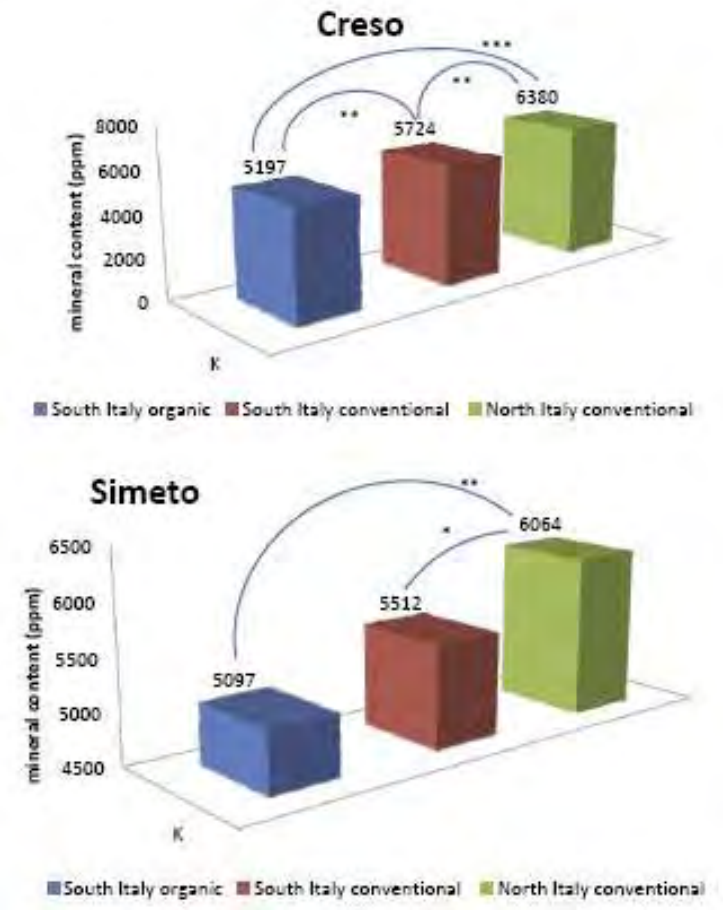
At the compound specific level P1 showed that this geographic influence was highlighted by variation in the specific levels of, for example, isoleucine contents (see above). As part of this figure the direct comparison with the organic comparator (in the south) is included and shows that for this region at least there is no significant difference in content.

At the micronutrient level, the three varieties Creso, Simeto and PR22D89 exhibited differential mineral contents depending on the genotype, location of growth and agronomic treatment (See below). Creso differed significantly in its Manganese content between organic treatment in South Italy (blue columns), conventional treatment in South Italy (red columns) and conventional in North Italy (green columns). Simeto and PR22D89 exhibited higher iron contents under organic treatment, slightly elevated manganese and a lower content in zinc.. Other differences were not significant. A difference in sodium concentration might be due to the location and treatment (Fig. 33) and to a lesser extent to the genotype, although it was less pronounced in PR22D89 (not shown).

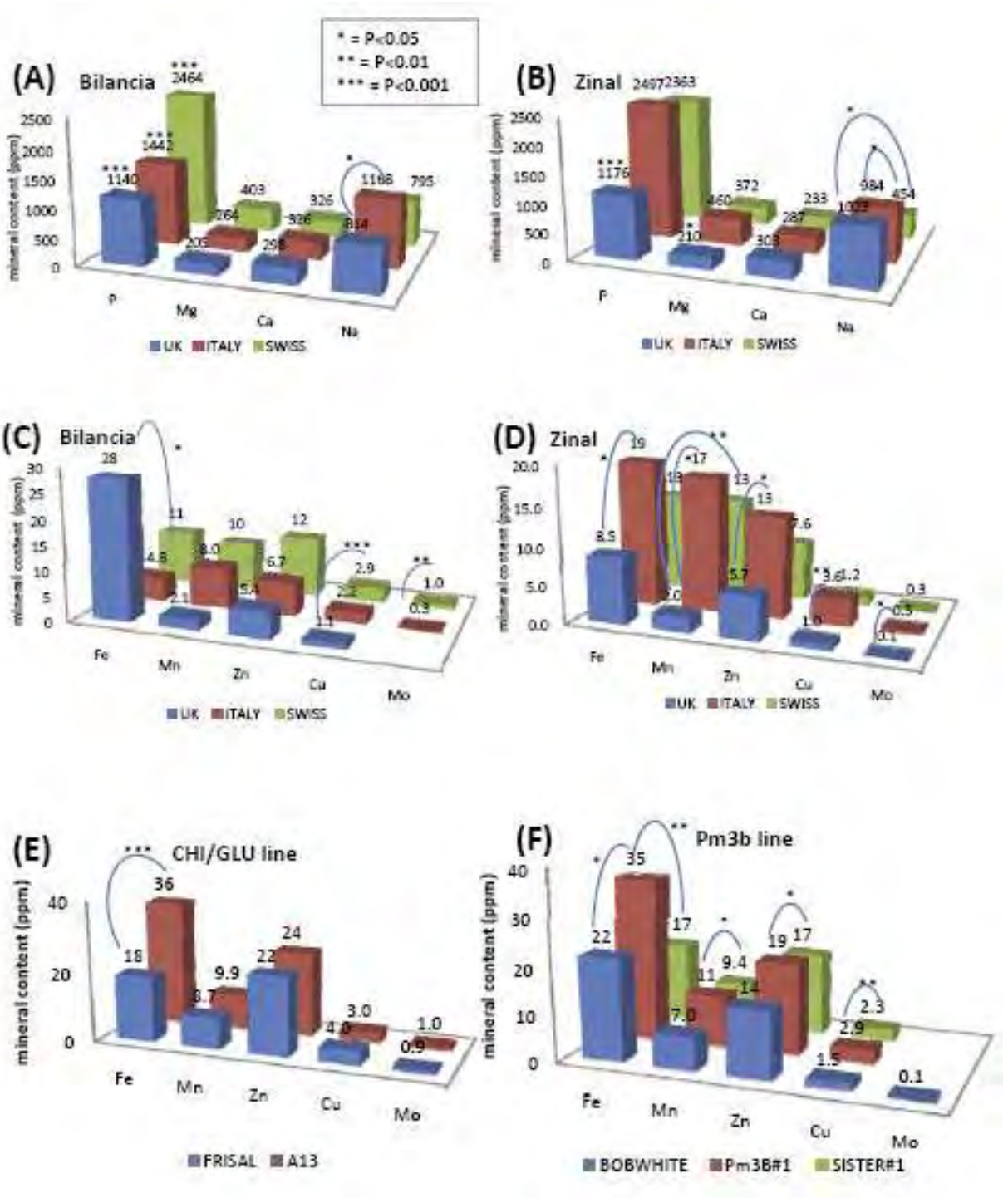


The selected micronutrient contents of durum wheat (cvs. Creso, Simeto and PR22D89) grown in north and south Italy and under conventional and organic agricultural regimes.

A difference in sodium concentration might be due to the location and treatment (Fig. 33) and to a lesser extent to the genotype, although it was less pronounced in PR22D89 (not shown).



The sodium contents of durum wheat (cvs. Creso and Simeto) grown in north and south Italy and under conventional and organic agricultural regimes.

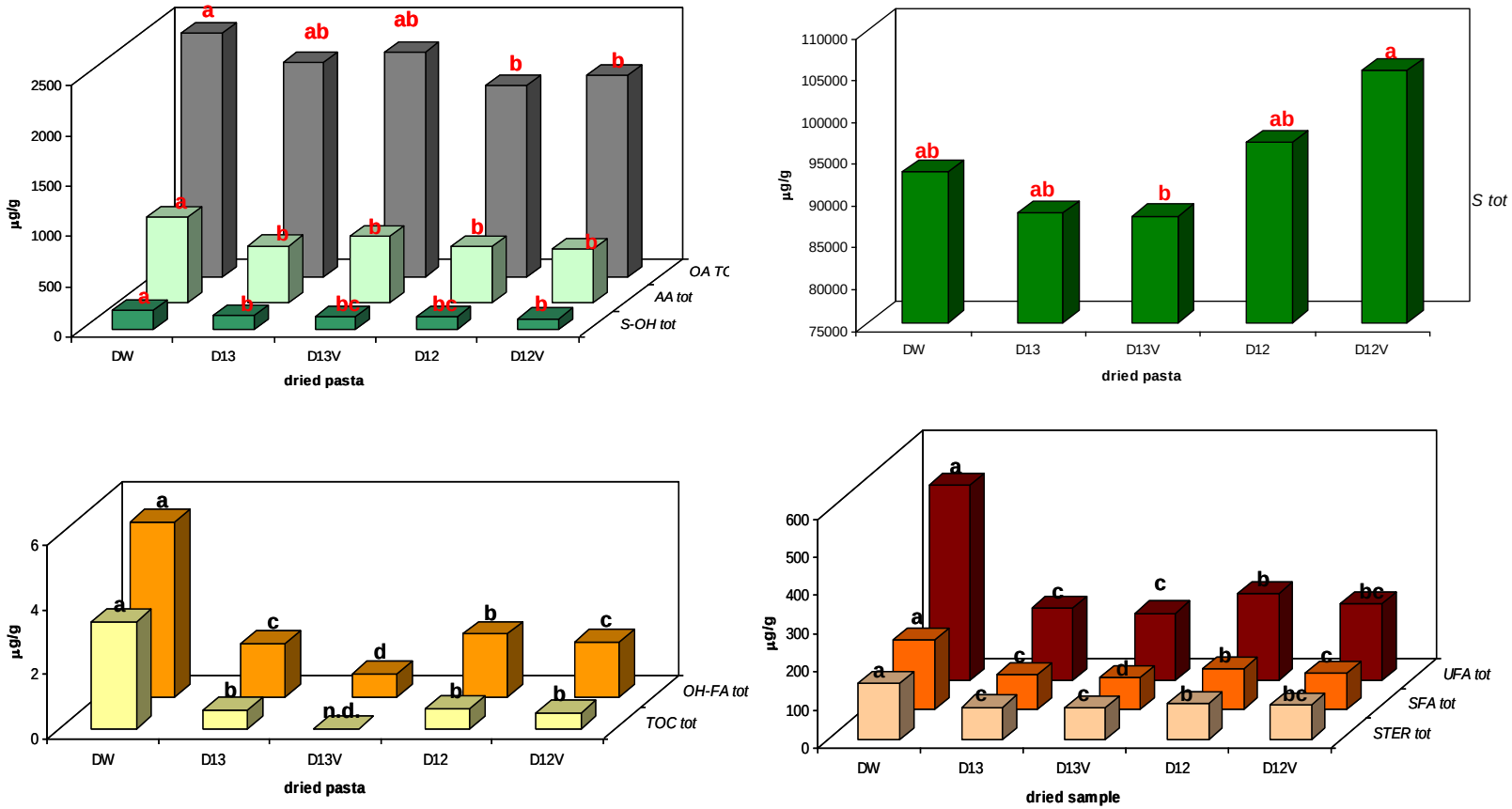


The selected micronutrient contents of bread wheat cultivars (Bilancia and Zinal) and GM lines with their associated parents and sister lines (Frisal, A13; Bobwhite, Pm3#1 and SISTER#1) grown in different locations.

As with the durum wheat bread wheat also experienced a location related impact on micronutrient content (see above). Among the macro-elements phosphorus was lowest in the UK, medium in Italy, and highest in Switzerland with a large step between Italy and Switzerland in Bilancia. For the cv Zinal the largest variation in phosphorus content was between U.K. and Italy. Sodium content was about the same (not shown). Among the trace-elements, iron content was most susceptible to the location, but not in the same direction in every variety. In the variety Bilancia iron was much higher in the U.K. whereas

it was lower in the U.K. in variety Zinal and in Mascot (not shown). Indeed the cv Mascot behaved for the macro-elements like Bilancia and for the trace elements like Zinal.

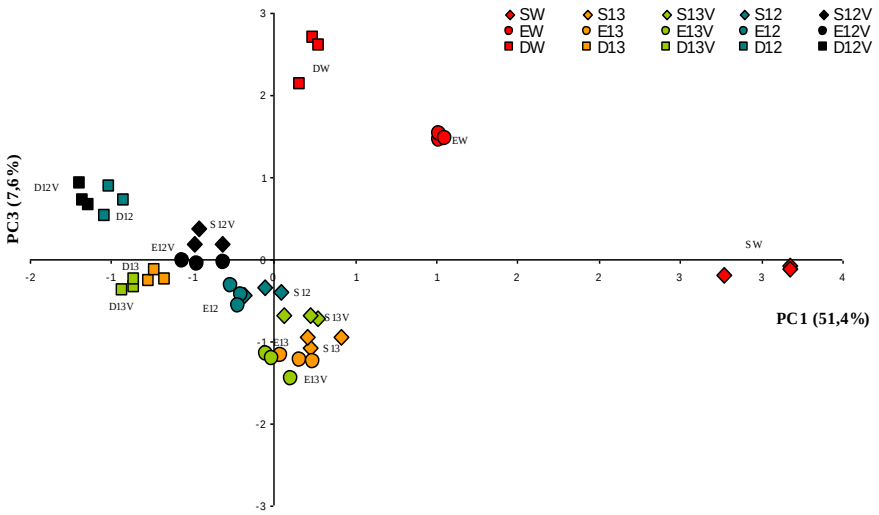
Two genetically engineered spring wheat lines were compared with their respective wild type and sister line where available. The Swiss elite line Frisal was genetically modified with a Chitinase and a Glucanase from barley for fungal defence and the bar gene as a selection marker, providing resistance against the herbicide Basta. Frisal wild type (Frisal) did not differ from the genetically modified line (A13) in its mineral content except of the higher iron content in the transgenic line. The old Mexican breeding spring wheat line BobWhite was supplemented with the powdery mildew resistance allele Pm3b#1 from wheat. The sister line lost the transgene by segregation. Wild type (BOBWHITE), transgenic line (Pm3b#1) and sisterline (SISTER#1) were compared. In this transgenic line, the iron content was higher as compared to wild type and sister line. Other minor differences were of lower significance. We have currently no explanation for the higher iron content in the modified lines, since the genes are different and the transformation events were independent and mediated by different selection regimes.



Variation in selected chemical components during durum wheat pasta preparation. - OA tot : Organic Acids total; AA tot: Amino Acids total; S-OH tot: sugar alcohols total; S Tot: sugars total; OH-FA tot: Hydroxy- fatty acids total; TOC tot: tocols total; UFA tot: unsaturated fatty acids; SFA tot: saturated fatty acids; STER tot: sterols total. DW = whole meal dried pasta; D13 = dried pasta 13% protein; D13V = dried pasta 13% protein + vitamins; D12 = dried pasta 12% protein; D12V = dried pasta 12% protein + vitamins

As with the other crops the associate processed products were also analysed. For durum and bread wheat this was pasta and bread, respectively. The pasta processing company, (P14, TAMMA Industries) provided five different sample sets: a set obtained from whole grain pasta processing, 2 sets from pasta processing run with 12% with and without the addiction of B group vitamins to improve the nutritional value, and 2 sets from pasta processing run with 13% with and without the addiction of B group vitamins to improve the nutritional value. Each sample set was made of three collecting point representing the key steps in the industrial pasta processing: semolina, extruded pasta and dried pasta. Figure 35 shows the total content for selected classes of compounds detected in dried pasta samples. The higher values were exhibited in the whole meal pasta with the exception of sugars where the higher content was found in pasta at 12% protein content plus vitamins. As part of the full nutritional analysis the tocopherols were assessed and it was found that the pastas with specified protein level (inclusion ±vitamins) had dramatically reduced levels. The explanation for this is not clear. To obtain a representation of the chemical composition and to evaluate the processing, the data were subjected to PCA (See above)

Whole grain samples differed significantly from the other samples. Furthermore, there are clearly three distinct groups representing the processing steps: semolina, extruded and dried pasta samples.



PCA of the GC-MS data derived from the following samples. SW= whole meal semolina; S13 = semolina 13% protein; S13V = semolina 13% protein + vitamins; S12 = semolina 12% protein; S12V = semolina 12% protein + vitamins; EW = whole meal extruded pasta; E13 = extruded pasta 13% protein; E13V = extruded pasta 13% protein + vitamins; E12 = extruded pasta 12% protein; E12V = extruded pasta 12% protein + vitamins; DW = whole meal dried pasta; D13 = dried pasta 13% protein; D13V = dried pasta 13% protein + vitamins; D12 = dried pasta 12% protein; D12V = dried pasta 12% protein + vitamins

P11 supplemented these analyses with more detailed carotenoid analysis and this showed that, in an analogous manner to the P5 measured tocopherol contents, there is a negative impact on total carotenoids accompanying vitamin supplementation of the dried pasta: D13V more so than D12V (see below). In the former lutein loss is the driver whilst the less pronounced loss in D12V is due to a reduction is zeaxanthin. Although not nutrients lutein is a required components for reducing the risk of age related macular degeneration and its

loss in, what is a staple food in Italy, is worth watching. This pattern is not replicated for the extruded samples.

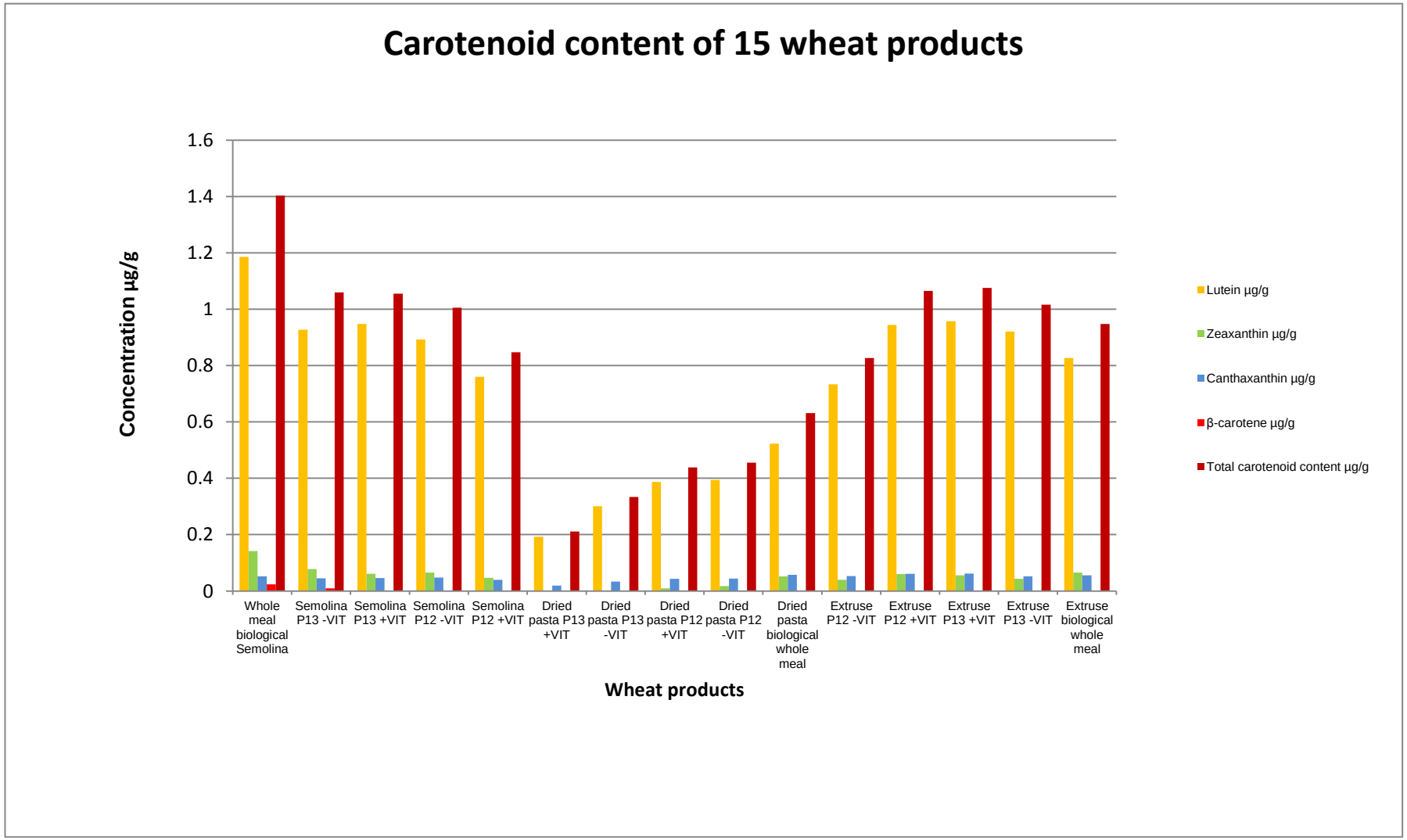
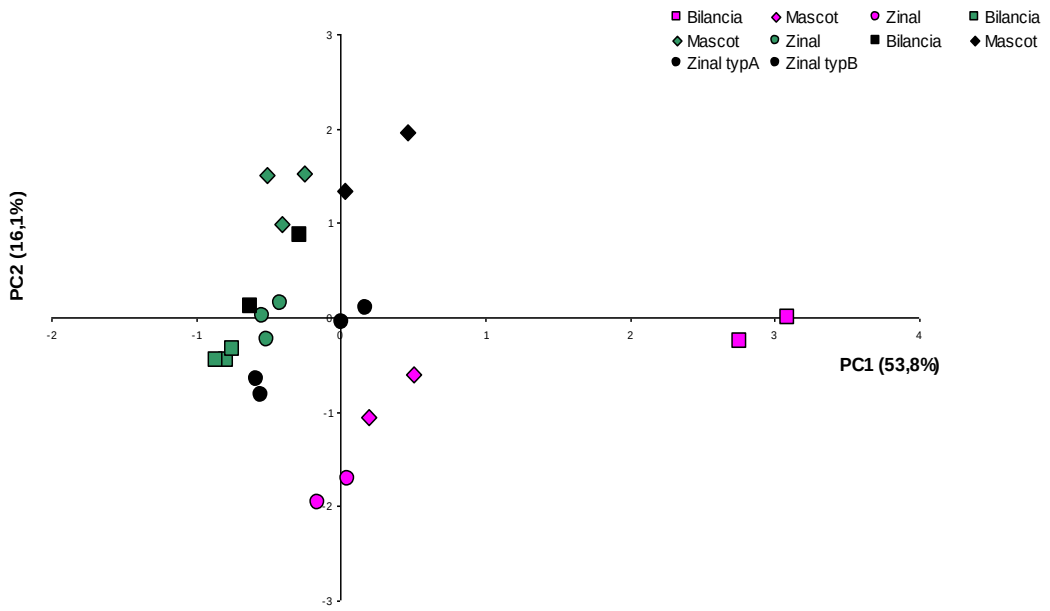


Figure 37: P11 derived carotenoid contents in processed durum wheat from P14.

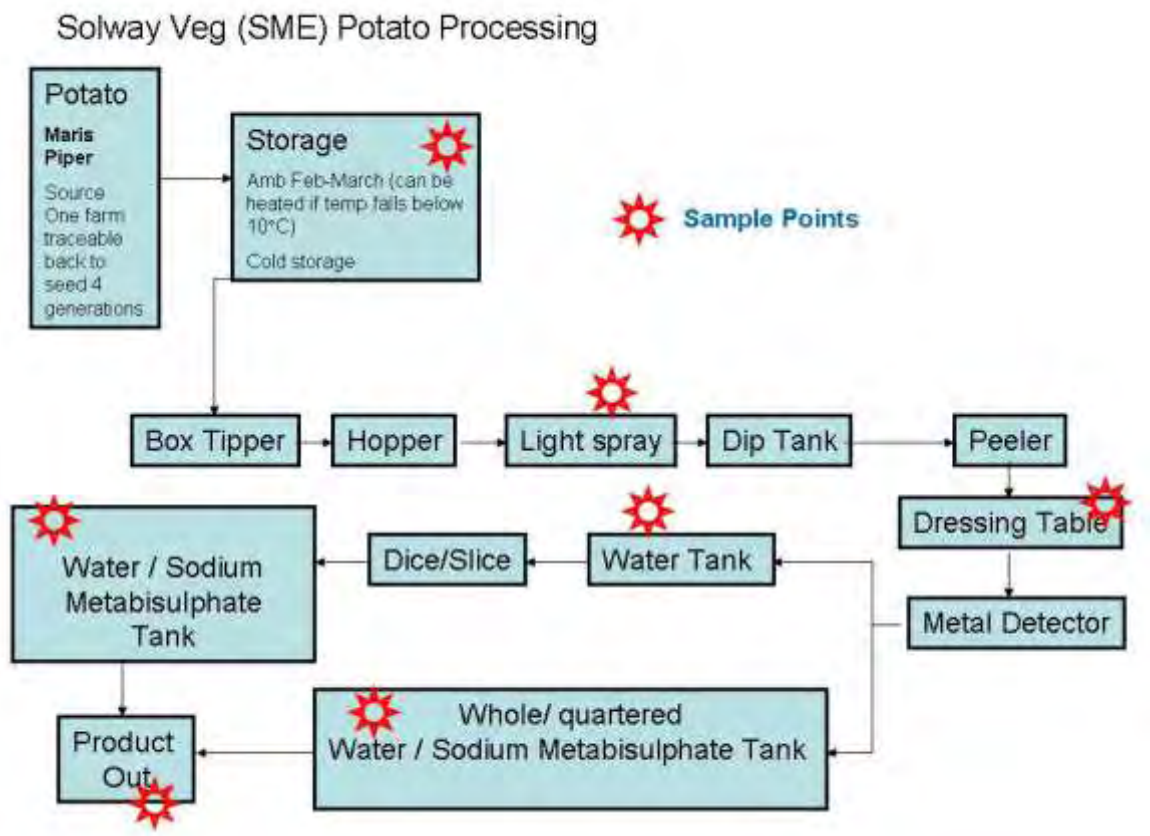


PCA of CSMS data derived from the following samples; Bread wheat grown in Italy (green), Scotland (pink) and Switzerland (black).

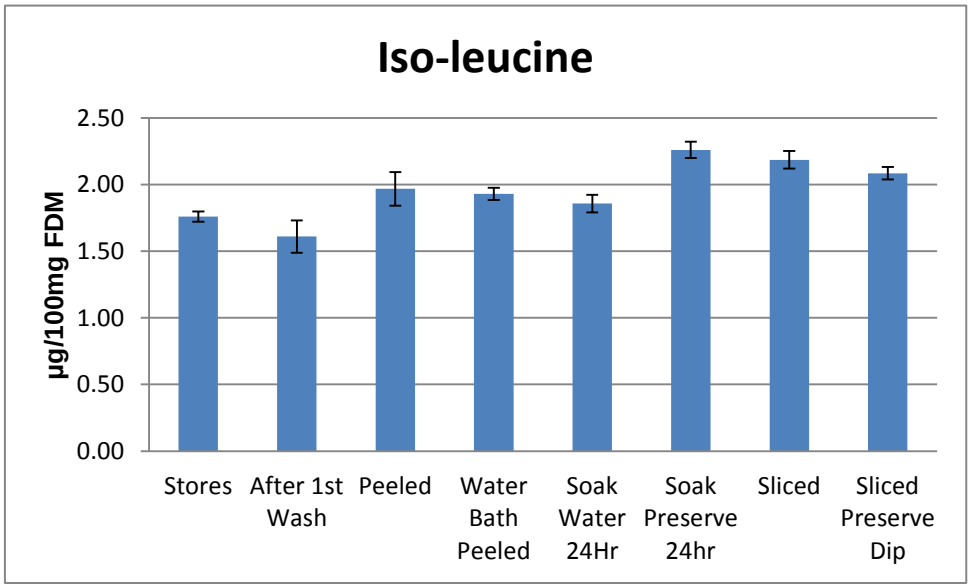
The effect of environment on the bread wheat cultivar metabolomes is shown graphically above. The graphic plots the scores for principal components 1 and 2, explaining for the 54% and 16% of total variance respectively. The metabolites correlated to PC1 were sugars, AAs, FA and OH-FA while those related to second component were mainly OA, TOC and alanine. The samples grown in Scotland differ from those grown elsewhere and this was more evident for samples of the cultivar Bilancia from Italian origin. Moreover, in general we also noted that the cultivar Zinal showed the lower content of tocopherols both in Swiss and UK samples.

Potato processing

Solway Veg Ltd (**P15)** is a vegetable processing and packing company based at Gretna Scotland. As part of DEVELONUTRI P15 agreed access to, and provision of material from, their potato processing line. P15 &P1 collected samples at multiple points throughout the standard potato processing line over a period of two days (29-30 January 2009) to generate two biological reps per sampling point. Samples were frozen under liquid nitrogen onsite at P15 and transported back to P1 for freeze drying and milling as described in the Potato section. Freeze drying of potato was done and the material circulated for analysis.



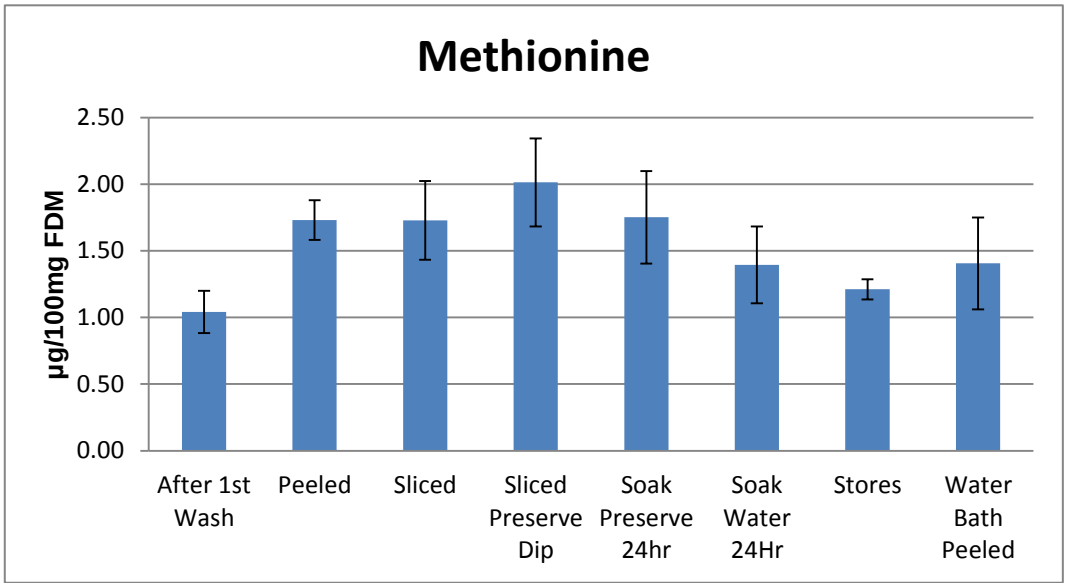
Schematic diagram of Solway Veg vegetable processing line and the points throughout this that were samples for potato metabolite analysis.



Changes in potato isoleucine content during passage though P15's processing plant.

The processed potato material was subject to a quantitative metabolomic approach and this showed that there some unexpected changes in amino acids during the whole process. Many of the amino acids did not show a significant change but the mean value (ug/100mg FDM) gradually increased throughout the process suggesting protein degradation is evident since biosynthetic processes are negligible.

Some of the other amino acids exhibited different patterns with, for example methionine exhibiting a peak at the slided dip stage (see below). The analysis of the data with respect to the other nutrient is being completed for publication.

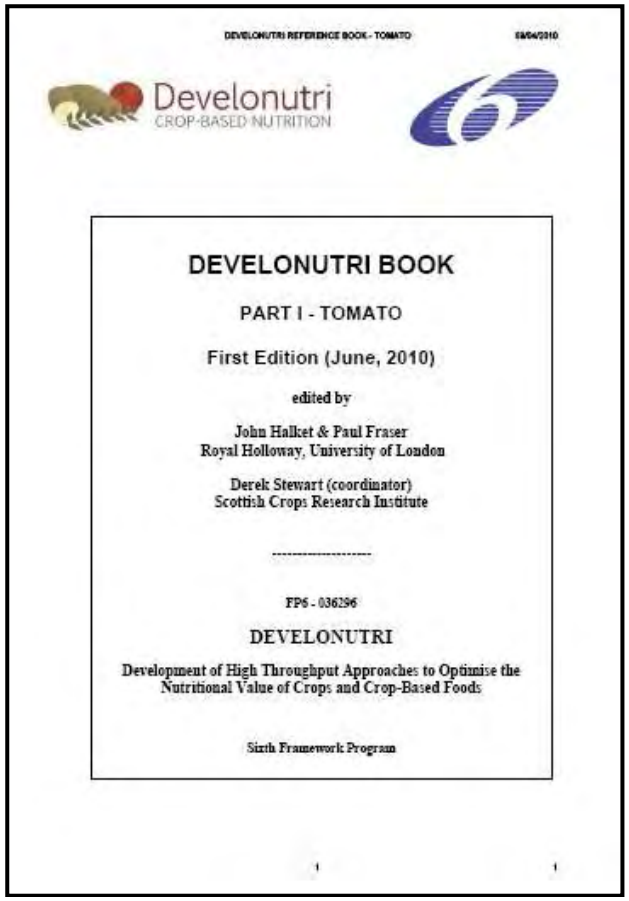


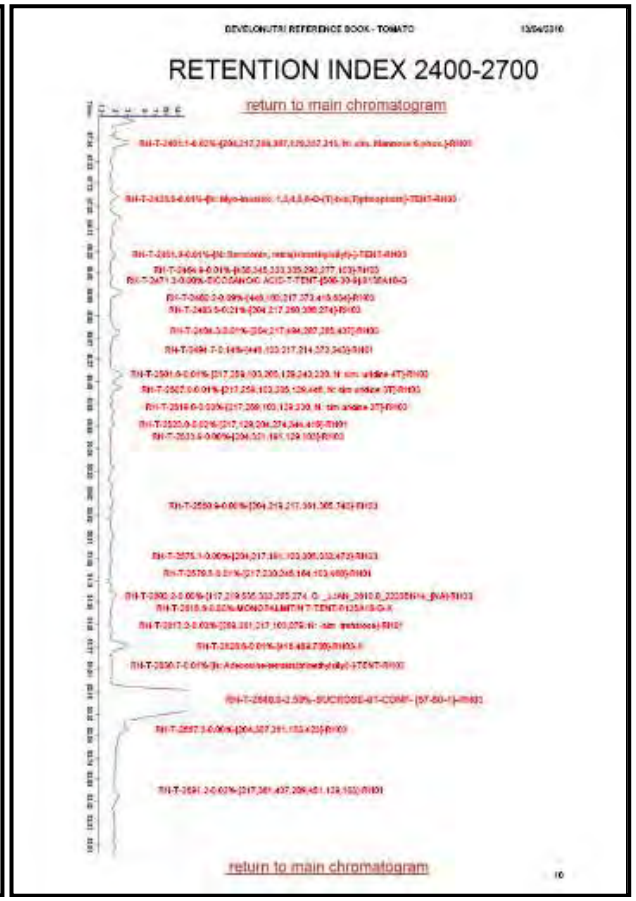
Changes in potato methionine content during passage though P15's processing plant.

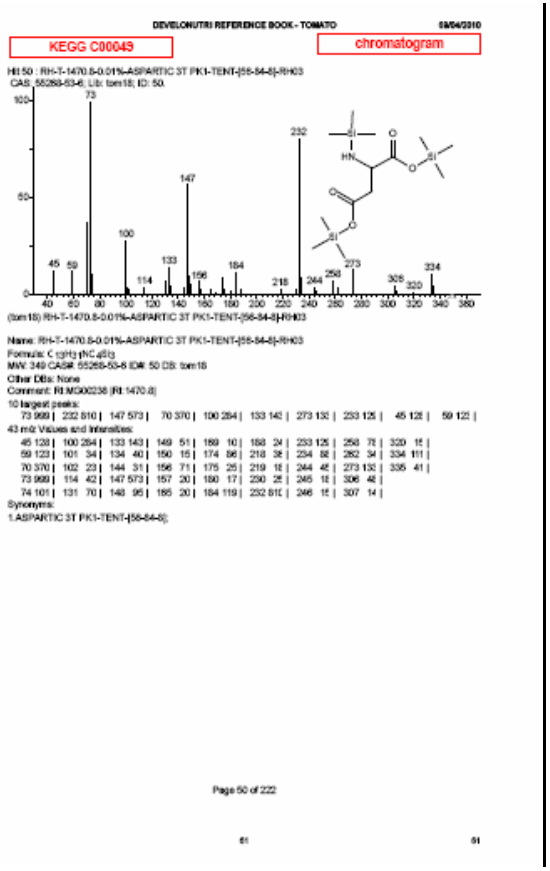
A deliverable identified at the start of the project was interaction with the other EU food related projects specifically META-PHOR, SAFEFOODS, EU-SOL, QLIF and TRUEFOODS. This has occurred on a repeated ad hoc basis for SAFEFOODS, EU-SOL and QLIF and more recently the EU FP6 and 7 capacities projects BarleyBread and BrainHealthFood and the Interreg IVB project Climafruit.. The nature of this interaction has been the exchange of expertise (BarleyBread, BrainHealthFood and Climafruit) generated via DEVELONUTRI into many of these project, co analysis (QLIF & SAFEFOODS) or simple assistance with regard to appropriate methodologies (BrainHealthFood). The interaction with META-PHOR, was less than planned, with respect to meetings, due to the staggered start dates but co publications have been written and submitted with several more planned

<u>Workpackage 2 -</u> Multiple metabolite nutrient and trace element database construction

Second generation Automated Mass Spectral Deconvolution and Identification System (AMDIS) databases were completed for tomato, potato and wheat following refinement based upon data files submitted by partners P1, P2, P5 and P9. A new database system was conceived and executed using the Adobe Acrobat Platform to contain the multi format analytical data (UV/Vis, MS, NMR, IR, etc) to be integrated with respect to the crop derived and associated food stuff (anti)nutrient and micronutrients (see below). A snap shot of this database for tomato showing the retention indices section, naming conventions etc. Within the book the chromatographic peaks are hyperlinked to their corresponding mass spectra giving a hitherto unrivalled picture of the tomato (and latterly potato and wheat) metabolome







Snapshots of the DEVELONUTRI tomato reference book generated from the analyses undertaken on the cumulative WP1 and WP4 derived samples.

Workpackage 3: Emerging technologies and their impact as high throughput screening approaches for plant breeding and metabolite and nutrient analysis.

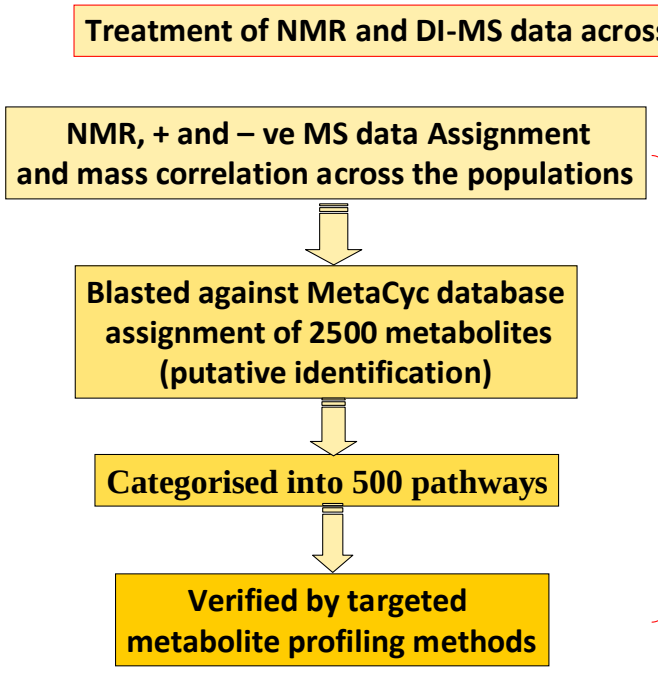
MALDI-ToF-MS. ***P2*** *& P12*

During the initial 24 months the MALDI-TOF-MS approach was evaluated and reported on. In the final year of the project MALDI-TOF/TOF was been evaluated and the advantages of a UPLC front end ascertained. In addition P2 has advanced data analysis methods for Direct infusion MS and their interpretation.

(i) MALDLI-TOF/TOF-MS as demonstrated with crude polar tomato extracts to have fingerprinting capabilities but the initial problem as found with MALDI-TOF was that quantification was the issue as a result this has limited its application to operating at a purely qualitative level.

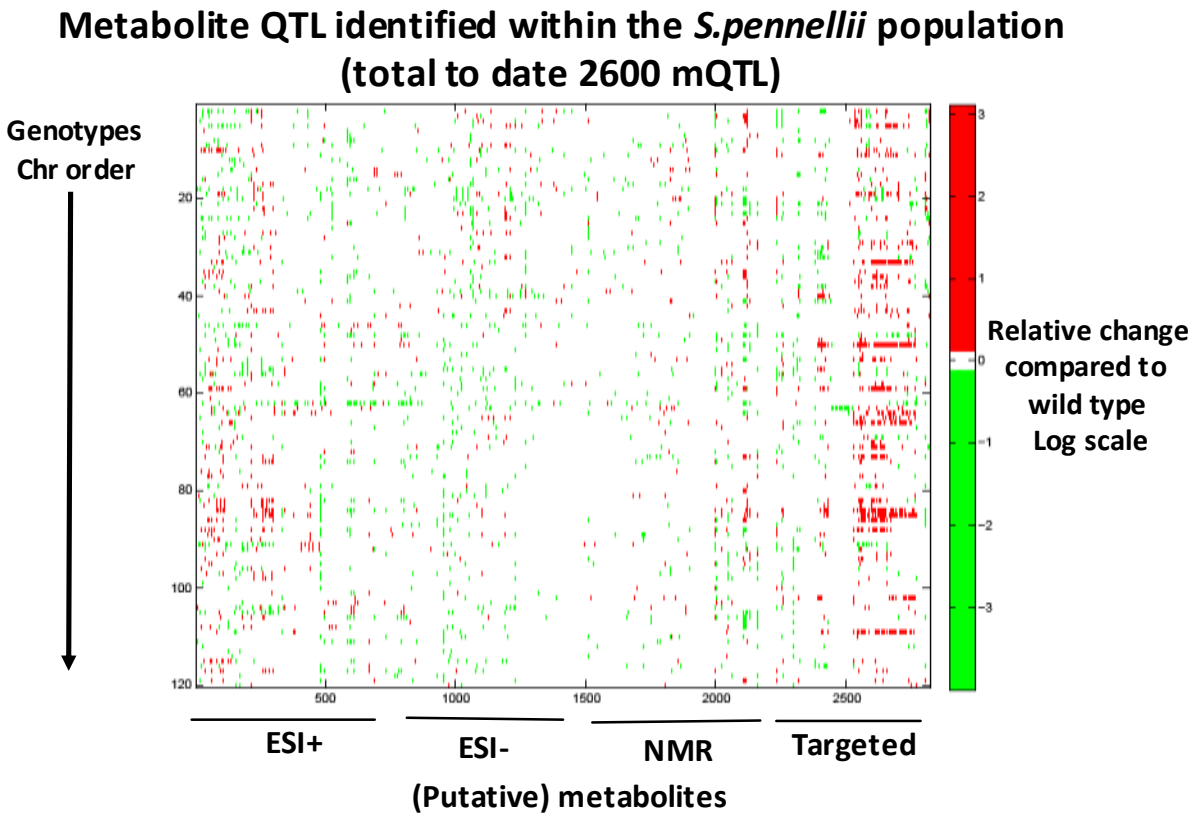
(ii) UPLC. This development in separation technology has been shown to be fit for purpose. Both with phenolic and carotenoid analysis the approach has greater reduced run times by 10-fold. For example, carotenoid analysis is now being performed in 6 mins compared to 60 mins. This technology has been of great benefit to the demonstration activities performed in WP4. The only drawback presently is that no C_{30} columns are available in the UPLC format therefore if detailed isomer profiles are required then the conventional approach is necessary.

(iii) Data analysis- With nominal masses acquired from direct infusion MS and the addition of NMR data, software we have shown that it is possible to establish correlations between (chemo)molecular species and assignment of identities. This approach has been demonstrated on the S.pennellii introgression collections in collaboration with EU Sol. Examples are provided in the figures below



IL	Number of pathways with changes	Mean percentage of changes per pathway
3--3	370	9.87427795
8--2	410	9.719486399
8--1--1	296	4.658395326
9--3--1	313	4.265588353
3930	259	4.072128201
1--1	285	3.285174847
2--4	293	2.798726132
3964	248	3.5815689
10--1	260	2.667015168
3920	250	2.805760992
3935	241	2.681874844
1--3	246	2.451544002
7--1	259	1.985865937
3--2	223	2.441957285
10--1--1	223	2.365885965
2--5	245	2.107716632
7--2	212	2.389473356
3951	212	2.230223322
2--1	204	2.264163335
9--3	221	2.062853962
3939	209	2.161178716
8--2--1	230	1.851186375
3922	218	1.989436224
3--4	194	2.085054547
3941	196	1.979938969
7--3	165	2.033330139
11--1	176	1.890571825
3957	192	1.683705274
1--1--3	187	1.714517925

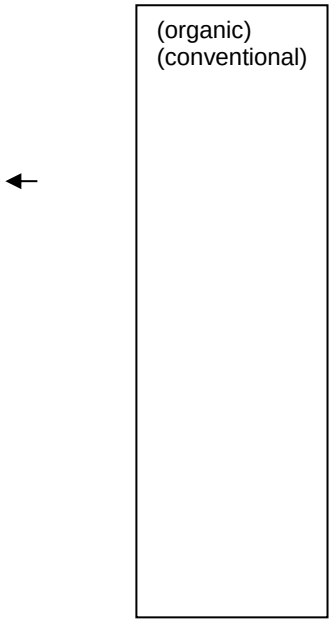
The workflow involved in the assignment of molecular species to be identified.



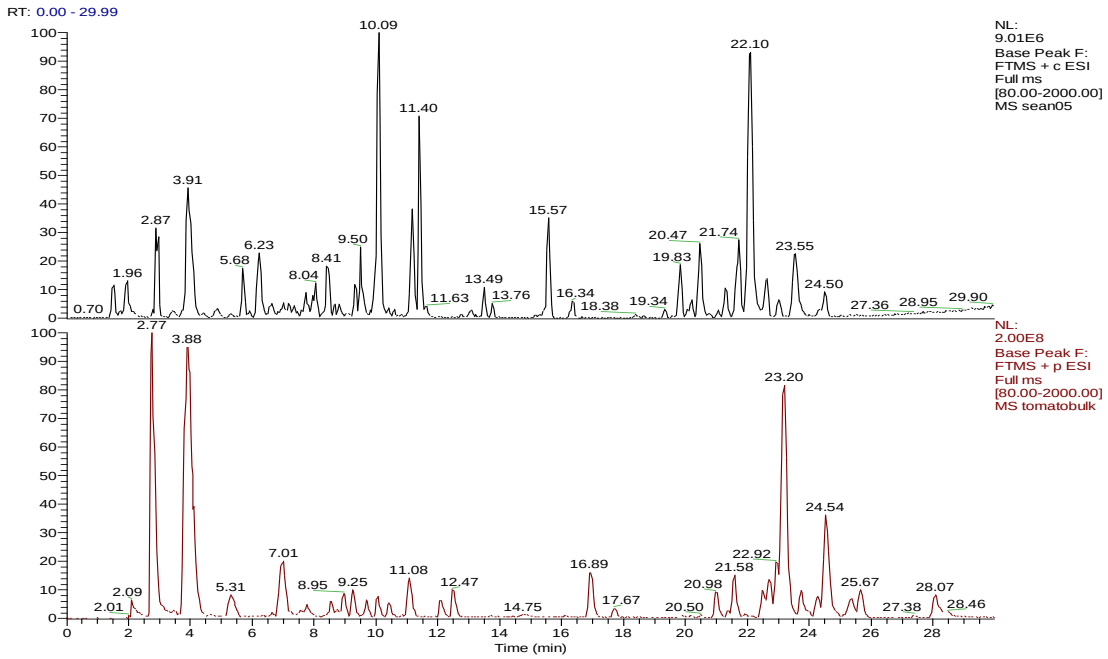
Heatmap illustrating the up and down metabolite QTLs identified in the *S.pennellii* populations using data integration from metabolite interrogative multi-platforms.

3.1.2 FT-MS. ***P3****.*

This section compares results from the LTQ FT Ultra system that is a fully integrated hybrid mass spectrometer consisting of a Linear Ion Trap Mass Spectrometer, LTQ XL, combined with a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer and the LTQ Orbitrap XL system. Samples of potato, wheat and tomato were analysed on both instruments using identical columns and solvent gradients (see below).

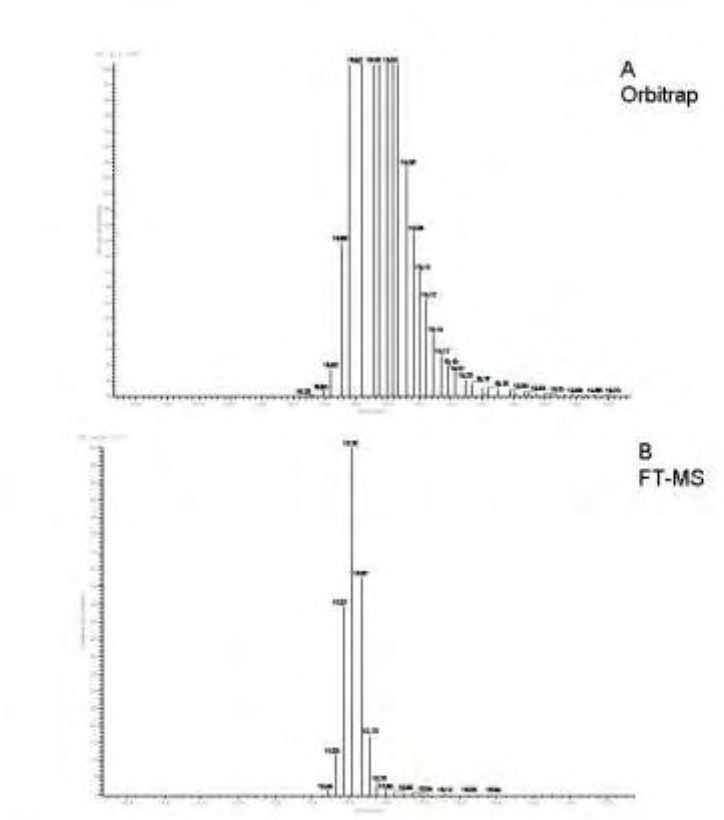


LC-MS analysis of potato (cv Desiree) using FT-MS (top) and Orbitrap (bottom) MS detection systems. (Both systems were run in positive ionisation mode and 80-2000 amu scan range). Note the FT-MS peaks are eluting approximately one minute earlier compared to Orbitrap due to slight differences in LC system.



LC-MS analysis of a pooled variety tomato sample using FT-MS (top) and Orbitrap (bottom) MS detection systems. (Both systems were run in positive ionisation mode and 80-2000 amu scan range).

To enable sufficient scans across an analytical peak (minimum 5 scans) the resolution of the FT-MS had to be reduced to 2000 and this enabled, on average, 8 scans over a 30 second peak. The faster scanning of the Orbitrap meant that it could be operated at much higher scan rates (6000) which gave on average 20 scans over a 30 second peak. The reduced number of scans for the FT-MS instrument means that its quantification was far less robust and reproducible compared to the analogous Orbitrap derived data



A peak in the (a) Orbitrap (b) FT-MS chromatograms showing individual scans acquired under a utilitarian run setup.

Comparison of the metabolites detected by FT-MS compared with the Orbitrap highlighted that the later reported on fewer metabolites. For example, positive mode LC-MS analysis of potato yielded 99 and 77 metabolites for the Orbitrap and TF-MS, respectively. This has significant consequences for nutritional analysis and impacts upon the ability to cover the required nutrient complement. For example, amino acid coverage is limited for FT-MS but with the Orbitrap is able to detect and quantify most of the amino acids under the same chromatographic conditions (see below)

Amino acid quantification by LC-Orbitrap

Compound	**Range (µg mL^{-1})**	**Linearity (R^2)**	**LOD (µg mL^{-1})**	**LOQ (µg mL^{-1})**
Arginine	20-2.5	0.999	0.6	1.200
Glutamine	80-2.25	0.998	0.04	0.064
Histidine	47-0.006	0.981	0.005	0.006
IsoLeucine	9.2-0.002	0.997	0.1	0.300
Leucine	110-0.11	0.998	0.03	0.050
Lysine	90-0.055	0.999	0.006	0.010
Methionine	80-0.009	0.984	0.01	0.020
Phenyl Alanine	120-0.004	1.000	0.004	0.007
Proline	83-0.005	0.997	0.007	0.008
Serine	150-0.58	0.999	0.6	0.650
Threonine	144-0.07	0.995	0.07	0.090
Hydroxyproline	128-2	0.999	4	6.00
Tryptophan	82-0.01	0.996	0.01	0.020
Tyrosine	86-0.01	0.994	0.011	0.021
Valine	83-0.005	0.998	0.001	0.002

The LTQ FT Ultra (FT-MS) does indeed have higher resolution capabilities than that of the Orbitrap but at a cost. It has a resolution of 100,000 and mass accuracy of <1ppb. The Orbitrap exhibits a similar resolution and, with the use of a "lock mass", has a mass accuracy in the region on ~1 ppm that was routinely attainable. However the LTQ FT Ultra had a relatively slow scan rate meaning that as co-eluting metabolites could be missed. Indeed with the addition of UPLC where we have seen previously that run time can be condensed by ~10-fold this further compounds the disadvantage of the FT-MS.

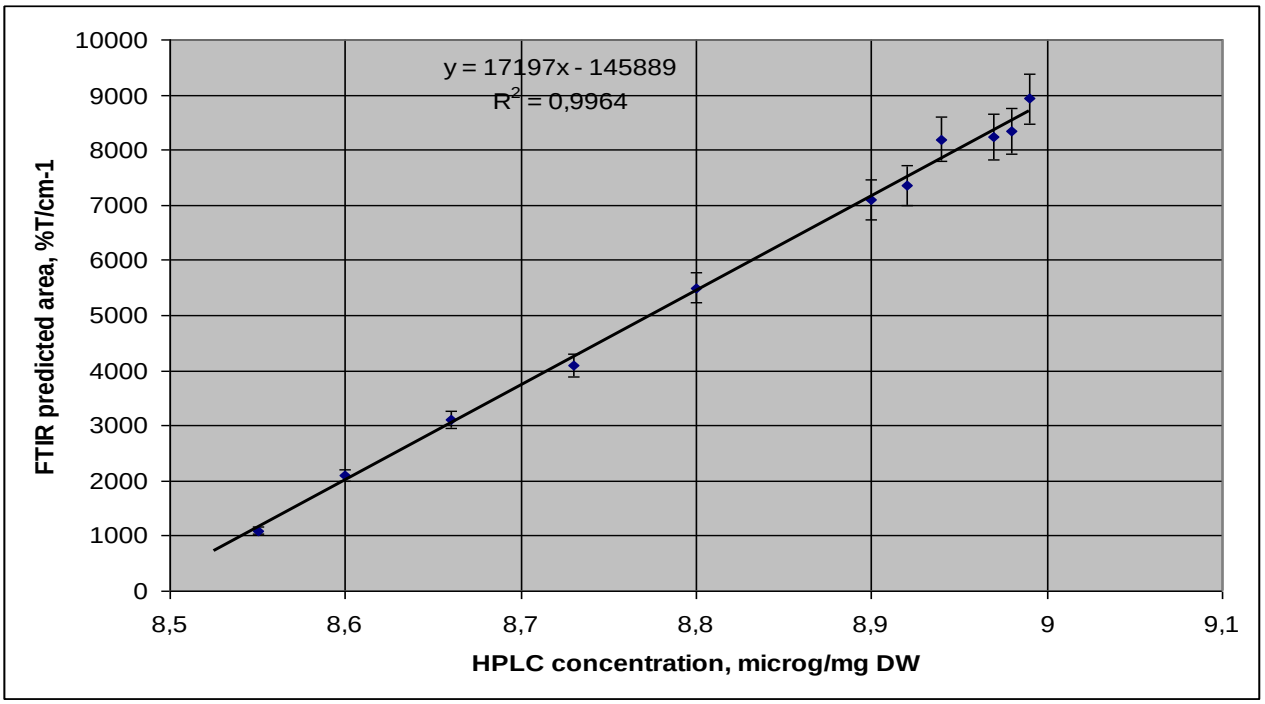
3.1.4 FT-IR. ***P1,*** *P6 & P8.*

The year two report highlighted the potential that FT-IR, a well established and widely used technique, held as a potential multiple nutrient analytical approach. P8 used two approaches; Diffuse reflectance infrared Fourier-transform (DRIFT), for freeze-dried powders and Attenuated Total Reflectance (ATR) for liquid extracts. Freeze-dried aliquots of standard metabolite/micronutrient mixtures were be mixed (in a dilution series) with an IR transparent support (KBr) and analyse by DRIFT spectroscopy. Mixtures spiked with selected metabolites/nutrients were also analysed to establish the sensitivity and the ability to discriminate using this approach.

FT-ATR was applied to the processed tomato samples generated by P6 (WP4). Using lycopene content as an example, it was shown that FT-ATR quantification was in good agreement (R^2 = 0.9996) with that obtained from HPLC but in a fraction of the time and cost (see below). Furthermore multiple nutrients were also shown (data not shown) to be accurately assessed and quantified simultaneously, such as fructose sucrose etc.

Average predicted IR lycopene values compared to average HPLC reference values

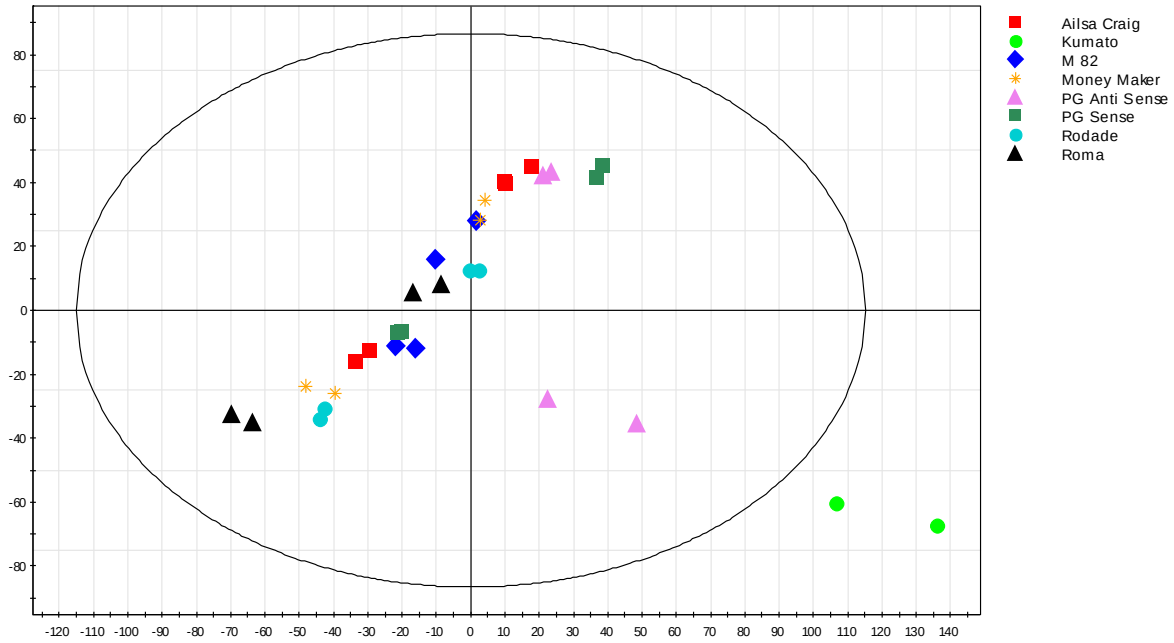
Sample Name and type	ATR □g/mg DW	HPLC □g/mg DW
Olka	8.55	8.57
Tukas	8.56	8.55
Tamek	8.55	8.49
Tat	8.63	8.77
Akfa	8.59	8.6
Burcu	8.6	8.62
Cappy	8.56	8.6
Dimes	8.59	8.64
Metro	8.55	8.62
Dunya	8.56	8.7
Migros	8.55	8.65
Sundried Vacuum packed whole	8.55	8.6
Yedi	8.58	8.67



The correlation of FT-ATR and HPLC derived data relating to the quantitative detection of lycopene in tomato products.

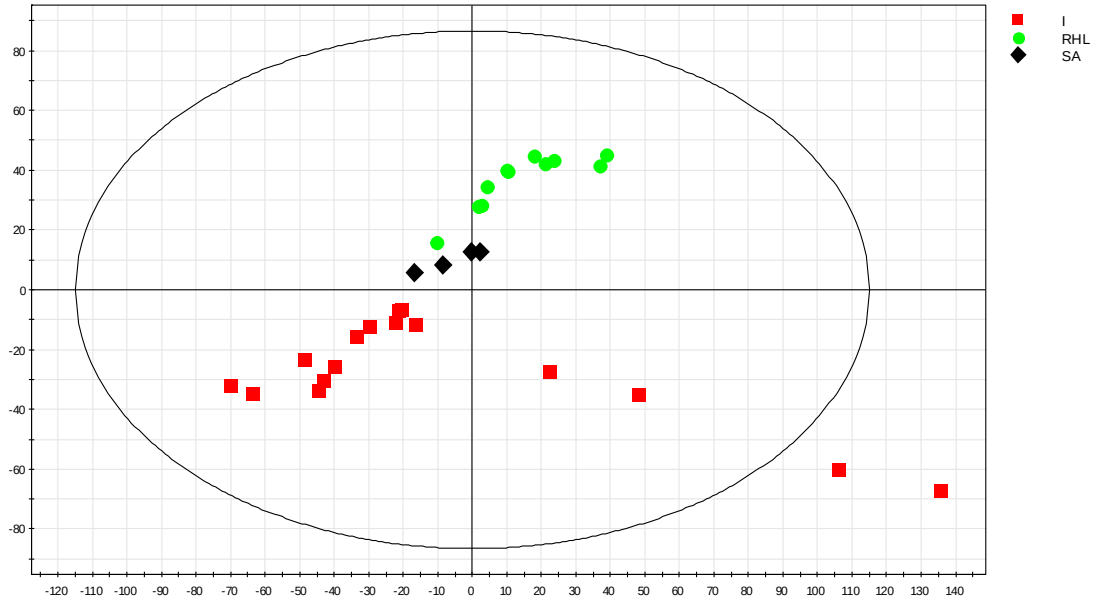
P1 also undertook an FT-ATR approach to full metabolome analysis using the sample identified in the table above. This showed that, like the other more specific analytical approaches (LC and GC-MS), that subsequent PCA of the spectral data could provide global metabolite information.

AFT-ATR and PCA failed to create any significant segregation between the tomato cultivars (see below) although they did not cluster together highlighting that there were different



PCA plot (score 1 v 2) of the FT-ATR data generated from the tomato cultivars (WP4).

However when labelled according to country of origin/growth there was a clear segregation (see below)

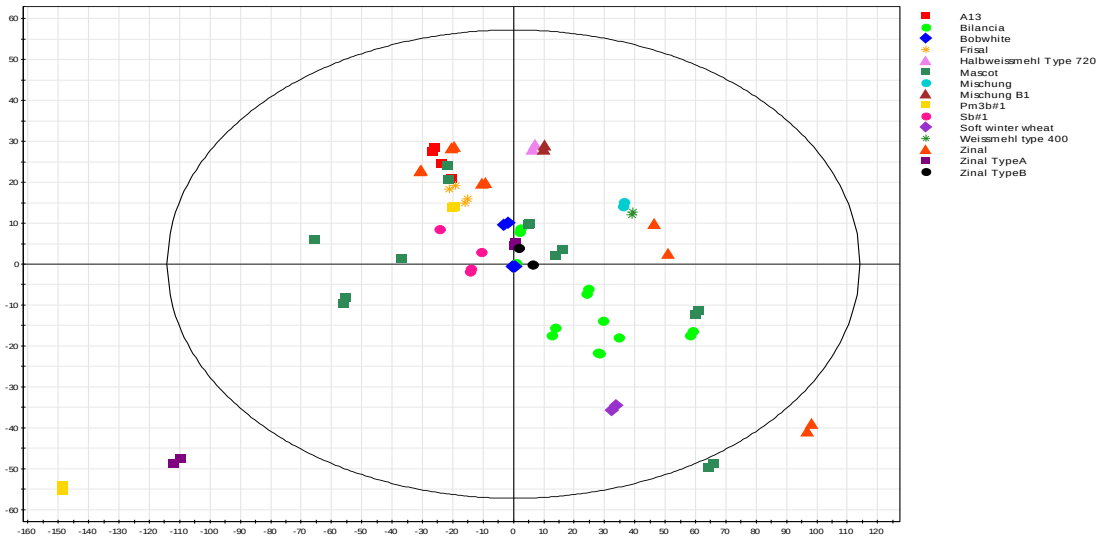


PCA plot (score 1 v 2) of the FT-ATR data generated from the tomato cultivars (WP4) and labelled according to country of growth.

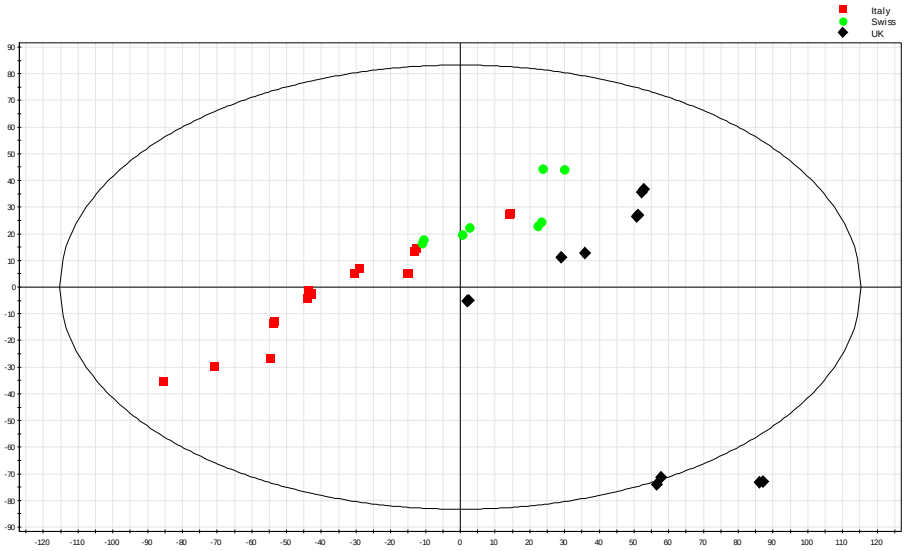
The basis of this segregation at the IR level was a trend (in score 2) of an increase in intensity in the fingerprint region to 1000cm^{-1} and at 3000 cm^{-1}, the later a C-H stretching region and a decrease in the 1200-1600 cm^{-1} and 2500 cm^{-1} from the cultivars grown in Italy to the UK. The interpretation of this in the context of metabolite/nutrient change is ongoing as they are correlated.

Bread Wheat

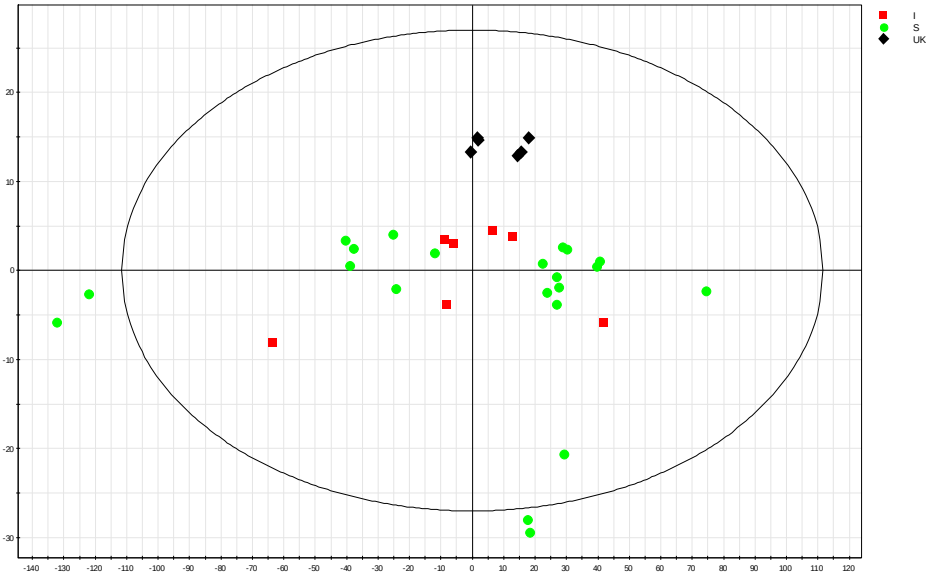
Commercially available cultivars representative of spring and winter wheat adapted to European conditions. One commonly grown cultivar from three European countries were selected for analysis; Bilanca (Italy), Mascot (UK), Zinal (Switzerland). The selected cultivars were grown in UK Italy and Switzerland. Genetically modified wheat from a two Frisal wheat lines (A13 and Sb#1) and a Bobwhite line (Pm3b#1) and their wild type were also included in the study. The PCA plot of all cultivars and GM lines showed no segregation between cultivars. As before, relabeling according to country saw the general pulling apart of the materials. This trend was also demonstrated in bread manufactured from the wheat however this was observed at high scores only.



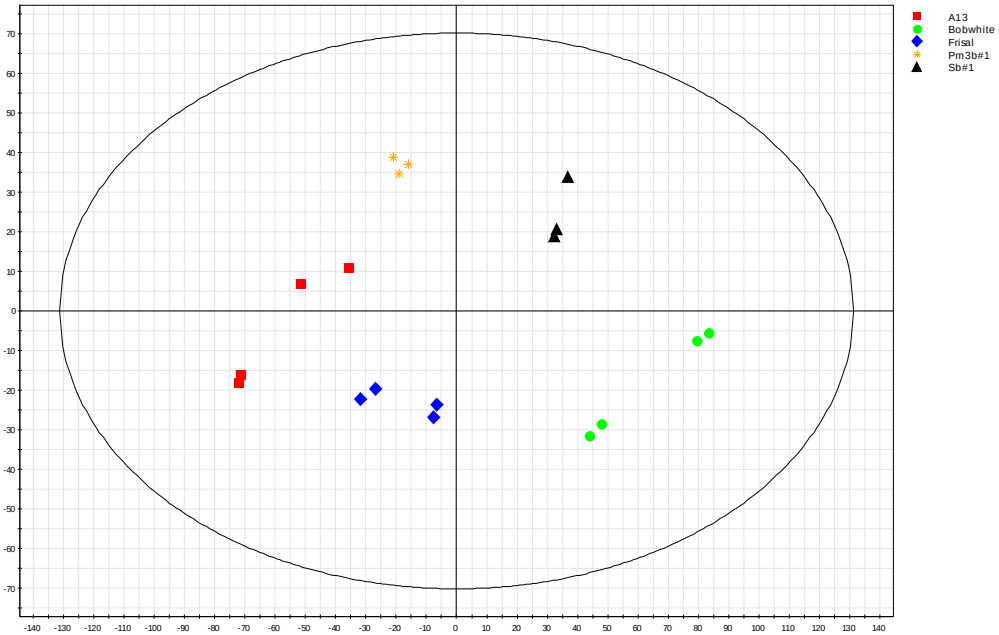
PCA plot (score 1 v 2) of the FT-ATR data generated from the bread wheat cultivars (WP4).



PCA plot (score 1 v 2) of the FT-ATR data generated from the bread wheat cultivars (WP4) but labelled according to country of growth.



PCA plot (score 1 v 5) of the FT-ATR data generated from the bread manufactured from wheat varieties Bilanca, Mascot, Zinal grown in UK Italy and Switzerland. The samples are labeled according to country of growth.



PCA plot (score 1 v 2) of the FT-ATR data derived from the bread wheat GM lines, and their parental and sister lines

Comparison of the parental, GM and sister bread wheat samples demonstrated that all the samples were separable and that Frisal, A13 and SB#1 had a slightly higher absorption in 1500-1600 cm^{-1} region. Whereas the parental Bobwhite exhibited a greater absorbance in the 1500-1600 cm^{-1} region compared with its associated GM, Pm3b#1 (see above). .

<u>Conclusions</u>

Cross-comparison between the MALDI-TOF/MS data and traditional technologies (GC-MS LC-MS and Direct Infusion-MS; as outlined in WP1) indicated that it was not possible to compare the two techniques accurately, although collectively the two techniques were complementary providing valuable identification of, for example, some carotenoids. Presently a MALDI-TOF/MS species-specific database is under construction. Cross comparability may be derived to a degree by the comparison of the molecular ($[M+H]^+$ and

$[M-H]^-$) and fragmentation (MS^n) ions derived from each approach. It is abundantly clear from the comparative studies outlined in WP1 that this emergent technology will be used in a supportive capacity to standard LC-MS when applied to food and raw material analysis. The deficiencies with respect to reproducible quantitative MS analysis mean that it would best be used to support LC-MS with respect to compound characterisation and identification. In fact its utility may rests in this ability first to identify mass descriptors (MS, MS^2, MS^3 etc) providing compound specific ions that can be used by the LC-MS systems for quantitative profiling.

It was also clear that in isolation FT-IR in its various forms has a clear utility in nutrient analysis and with appropriate calibration can simultaneously quantify multiple nutrients simultaneously over a broad range and to a high degree of reproducibility. However when used as an unbiased analytical approach it falters due to the lack of specificity associated with IR absorbance regions. Its real utility lies in the combination with one of the other quantitative technologies (LC and GC-MS) where a calibration can be established and subsequently the IR approach is used to more rapidly quantify nutrient content or change.