Develonutri Year 2 report

Work performed and main achievements of the period

The second reporting period is perhaps the busiest period for experimental work in the project with the significantly large ring testing completed, the biological material generation now, or about to, bear fruit and the true integration of the SMEs into the project via access to process crop materials.

In WP1, wherein lay the responsibility for completion of delivering on partner supply of chemical standards, inter-lab calibration, validation and ring testing of profiling approaches, progress was beyond that expected with the data levels accrued beyond expectation. The outputs from the ring tests highlighted some interesting points with many sources of variation established. For example, the preliminary mineral ring tests highlighted the sample preparation method utilised as the single grates variable in the analyses, irrespective of the analytical technology used. This was corroborated once unity on sample preparation was adopted in a secondary mineral ring test leading to much more uniform mineral contents being reported. The ring testing of the MS based analytical (metabolomic) approaches, using centrally prepared (± nutrient spike) representative crop samples are producing encouraging results and are well on their way to validating the faith shown in them as accurate, robust and quantitative approaches to high throughput food compositional technologies. The cross comparability with the old or traditional methods has highlighted, in all cases, the greater breadth of metabolite coverage and detection level, that the MS-based systems exhibit. These developments are now being applied to real-life" samples; biological material grown under normal condition, varying agricultural practices and on samples derived from the collaborating SME processing lines.

In WP2, first and (in a few months) second generation Automated Mass Spectral Deconvolution and Identification System (AMDIS) software is being constructed that will allows 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. The first generation version has been trialled amongst project partners and the data from this has been fed back for iterative software updates and refinements. Validation of AMDIS utility is being assured by comparison against other deconvolution and machine on-board software. In collaborations with NIST the adaptation of AMDIS for LC-MS based applications is underway based on multi project data but with a significant input from the data derived from DEVELONUTRI.

In WP3 the aim is to establish the efficacy of emergent technologies as future approaches for high throughput screening for metabolites and nutrients in the target crops and foodstuffs. In year 2 the main focus has been the assessment of LC-FT-MS systems. The high accuracy systems (LC-ICR-FTMS) were shown to be extremely valuable in a food scenario due to their ability to generate molecular formulae (and hence putative structural hits) for all compounds separated by chromatography and deconvolution. However the scan times required to ensure this molecular accuracy were significant meaning that LC-ICR-FTMS could not operate as a high throughput technology. Conversely, the comparison with the LC-FTMS systems, in particular the Orbitrap, showed that these systems were high throughput, and that, via the generation of (deconvoluted) peak molecular and MS^n fragmentation ions, they were able to identify and characterise known and unknown compounds when integrated with a standards database (AMDIS). The utility of electrochemical (16 channel coulometric) detection for metabolites was tested and

definite promise was shown with the potential for relatively inexpensive, broad range metabolite detection where MS-based systems were not available. Further, these electrochemical detector systems also have an advantage over the MS-based systems in that they will readily covalently ionised molecular species; $R\text{-}SO_4^-$, $R\text{-}PO_4^-$ etc. FT-IR was utilised and probed as a first screen pass systems for metabolite/compound changes in food systems and using a solid-in-solid dilution approach, showed promised as a quantitative analytical technology. This will be fully probed in year three wherein the MS metabolite data will be used to construct and asses FT-IR based regression curves.

In WP4 the biological material upon which the project relies have been provide by the expert partners each for all analyses. This year has seen considerable effort in this respect with the (often repeated) circulation of potato (SCRI), tomato (RHUL) and wheat (CRA-CRC) for the analytical ranging and ring test studies. The biological material for the filed testing of metabolite determination has largely been completed with a range of varieties and GM materials encompassing the broad range of variability the consumer is likely to see on the open global market. For potato this mean 6 varieties targeted at boiling, potato chips and French fries well as access to a potato processing line via SME collaboration. In addition an GM line has been gift aided from NOFORISK and is a common cultivar down-regulated with respect to the gene Solanosyl glucosyl transferase-1 (SGT-1).

For tomato, six varieties and two GM lines, modified with respect to a poly-galacturonase (PG) gene by either antisense RNA technology or homology-induced silencing, have been grown and will be circulated to the project partners for analyses. Six varieties of durum wheat, chosen for their popularity and high yield/quality characteristic, have been grown at three sites across Italy and the associated grain has since been harvested milled and circulated to the project partners. For bread wheat six cultivars, chosen as representative of spring and winter wheat, have been grown at three partner sites. The associated grain is in the process of milling and circulation. The corresponding GM wheat trials suffered from environmental activist intervention and damage but it is hoped that enough grain will be available for comparative metabolomic analysis. In addition, the furore at the national level surrounding the GM wheat activists meant that the associated SME (P13) withdrew from the project. Means to address this via lab milling or the integration of another SME are under way

In WP5 the organisational issues including IP, outreach, knowledge transfer etc have being dealt with. As an example of the knowledge transfer aspect the project has already had been highlighted and discussed on several UK national television and radio interviews. A project website is being built at www.develonutri.org. In addition, a broad brush article on the project was published in the journal of the European Parliament (http://www.theparliament.com/EN/). Furthermore several papers and presentations focussing on, or utilising data derived from, DEVELONUTRI have taken place.

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

WP1 - In WP1, wherein lay the responsibility for completion of delivering on partner supply of chemical standards, inter-lab calibration, validation and ring testing of profiling approaches, progress was beyond that expected with the data levels accrued beyond expectation. The outputs from the ring tests highlighted some interesting points with many sources of variation established. For example, the preliminary mineral ring tests highlighted the sample preparation method utilised as the single grates variable in the analyses, irrespective of the analytical technology used. This was corroborated once unity on sample preparation was adopted in a secondary mineral ring test leading to uch more uniform mineral contents being reported. The ring testing of the MS based analytical (metabolomic) approaches, using centrally prepared (± nutrient spike) representative crop samples are producing encouraging results and are well on their way to validating the faith shown in them as accurate, robust and quantitative approaches to high throughput food compositional technologies. The cross comparability with the old or traditional methods has highlighted, in all cases, the greater breadth of metabolite coverage and detection level, that the MS-based systems exhibit. These developments are now being applied to real-life" samples; biological material grown under normal condition, varying agricultural practices and on samples derived from the collaborating SME processing lines.

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Section 2 – Workpackage progress of the period

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

In WP1, wherein lay the responsibility for completion of delivering on partner supply of chemical standards, inter-lab calibration, validation and ring testing of profiling approaches, progress was beyond that expected with the data levels accrued beyond expectation. The criteria analysed for were;

1) Validation of response linearity vs concentration for a group of standards.
2) Validation of response linearity vs amount of plant material extracted for both aqueous and non-polar fractions.
3) Validation of response linearity vs volume of aqueous extract derivatised.
4) Validation of response linearity vs injection volume.
5) Validation of extraction and analysis reproducibility.
6) Validation of extraction and analysis reproducibility (multiple injection and/or sample).
7) Validation of extract stability whilst on the autosampler tray (where appropriate).
8) Estimation of sampling errors via repeat injection of samples or repeat analyses.

This generated, for the most part data such as that for the HPLC analyses of phenolic acids and sterols as performed by P8.

Compound	**Range (µg mL^{-1})**	**Linearity (R^2)**	**LOD (µg mL^{-1})**	**LOQ (µg mL^{-1})**
Chlorogenic acid	0-2000	0.9998	0.17	0.60
Rutin	0-2500	0.9999	0.06	0.12
Apigenine	0-25000	0.9952	8.00	26.70
Lycopene	0-5000	0.9995	0.17	0.60
Ferulic acid	0-1200	0.9997	0.40	1.22
α-Tocopherol	0-1000	1.000	1.13	3.76
Sitosterol	0-200	0.9956	0.04	0.13

This was fine for the traditional analyses but extension to the emergent profiling technologies identified some problems. P2 and P8 vigorously trialled MALDI-TOF-MS. (These studies were initiated as part of WP3 directed research into emergent technologies but reported here due to comparison to the mainstay of WP1; LC-MS). They showed that this technique was in reality best suited to chemical fingerprinting and exhibited deficiencies with respect to reproducible quantification. This restricts its suitability as a global quantitative profiling method. However, MALDI-TOF--S has extensive utility in a support capacity to LC-MS based profiling as the MS^n capabilities assist in characterisation and identification of unknown compounds and in the absence of LC-(ICR)-FTMS could fill this requirement.

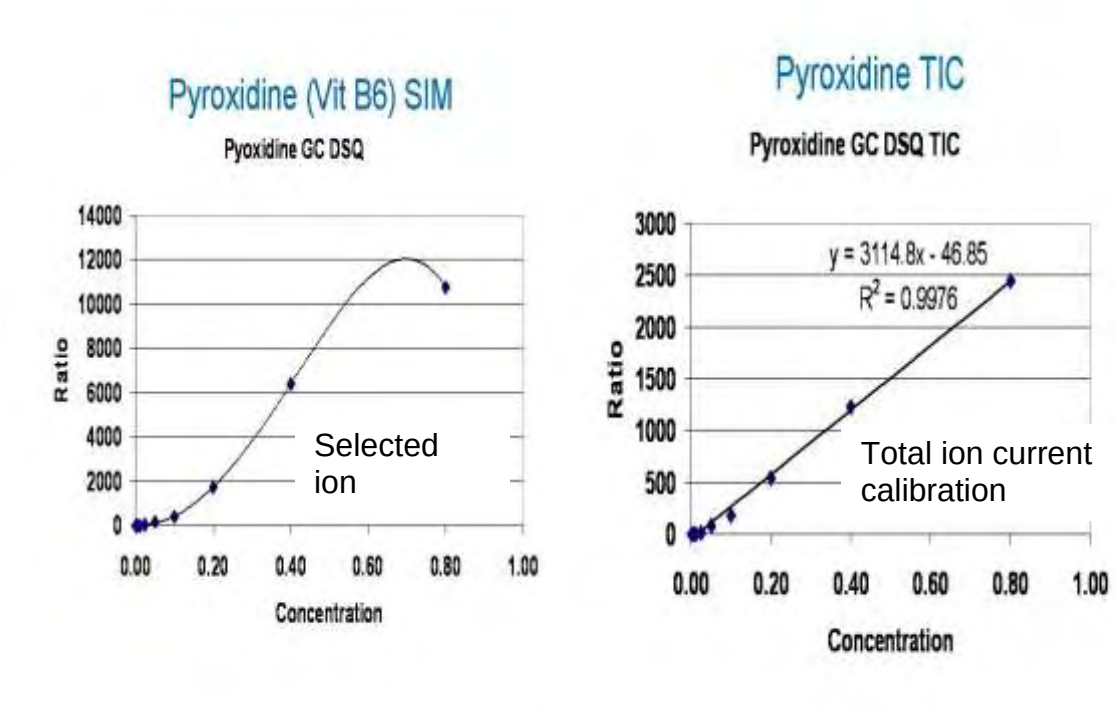
Extension to the metabolomic LC and GC-MS systems to illustrate accuracy and robustness also highlighted some problems. For example P1's linearity testing on GC-MS show a machine dependent problem with compound response lineraity

The outputs from the ring tests highlighted some interesting points with many sources of variation established. For example, the preliminary mineral ring tests highlighted the sample preparation method utilised as the single grates variable in the analyses, irrespective of the analytical technology used. This was corroborated once unity on sample preparation was adopted in a secondary mineral ring test leading to much more uniform mineral contents being reported. The ring testing of the MS based analytical (metabolomic) approaches, using centrally prepared (± nutrient spike) representative crop samples are producing encouraging results and are well on their way to validating the faith shown in them as accurate, robust and quantitative approaches to high throughput food compositional technologies. The cross comparability with the old or traditional methods has highlighted, in all cases, the greater breadth of metabolite coverage and detection level, that the MS-based systems exhibit. These developments are now being applied to real-life" samples; biological material grown under normal condition, varying agricultural practices and on samples derived from the collaborating SME processing lines.

Workpackage objectives (12-24 mths)

Within the work package there are several specific objectives that start but do not necessarily finish in this reporting period (12-24 mths). In essence the overall aim is to WP1 is the provision of known metabolites and nutrients in wheat, potato and tomato and the coordinated analysis and quantification of these using the existing technologies (AOAC methods, LC-RU, UV, PDA, ICP, etc) and comparison with the profiling approaches (LC-$MS^{(n)}$ CC-(ToF)-MS, NMR) across the partners. As part of WP1 the plan was to establish validated profiling approaches to complex metabolite/nutrient mixtures through ring-testing and to subsequently apply validated approaches to biological materials provided by WP4.

Extensive method development has been performed with both LC-MS and GC-MS to illustrate accuracy and robustness. Examples of P1's linearity testing on GC-MS are provided in Figure 1.



The GC-MS calibration curves (P1) for pyridoxine (Vit B6) derived from selected ion response (left) and area under the peak in total ion current (TIC, right) modes

These two graphs derived from the exact same raw GC-MS data clearly show the potential problems associated with using MS-based systems for metabolite quantification. When the selected ion, compound specific, method is used then straight line calibration is only evident at lower concentrations. Indeed if this method was the sole one used then for higher concentrations there is the situation where two potential answers could be derived for the same response reading. This problem arises due to ion suppression in the MS at higher concentrations and is very ion/fragment specific. However, calibrations based on area under the TIC-derived peak exhibit a very acceptable straight line response across a large concentration range.

Two points can be highlighted from this. Firstly, that the utility of MS-based quantification is vindicated with provisos regarding the quantification method. Secondly, the quantification needs to be probed to see what (compound specific) ions/fragments are used for the quantification, which would be the case for overlapping peaks, or whether a TIC-based "area under the peak" method is sufficient. This will depend on the complexity of the sample as using the latter approach for reporting on peaks in a complex biological sample will invariably lead to misreporting (overestimation) for some components due to co-elution/peak overlapping.

The ringtesting of SOPS was a major activity in year 2. The validations of these were assessed by a series of experiments outlined below.

Validation ring testing protocol for organic small molecular weight (<2000 amu) molecules

Expt.1.

1. Five 100mg aliquots for each sample are weighted out and placed into independent tubes/vessels.
2. Methanol (1ml) is added the suspension mixed (vortex) 3 times for 3 second bursts.
3. Water (1ml) is added the suspension mixed (vortex) 3 times for 3 second bursts.
4. Chloroform (3ml) added the suspension mixed (vortex) 3 times for 3 second bursts.
5. The suspension is incubated at 4^{o}C for 30 mins.
6. The mixture centrifuged at 3,000 rpm for 5 min.
7. Aqueous (hyperphase) removed and placed in a clean tube/vessel for subsequent analyse of polar compounds.
8. Chloroform (hypophase) removed and placed into a clean tube/vessel for subsequent analyse of non-polar compounds.
9. Non-polar chloroform fraction is dried and stored at -20^{o}C (in aliquots if appropriate).
10. Stored aqueous phase at -20^{o}C (in aliquots if appropriate).
11. The polar and non-polar fractions are then analysed on your analytical/derivatisation platforms available.
12. Determine only the predominant bio-active or anti-nutrients on each system (to keep everything simple) if you have isomers just the predominant isomer.
13. Determine the amounts of the predominant compounds firstly by (i) dose-response curve, (ii) relative to an internal standard ADDED TO THE EXTRACT after extraction and prior to running on the analytical platforms/ derivatisation.
14. Report the values as micro grams per milligram dry weight (□g.[mg dry weight]$^{-1}$), not as an average but individually.

Expt. 2.

P2 (provider) sent one large bulk extraction for each plant material. Aliquots of the extractions were generated to give each of the project RTD partners' five dried polar and non-polar fractions. The levels of the predominant bio-actives and/or anti-nutrients were determined as described for Expt. 1 following points 11 to 15.

Expt. 3.

P2 sent four spiked freeze-dried aliquots of each plant material for each partner. The levels of components were determined as described in Expt 1 following points 11 to 15.

A selected example (phenolics) of the data derived from the LC-MS analysis as performed by P8 is provided below.

LC-MS determination of phenolic compositional present in the ring test biological samples analysed following the validation ring testing protocol described above. Analyses performed by P8.

P1 = Wheat/Exp.2	Ferulic acid (mg/l)	Apigenine (mg/l)	Ferulic acid /sample (mg/l)	Apigenine/sample (mg/l)
	16.78408	1.668022	0.08392 ±0.0033	53.401 ± 0.082
	19.08969	1.01651	0.0954484±0.0033	50.8256± 0.082
	16.81219	0.9602	0.0840609±0.0033	48.014332± 0.082
	16.80640	1.16033	0.084032±0.0033	58.0165± 0.082
	16.67571	1.01735	0.0833785±0.0033	50.8675± 0.082
	16.74274	1.14174	0.0837137±0.0033	57.087± 0.082
P1' = wheat/exp.3 control	17.42122	1.022286	0.0871061±0.0033	50.143± 0.082

P2 = Potato/Exp.2	Ferulic acid (mg/l)	Chlorogenic acid (mg/l)	Ferulic acid /total sample (mg/l)	Chlorogenic acid/total sample (mg/l)
	20.90960	292.86452	0.104548 ±0.0126	1.4643226 ±0.005
	21.77959	292.85856	0.1088979±0.0126	1.4642927±0.005
	22.46849	295.56120	0.1123424±0.0126	1.477806±0.005
	22.03190	296.24930	0.1101595±0.0126	1.4812465±0.005
	22.39340	294.96131	0.111967±0.0126	1.4748065±0.005
	22.23276	296.10046	0.1111638±0.0126	1.4805023±0.005
P2' = Potato/exp.3 control	20.39834	206.14763	0.1019917±0.0126	1.0307381±0.005

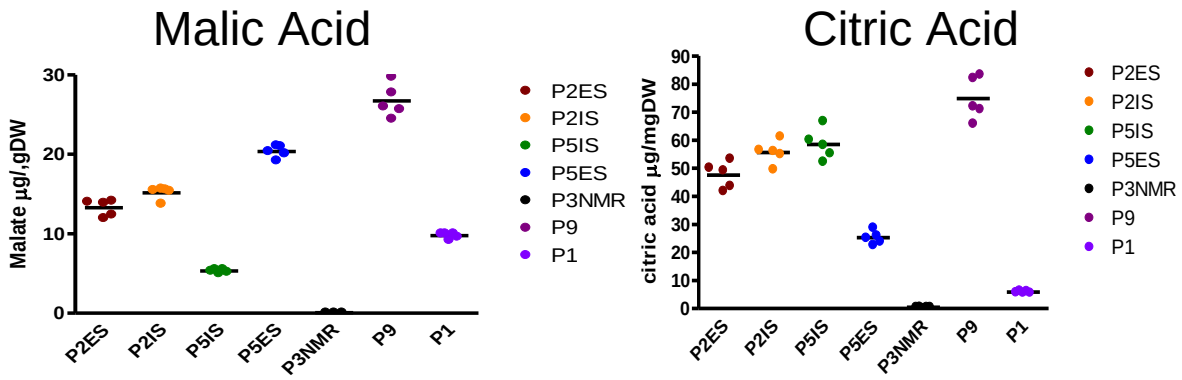
P3 = Tomato/Exp.2	Rutin (mg/l)	Chlorogenic acid (mg/l)	Rutin /total sample (mg/l)	Chlorogenic acid/total sample (mg/l)
	227.36450	227.36518	1.3368225 ±0.0046	1.3668259 ±0.016
	224.62340	273.13998	1.123117±0.0046	1.3656999±0.016
	225.98864	279.10754	1.1299432±0.0046	1.3955377±0.016
	226.17443	283.87769	1.1308771±0.0046	1.4193884±0.016
	225.15131	277.66186	1.1257565±0.0046	1.3883093±0.016
P3' = Tomato/exp.3 control	213.85827	263.58556	1.0692913±0.004	1.3179278±0.016

Other examples of how this unified extraction worked is highlighted below and this shows how the same chromatographic system but a different detectors (MS and DAD) can produce generate different results although in the same ball park.

Analysis of carotenoids in different tomato varieties by P6

	Lutein (µg/g)		β –carotene (µg/g)		Lycopene(µg/g)
r^2	0.99942	0.99724	0.99977	0.99913	0.99957
Sample ID	**DAD**	**MS**	**DAD**	**MS**	**DAD**
Kumato	14.5	15.2	11.6	10.6	211.3
Ailsa Craig	17.6	18.4	49.4	47.6	2377.7
Money Maker	7.9	7.9	18.8	17.8	376.9
PG Sense	11.8	10	33.6	26.7	1201.9
PG Anti	13.6	10.1	39	25.3	1344
M82	11.1	10.5	16	27	1012

In the case of the GC-MS profiling the composition over 60 metabolites was similar however quantitative variation was found between partners. For example, the figure below for malic and citric acid demonstrates this. Variability has now been addressed for P5 and P3, while P1 is addressing the robustness of their GC-MS platform. The variation in response for P1 has since been shown to be largely derived from the comparatively truncated GC runtime leading to variation in assigning peak areas in post-acquisition processing. This will be addressed in the analyses of year 3 material.



Examples for the levels of malic and citric acid estimated from select partner GC-MS profiles

Our conclusion for the organic small molecule platforms are that a validated procedure can be used with a harmonised extraction. Further work is required by several partners to improve the accuracy and robustness of their platforms and post-acquisition method. This aspect is being addressed.

The aim of the extraction procedure was universal extraction of many compounds simultaneously and this principal was achieved. However we must be clear that the

extraction procedure was not optimal for all compounds. Ring-testing of targeted methods, for example carotenoids, was successful between partners P2, P6, P7, and P8,

With respect to the micronutrient (mineral) testing the following protocol was applied as a validation ring-test. This follows on from problems evident in the first attempt at mineral content ring testing where it was evident that the partner specific methods gave a significantly large margin of variation predominantly due to the mineral extraction and preparation protocols. To counteract this the following single protocol was devised to be used by all partners actively analysing mineral content in the samples.

Protocol for DEVELONUTRI mineral ring test (tomato, potato and wheat samples)

1. Preparation of the samples
    a. Prior to digestion dry the samples at 100°C for 24 h
    b. Weigh approximately 500 mg per sample
    c. Make 3 repetitions per sample
    d. Wash all material with HNO_3 and HCl prior to use
    e. In addition to the crop samples weigh in 3 times 500 mg of the NIST 1568a standard which I will distribute to you

2. Digestion procedure
    a. Add 3 ml of HNO_3 and 1 ml of H_2O_2 to each digestion vessel
    b. Use the following microwave program:
        i. Heat to 70°C in 20 minutes
        ii. Heat to 130°C in 40 minutes
        iii. Heat to 200°C in 30 minutes
        iv. Leave at 200°C for 20 minutes
    c. Add an internal Ytrium standard (10 ppm) to every sample
    d. Dilute the digested samples with 0.1% HNO_3 to a volume of 25 ml

3. ICP-OES measurements
    a. Measure each mineral at least at two different wavelengths (at the one indicated below and at a second one depending on your machine and your preference)
    b. Make three technical replicates/measurement

Element	Wavelength (nm)
Ca	315.887
Cu	324.754
Fe	238.204
K	769.896
Mg	279.553
Mn	257.610
Mo	202.032
Na	588.995
P	178.287
Zn	213.856

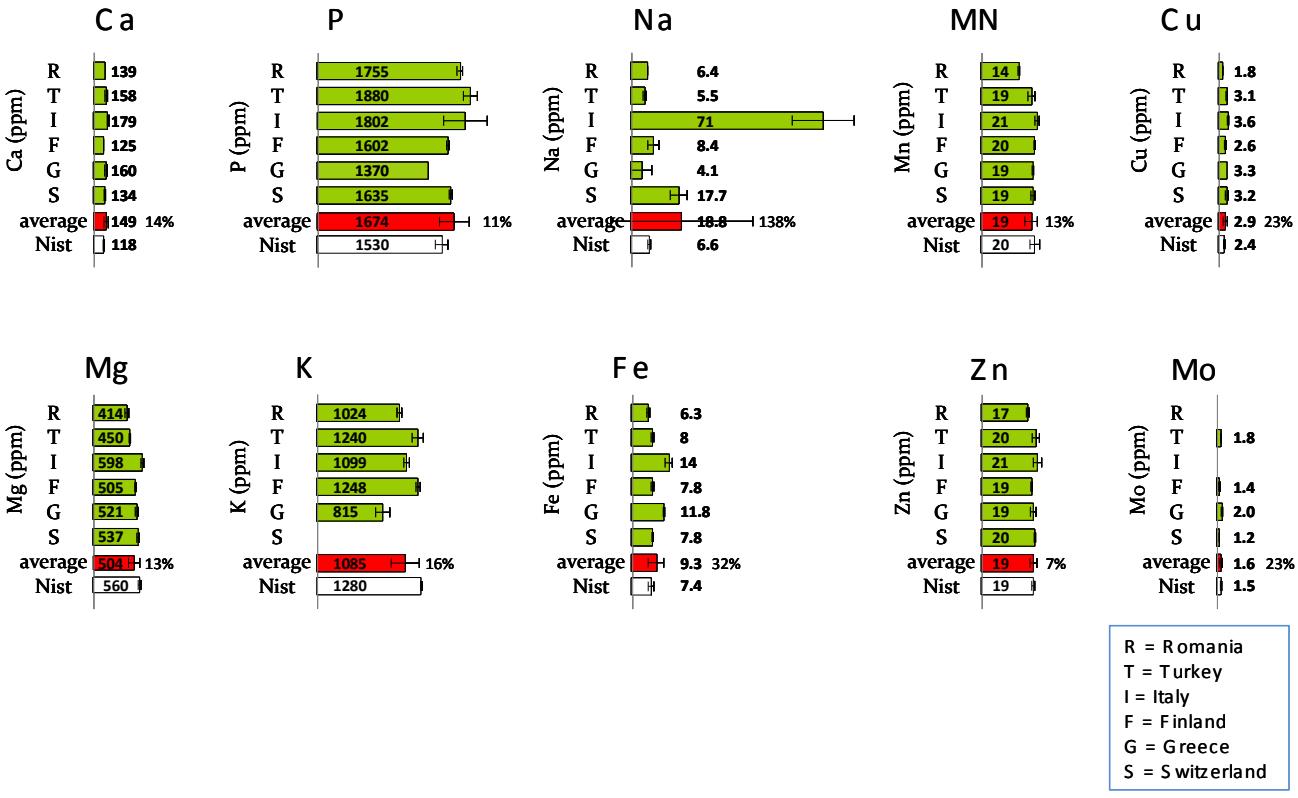
4. Analyzing the data
    a. Use the following formulas to determine the limits of detection and quantification
        i. Limit of detection: 3*stdev (blind)/slope (calibration line)
        ii. Limit of quantification: 10*stdev (blind)/slope (calibration line)

The above protocol for the analysis of minerals was developed and distributed to all the partners involved in this activity. The elements measured were Cu, Ca, Fe, K, Mg, Mn, Na, P, Zn. Since the Se and Mo concentrations were too low to be detectable with ICP-OES, they were omitted from the ring test. Homogenous samples have been distributed with samples of wheat, lyophilized tomato and potato samples, plus aliquots of the NIST

rice standard 1568a (https://www-s.nist.gov/srmors/certificates/view_cert2pdf.cfm?certificate=1568A).

The results of this second ring test were collected and evaluated and provided largely consistent data. Values of all groups reported close to the NIST standard with the exception of sodium in one group (P5), which might have been due to a Na contamination from glass vessels. In contrast to the NIST standard, the measurement of the crop samples provided selected significant variation, e.g. the Fe content as reported by P8. Not all the mean values met the USDA standard. As a result of the partner specific variability the decision was made that the demonstration measurements of the minerals should be confined to P1, P4 and P5.

NIST measurements



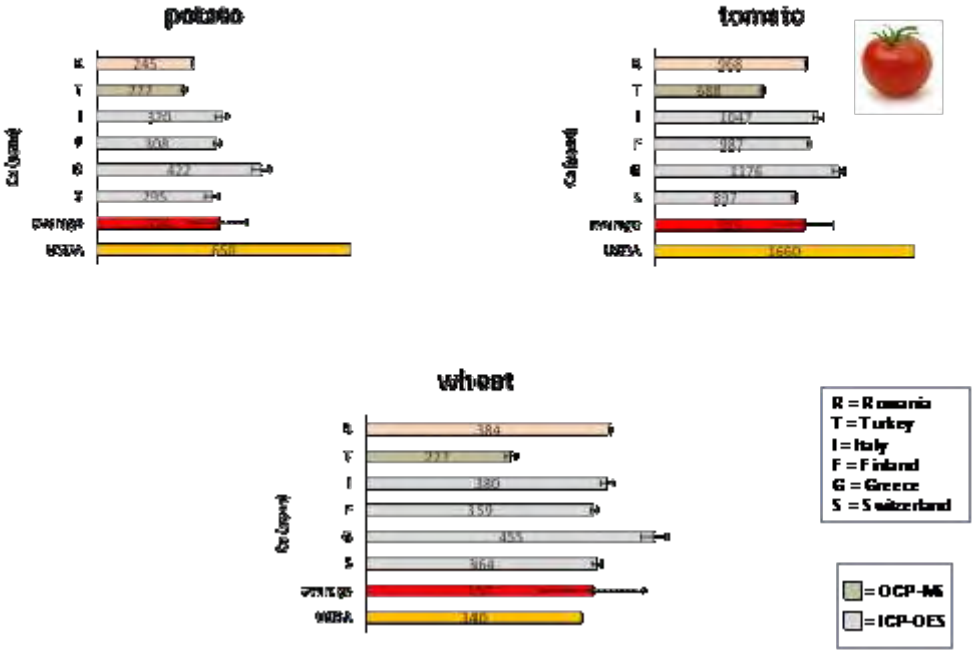


Figure 3. The selected mineral contents of the NIST rice standard (upper) and representative Ca contents of the ring test potato, tomato and wheat material as analysed by partners P4-8 & P10.

Workpackage 2

WP2 had a heavy commitment to improve the quantitative capability of the NIST AMDIS software (Automated Mass Spectral Detection and Identification System) for high throughput metabolic profiling. Indeed using it facilitated the construction of a tomato metabolite book and the other crop ones will be forth coming as time and funding allows

An example of the LC-MS, FTIR and ICP-AES annotation used by P8 for their database to identify (anti)nutrients in the target crops and products: potato, wheat and tomato.

Table 5. The LC-MS, FTIR and ICP-AES annotation used by P8 to identify (anti)nutrients in the target crops and products: potato, wheat and tomato.

Compound Class	Compound	Molecular weight	LC/MS	FT-IR	Calibration LC/MS	Molecular Formula
Phenolic Acids	Ferulic Acid	194.18	X	X	X	$C_{10}H_{10}O_4$
	Lycopene	536.88	X		X	$C_{40}H_{56}$
	Rutin	610.53	X	X	X	$C_{27}H_{30}O_{16}.3H_2O$
	Chlorogenic acid	354.31	X	X	X	$C_{16}H_{18}O_9$
	Lutein	568.9	X		X	$C_{40}H_{50}O_2$
	Apigenine	397.636	X		X	$C_{27}H_{43}NO$
Sterols	Sitosterol	414	X		X	$C_{29}H_5O$
	Cholesterol	386		X		$C_{27}H_{46}O$
Vitamins	B1 Thiamine HCL	265.35	X	X	X	$C_{12}H_{17}N_4OS;$
	B2 Riboflavin	376.36	X	X	X	$C_{17}H_{20}O_6N_4;$
	B3 Niacin (Nicotinic Acid)	123.6	X		X	$C_6H_5NO_2;$
	B5 Pantothenic Acid	219.23	X		X	$C_9H_{16}O_5N$
	Vitamin B6 Pyridoxine	169.18	X	X	X	$C_8H_{11}NO_3$
	Vitamin B11/ Folic acid	441.40				$C_{19}H_{19}N_7O_6;$
	Vitamin B12/cobalamine	1355.4	X		X	$C_{63}H_{88}CoN_{14}O_{14}P$
	Vitamin C	176.13	X	X	X	$C_6H_8O_6$
	Vitamin E/ alpha tocopherol	430.7	X		X	$C_{29}H_{50}O_2$
	Vitamin H Biotin	244.13	X	X	X	$C_{10}H_{16}N_2O_3S$
	Vitamin K1 Phylloquinone	450	X		X	$C_{31}H_{46}O_2$

GC-MS derived database reporting for the ring test tomato, potato and wheat samples as reported by P1

Name	RT	Identifier Masses	Tomato	Potato	Wheat
L-Alanine (diTMS)	1.12	116	X	X	X
OxalicAcid (diTMS)	1.33	190.00,219.00	X	X	X
L-Valine	2.17	144	X	X	X
Leucine (diTMS)	2.68	158	X	X	X
Niacin(NicotinicAcid)(TMSe)	2.83	180,136,106,78,195	O	O	O
L-IsoLeucine(diTMS)	2.85	158	X	X	X
L-Proline (diTMS)	2.87	142	X	X	X
Glycine (tritms)	2.94	174	X	X	X
SuccinicAcid (diTMS)	3.03	247	O	O	O
L-Serine(triTMS)	3.35	204	X	X	X
L-Threonine (triTMS)	3.51	218.5	X	X	X
b-Alalnine	3.78	174,248	X	X	X
MalicAcid (triTMS)	4.15	233	X	X	X
L-Methionine	4.30	176	X	X	X
L-AsparticAcid	4.32	232	X	X	X
GlutamicAcid(triTMS)	4.83	246	X	X	X
L-PhenylAlaline	4.84	192,218	X	X	X
Asparagine(4TMS) ?	4.86	188	X	X	X
Asparagine(triTMS)	5.07	116	X	X	X
L-glutamine(triTMS)	5.35	227	X	X	X
Glutamine	5.54	156	X	X	X
CitricAcid	5.74	273,363,465	X	X	X
QuinicAcid	5.89	345	X	X	X
d-fructoseoxime(5TMS)	5.95	307	X	X	X
Fructoseoxime(5TMS)	5.99	307	X	X	X
Mannose	6.01	319	X	X	X
Galactose	6.02	319	X	X	X
unox glucose	6.04	204	X	X	X
GlucoseOxime(5TMS)	6.05	319	X	X	X
unoxglucose	6.07	204	X	X	X
Pyridoxine(triTMS)	6.10	147,290,295,370	O	O	O
p-CoumaricAcid(TMS)	6.12	219,249,293,308	O	O	O
Histidine(triTMS)	6.15	154254	X	X	O
L-Lysine	6.16	174	X	X	O
L_Tyrosine	6.23	218	X	X	X
PantothenicAcid(perTMS)(DSQ)	6.26	291,247,201,157,103	O	O	O
Unoximated Glucose	6.39	191,204	X	X	X
3,4,DimethoxycinnamicAcid (caffeic)	6.65	191,221,265,280	O	O	O
FerulicAcid	6..9	338,308,249,293,147	O	O	O
CaffeicAcid(triTMS)	7.00	219.396	O	O	O
Tryptophan	7.25	202,291	X	X	X
Biotine	7.95	241,189,204,147	O	O	O
Rutin	8.19	204,217,129,147	O	O	O
Thiamine	8.45	194	O	O	O
Sucrose	8.65	217,361	X	X	X
Maltose-O-methyloxime(TMS)	8.96	204,217,361	X	X	X
ChlorogenicAcid(breakdownProd) QUINIC Acid(TMS)	10.01	255,345	X	X	X
AlphatocopherolAcetate	10.23	165,430,472	O	O	O
Stigmasterol(TMSe)	10.55	255,129,83,484	O	O	O
BetaSitosterol(TMSe)	10.66	128.75,356.82,395.80,485.81	O	O	O
Raffinose(TMS)	10.94	361,437,451	X	X	X

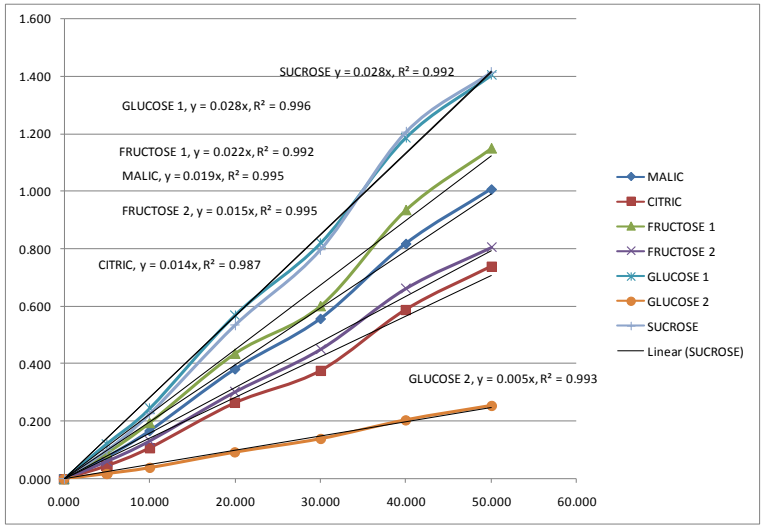
X denotes detected **O** denotes not detected

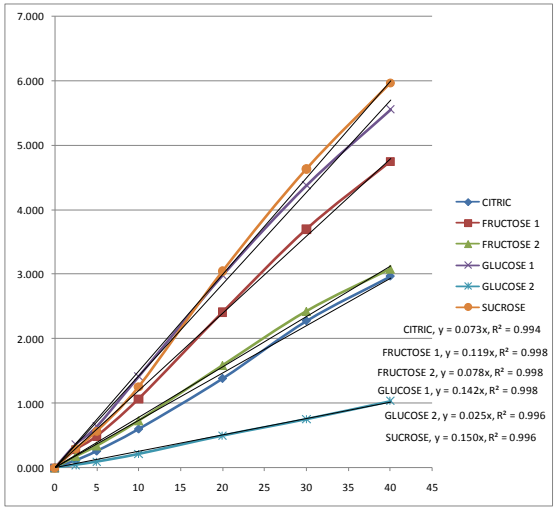
Representative LC-MS-derived database reporting for the ring test potato samples as reported by P1

POTATO LC-MS

	M/Z	MS2	Formula	ADDUCT	NAME
1.49	131.0353	121,108,93			
1.59	147	130,84	C6H14N2O2	[M+H]1+	Lysine
1.85	381.0801	201,219,264	C12H22O11	[M+K]1+	***
1.88	120.06	101.93,73.96	C4H9NO3	[M+H]1+	Threonine
2.81	132.1		C6H13NO2	[M+H]1+	Leucine
2.81	150.06	133.98,104.94	C5H11NO2S	[M+H]1+	Methionine
2.83	132.1	84.17,112.24	C6H13NO2	[M+H]1+	Iso leucine
2.8	170.08			[M+H]1+	Pyroxidine (Vit B6)
4.3	166.086	121,120,150	C10H10O5	[M-CO2H+H]1+	5-hydroxy-ferulic-acid
5.12	220				Pantothenic acid (Vit B5)
5.84	163.0385	145,135,107			
6.33	123.0438				
5.84	251.1387	234,163,89	C13H18N2O3	[M+H]1+	N-Caffeoylputrescine
6.26	392.2908	216,347,177			
6.83	531.3187	294,293,222	C27H46O10	[M+H]1+	L-Oleandrosyl-oleandolide
7.33	355.103	163,145,285	C16H18O9	[M+H]1+	Chlorogenic acid
7.36	205.0971	189,188,160	C11H12N2O2	[M+H]1+	Tryptophan
7.82	265.1545	249,178,177	C14H20N2O3	[M+H]1+	Subaphyllin
7.99	822.3038	322,339,304			
8.57	474.2607	223,222,224	C21H42NO7P	[M+Na]1+	1-16:1-lysophosphatidylethanolamine
9.13	387.2011	225,369,368			
9.25	773.2151	593,726,755			
9.51	289.0706	271,243,153			
9.75	412.2079	184,394,125			
9.85	903.257	742,271,741			
10.01	445.1342				
10.16	369.118	177,145,351			
10.31	695.366	222,681,294	C36H56O14	[M-H2O+H]1+	
10.67	887.2625	726,271,725			
10.82	917.2728	755,271,433			
11.03	506.2532	373,211,487			
11.13	611.16		C27H30O16	[M+H]1+	Rutin
11.14	366.3478				
11.22	464.1587	163,285,316			
11.51	595.1665				
11.64	822.486				
11.8	866.4905	851,722,723	C45H73NO16	[M-H2O+H]1+	Solasonine
11.86	207.7		C11H12O5	[M+H]1+	Sinapic acid
11.88	312.123				
11.92	165.05		C9H8O3	[M+H]1+	p-Coumaric acid
12	344.2431	102,123,184	C18H30O5	[M+NH4]1+	2,3-dinor-PGE1
12.15	638.3084	456,293,474			
12.3	368.3636	323.216,126			
12.4	448.218	269,431,233			
12.48	1009.146	991,986			
12.65	1051.571	539,417,928			
13.15	859.4236	707,723,398			
13.56	868.5075		C45H73NO15	[M+H]1+	Solanine
13.8	852.5123	707,798,709	C45H73NO14	[M+H]1+	Chaconine
14.24	938.5135	895,835,735	C46H77NO17	[M+Na]1+	Tylosin ???
14.47	344.1492	207,175,147			
14.68	314.1388	177,145,117			
14.72	1059.201		C24H22O13	[2M+Na]1+	Apigenin 7-(6''-malonylglucoside)
14.91	584.3586	184,412,567			
15.13	1072.228				
15.35	1077.224				
15.85	550.3149				
16.51	918.416	902,547,546			
16.74	552.3302	184,535,412			
17.28	348.2745		C18H34O5	[M+NH4]1+	9,12,13-TriHOME
17.59	532.3194	516,499,184			
18.84	536.3354	449,375,481			
19.19	607.2658	396,295,397			
19.52	552.3303	535,184,443			
19.9	534.1396	184,516,517			
20.66	595.2885	578,577,371			
20.92	214.0894				
21.79	694.4022	609,660,635			
21.97	597.3044	616,581,536			
22.26	476.2776	337,460,306	C23H42NO7P	[M+H]1+	1-18:3-lysophosphatidylethanolamine
22.47	518.3253	184,501,185	C26H48NO7P	[M+H]1+	1-18:3-lysophosphatidylcholine
23.18	598.3264	564,356,582			

P12 undertook a significant amount of work and interacton with NIST to develop AMDIS for the GC-MS analysis of food crops. AMDIS has the advantage of enabling data from all manufacturers data systems to be processed irrespective of the instrument manufacturer thereby facilitating inter-laboratory comparisons. AMDIS also requires less user intervention, for example setting of integration parameters. Indeed the degree of linearity achieved using AMDIS for increasing amounts of ring-test analytes in tomato, potato and bread wheat samples is highlighted below,





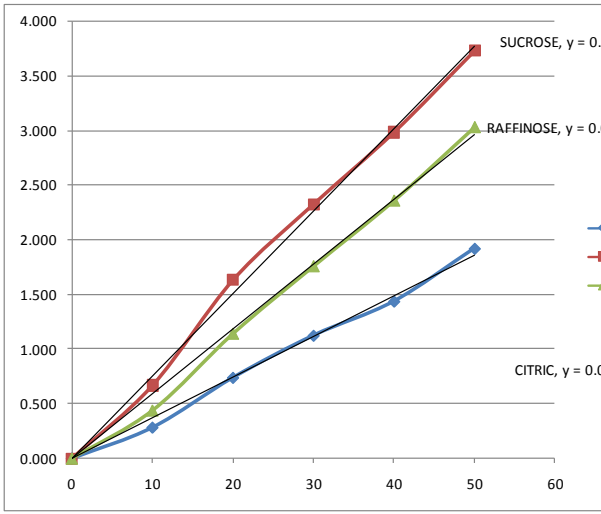
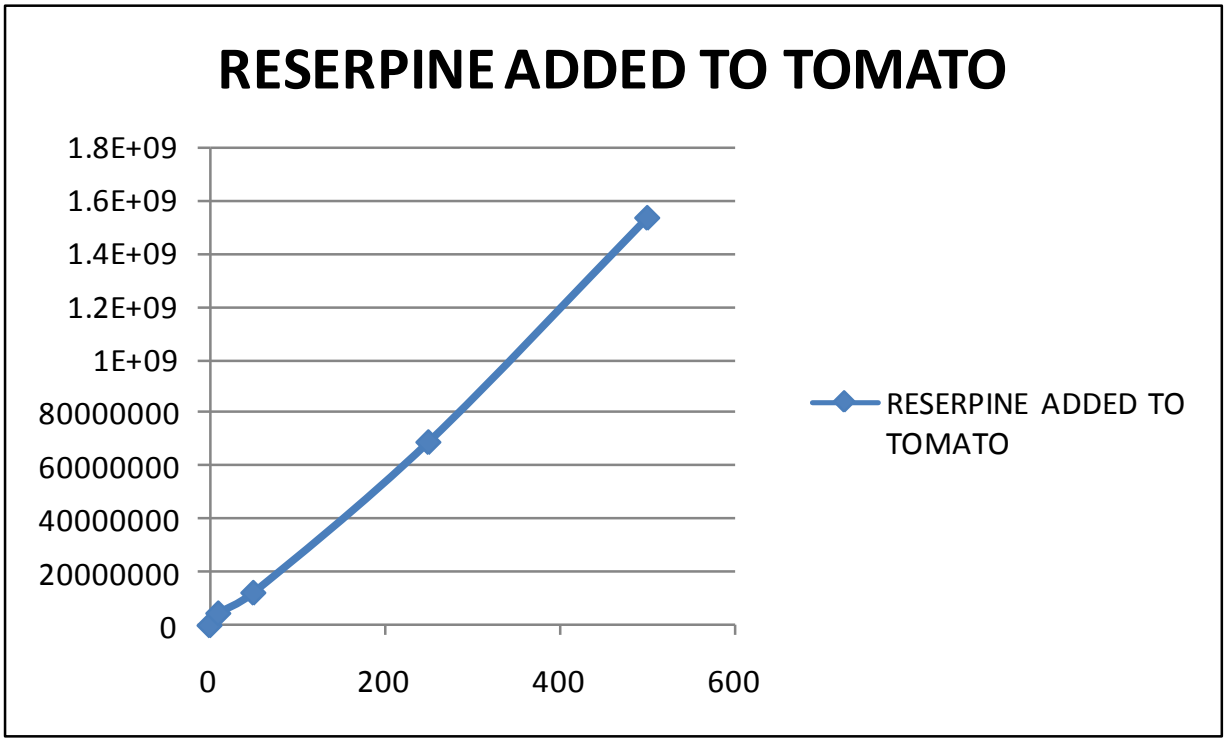


Figure 9
Graphic representation of the degree of linearity achieved for increasing amounts of ring-test analytes in tomato, potato and bread wheat samples derived from GC-MS and identified by AMDIS.

It was always known that the construction of LC-MS libraries/databases would be problematic due to the inherent influence that the elution solvents have on the molecular and fragmentation ions as well as the very fragmentation processes themselves. Furthermore the solvent can significantly influence the degree of ionisation in the MS thereby potential limiting lower detection limits for some compounds. However, progress wasas been made on this topic and several electrospray ionization libraries of key analytes (mainly flavanoids) have been constructed for evaluation purposes, including AMDIS. These have been prepared using both ion trap and quadrupole mass spectrometers.

At the partner level in house mass spectral libraries have been accrued with a view to integrating these into the LC-MS AMDIS database. The in house databases contain >300 polar and non-polar compounds from a ranges of extractions, including those generated within this project acquired in both positive and negative ESI ionization. The databases contains R_ts, accurate masses, mass errors, signal/to/noise ratios, masses of the most abundant fragment ions and putative identification based on the interrogation of the web-based systems, such as the METLIN database, using the XCMS software, available on the Bioconductor project website, as well as the on board structural prediction systems. In many cases the identities of selected compounds are being confirmed through comparison of experimental MS/MS data with fragmentation patterns generated by the MASSFRONTIER, Metalign etc software, as well as by comparison of the RTs and MS/MS data with those of authentic standards.

lthough AMDIS was designed for GC/MS, preliminary results for LC/MS with very different noise characteristics, are encouraging. For example the results from analysis of different amounts of reserpine (often used as an internal standard) spiked into tomato samples highlighting the utility of AMDIS for this approach.



The determination and quantification of the LC-MS external standard resperpine spiked into a ring test tomato samples

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

In this project year WP 3 focussed on the high end MS systems and FT-IR as a measn to facile profiling

Both P3 and P1 evaluated FT-MS and related platforms for their effectiveness as emerging technologies in the analysis of plant (food) metabolites. The low ppm mass accuracy typically achieved from FT-MS instruments allowed the acquisition of putative molecular formulas on the basis of accurate masses alone. Assuming that the technology can process effectively this should be ideal for high throughput metabolomics applications.

Lengthy experimental assessment showed the FT-ICR-MS to be less sensitive and with a limited dynamic range with respect to the Orbitrap. Also, the ultra high-resolution typical of ICR instruments (>1000000) was only reached with scan times incompatible within chromatographic profiling timescales and therefore not appropriate for LC-MS profiling of plant metabolites; i.ei it was not a instrument with the high throughput to do multiple quantitative food analyses.

Aassessment of the Orbitrap (LC-FTMS) system was undertaken by both P3 and P1 separately to ensure full exploration of the system. At P3 comparative evaluation of different platforms was performed using both pure standards and total extracts from the plant materials generated by WP1 and WP4. Mixtures of several standards, relevant to the profiling of plant extracts, were prepared for LC- or direct infusion-FTMS analysis. An example of LC-FTMS (Orbitrap) analysis of a mix of pure standards is highlighted below.

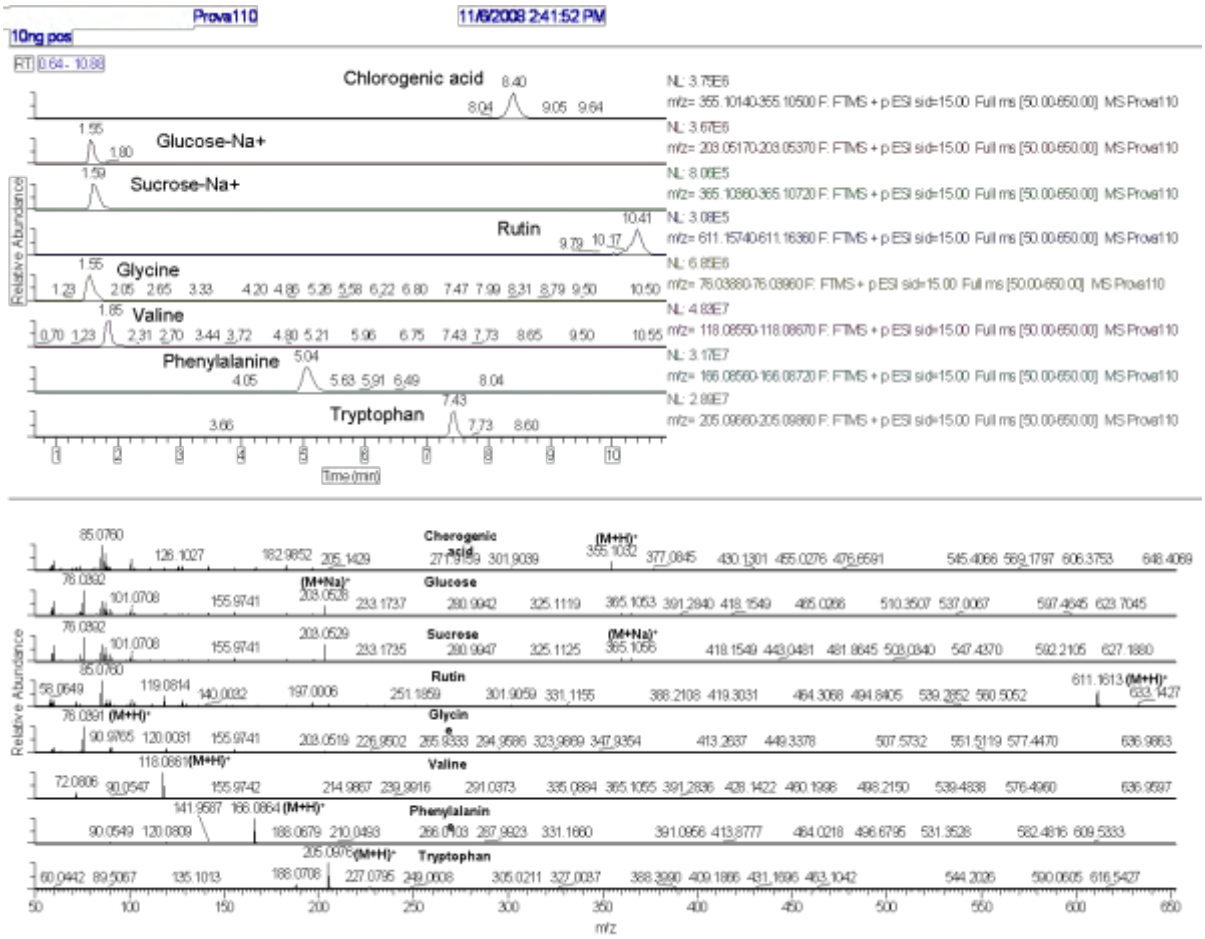


Fig. 11. LC-ESI-FTMS analysis of a mix of pure standards. In the upper panel, EICs (extracted ion chromatogram) of selected compounds and in the bottom panel, the corresponding high-resolution spectra. Glucose and Sucrose preferentially formed adducts with sodium $(M+23)^+$.

Mixtures were analysed both in positive and negative ion mode. Overall, and following multiple replication, a high reproducibility of retention times was observed. Adopting a resolution of 30000, mass accuracies were routinely below 5 ppm. Standards mixtures included the following: LC-ESI-FTMS (positive and negative): amino acids, organic acids, phenolic acids (chlorogenic, caffeic), mono- and disaccharides, and glycosylated flavonoids (rutin).

Standard mixtures were also analysed by APCI-FTMS (soft ionisation approach) to ensure capture of compounds recalcitrant to ionisation. In this case the analysis was mainly focused on carotenoids. Spectra of representative compounds were acquired as direct infusion in positive and negative mode. An example of a full MS/MS spectrum of β-carotene by $APCI^+$ is shown below. Fragment ions of m/z 399, 413, and 455 were also found to be common of several carotenoids (lutein, lycopene) and represent successive fragmentations of the isoprene chain. In fact the use of carotenoids as a test set was doubly useful since it both established that FT-MS did detect metabolites with good accuracy but also, and importantly with respect to food analyses, it easily detects and characterises long chain alky molecules, such as carotenoids, which are notoriously difficult to characterise using LC-MS systems due to their poor ionisation behaviour.

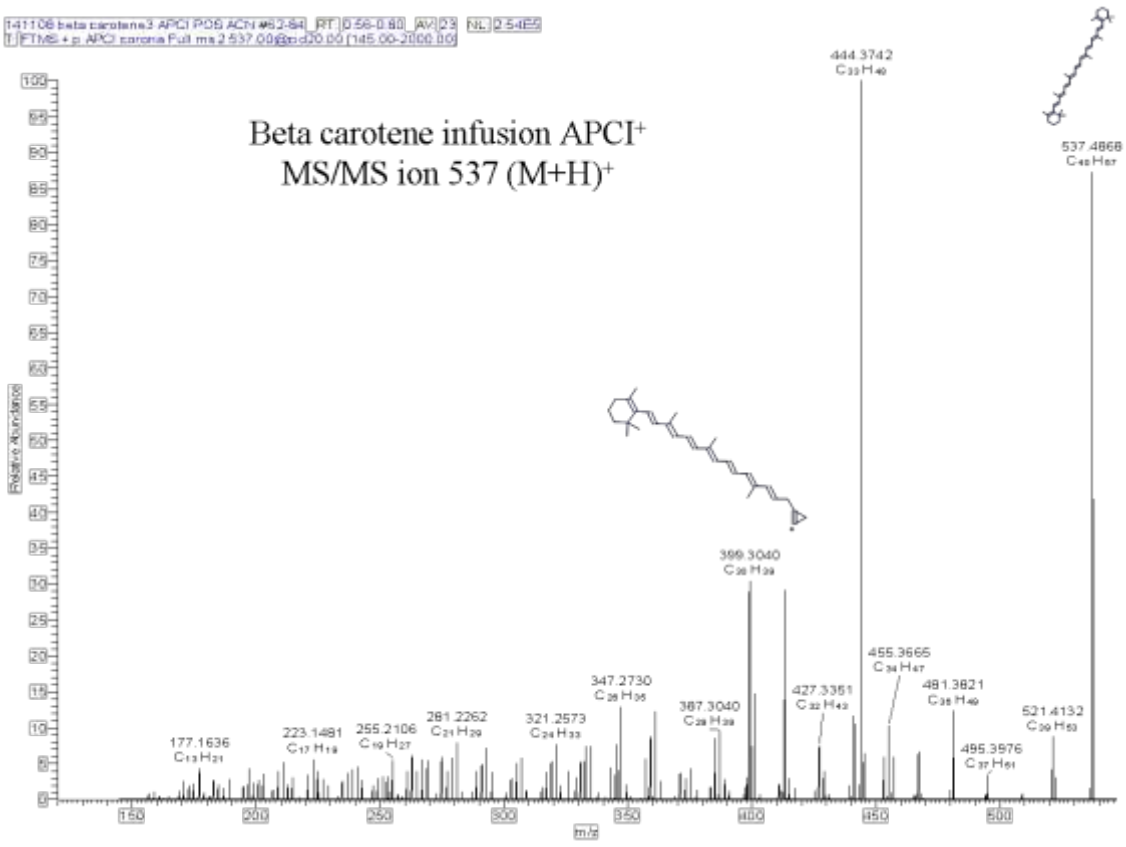


Fig. 12 FTMS APCI+ Full MS/MS of β-carotene (direct infusion). Putative molecular formulas of the most abundant ions are indicated below each mass. A proposed structure for the fragment ion at m/z 399 is shown.

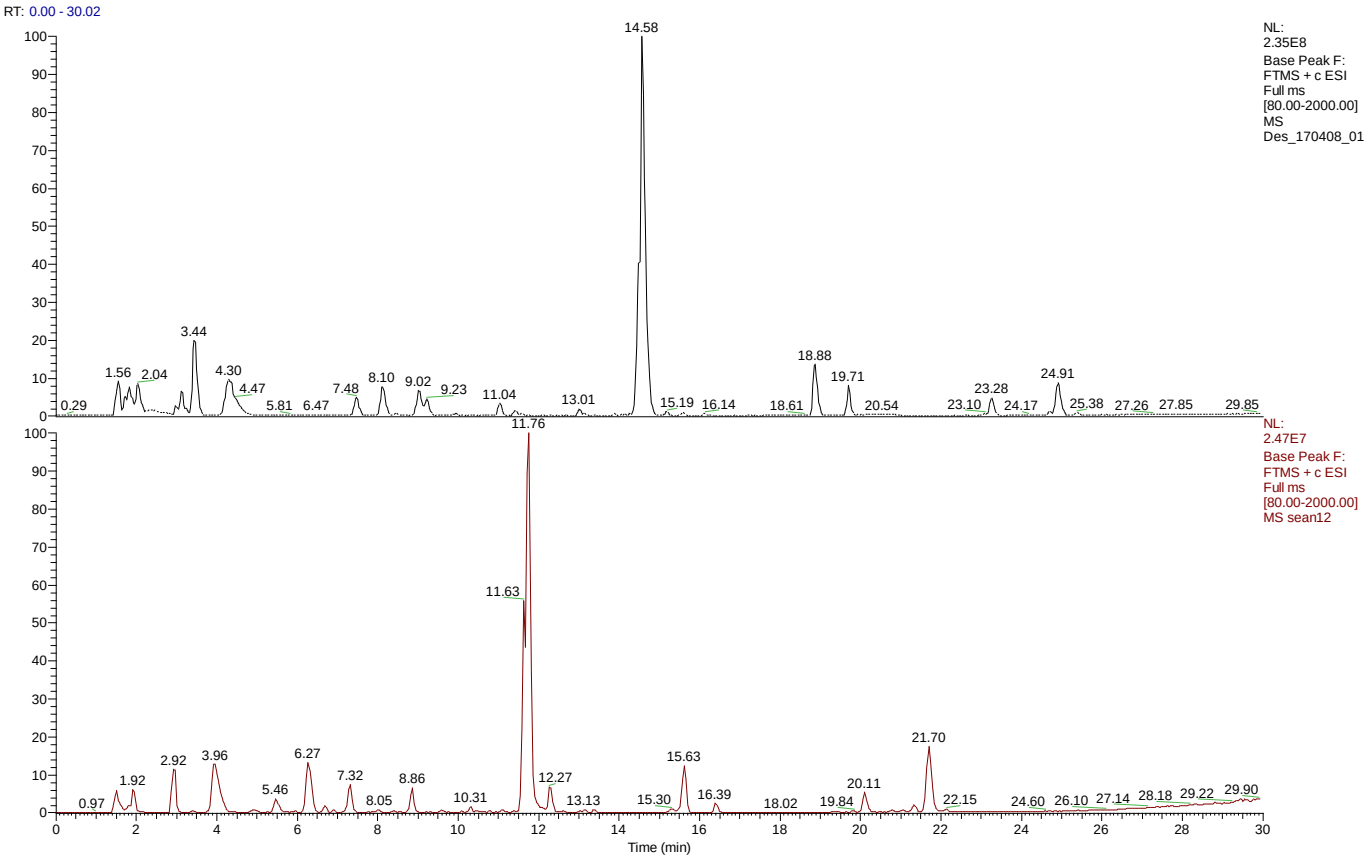
Evaluation of FTMS as emerging technology was also assessed through analyses of complex extracts from the biological materials provided by WP2and WP4. In particular, polar metabolites were extracted from freeze-dried potato powders according to a protocol provided by P1, which was further optimized with regards to the mobile phase composition, chromatographic conditions and ESI source settings (stored in the tune file). Polar extracts were analysed with LC-ESI-FTMS both in positive and negative mode, and mass accuracy was tested in both modes with or without the use of specific ions as mass locks. In the former case, the average mass error was < 2 ppm; in the latter case (external calibration) errors < 5 ppm were routinely obtained. FT mass accuracy was also tested for stability every other day and resulted to be < 2 ppm for up to 15 days without the need for recalibration.

P1 also undertook a comparison between the Orbitrap and FT-MS systems but confined the comparison to Thermos systems. Both are essentially hybrid instruments, i.e. a dual MS instruments, the front ends (chromatographiy) are an LTQ XL with the difference between the two instruments second MS. The Orbitrap couples the LTQ XL with a linear ion trap and the patented Orbitrap™ FT-MS. The LTQ FT Ultra combines the LTQ XL Ion Trap MS and Fourier Transform Ion Cyclotron Resonance mass spectrometer. This gives repeatedly greater resolution and sensitivity coupled with ppm mass accuracy as outlined above in the studies of P3. It was key to have similar front ends to assess the MS technologies and therefore only the data output of the backend mass spectrometer was compared.

Methanol/water extracts were made from the ring test potato, tomato and wheat freeze dried powders and analysed on each instrument under identical chromatographic conditions (see below for potato).

The numbers of metabolites detected reproducibly by the Orbitrap system gave the following: potato (80) tomato (40) and wheat (34). The LTQ FT ultra system reported potato (57) tomato (63) and wheat (29). The Orbitrap system detected more metabolites for potato and wheat extracts however, the LTQ FT ultra detected more metabolites for tomato. It should be noted that each instrument detected metabolites that the other did not; i.e. there is a clear sensitivity and selectivity differential.

It is abundantly clear from the comparative data outputs that the LTQ-ICR-FTMS is extremely useful in providing putative (high significance hit) molecular formulae that, when interpreted by an experienced phytochemist/food chemist, would provide candidate structures for the compounds/peaks. However as for the studies of P3 the throughput (scan time) required to do this is not trivial and in reality should be confined to complete unknowns. The output of the Orbitrap is a list of molecular ions fragments and adducts which through the process of standard comparison and database construction will be sufficient to provide characterisation and identification of the compounds. In addition, this is all done in real time validating the use of the Orbitrap as a high throughput profiling system.



Example of the chromatograms of an extract from a freeze dried potato extract run on an Orbitrap (top) and LTQ FT Ultra (bottom). Both systems were run in positive ionisation mode and 80-2000 amu scan range.

The comparative outputs from a potato extract prepared according to the DEVELONUTRI SOP (1st annual report) and analysed on Thermo LTQ-FTMS (Orbitrap) and LTQ-ICR-MS.

Potato

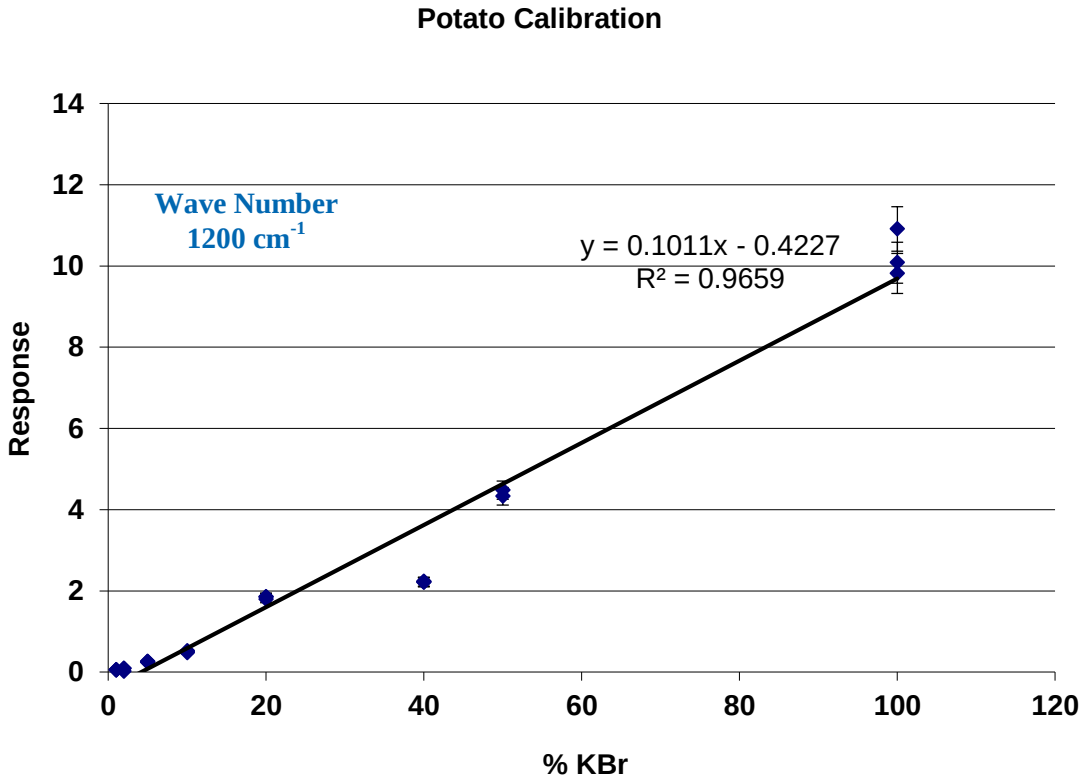
ORBITRAP			FTMS				
	MS2	Retention Time	M/Z	Formula	adduct	mass
1.49	151.0353	121,108,93					
1.59	147	130,84			$C_6H_{14}N_2O_2$	[M+H]1+	
			1.51	192.0617	C6H9N4O2	[M+Na]1+	192.061772
1.85	381.0801	201,219,264	1.78	381.0791	C12H22O11	[M+K]1+	381.079373
1.88	120.06	101.93,73.96			$C_4H_9NO_3$	[M+H]1+	
2.81	132.1				$C_6H_{13}NO_2$	[M+H]1+	
2.81	150.06	133.98,104.94			$C_5H_{11}NO_2S$	[M+H]1+	
2.83	132.1	84.17,112.24			$C_6H_{13}NO_2$	[M+H]1+	
2.8	170.08					[M+H]1+	
			1.96	200.0716			
3.53			2.96	200.0716			
			3.37	286.1436			
			3.56	224.0763	C7H10O7	[M+NH4]1+	224.076481
			3.74	328.1389			
4.3	166.086	121,120,150	4.10	166.0615	C10H10O5	[M-CO2H+H]1+	166.061899
5.12	220						
5.84	163.0385	145,135,107					
6.33	123.0438						
			4.51	220.1178	C9H17NO5	[M+H]1+	220.117951
5.84	251.1387	234,163,89	4.96	251.1388	C13H18N2O3	[M+H]1+	251.13902
6.26	392.2908	216,347,177					
6.83	531.3187	294,293,222	5.55	531.3171	C27H46O10	[M+H]1+	531.316377
7.33	355.103	163,145,285	5.96	355.1021	C16H18O9	[M+H]1+	355.102362
7.36	205.0971	189,188,160	6.27	205.0970	C11H12N2O2	[M+H]1+	205.097155
7.82	265.1545	249,178,177	6.68	265.1545	C14H20N2O3	[M+H]1+	265.15467
7.99	822.3038	322,339,304					
8.57	474.2607	223,222,224	7.32	474.2593	C21H42NO7P	[M+Na]1+	474.259113
			7.78	472.2439	C23H35N3O6	[M+Na]1+	472.241808
9.13	387.2011	225,369,368	8.00	387.2011			
9.25	773.2151	593,726,755					
9.51	289.0706	271,243,153					
9.75	412.2079	184,394,125					
9.85	903.257	742,271,741					
10.01	445.1342						
10.16	369.118	177,145,351					
			8.55	389.1552	C6H13N3O3	[2M+K]1+	389.154542
10.31	695.366	222,681,294	8.82	695.3641	C36H56O14	[M-H2O+H]1+	695.363722
10.67	887.2625	726,271,725	9.19	887.2598			
10.82	917.2728	755,271,433	9.36	917.2702			
11.03	506.2532	373,211,487					
11.13	611.16				$C_{27}H_{30}O_{16}$	[M+H]1+	
11.14	366.3478						
11.22	464.1587	163,285,316					
11.51	595.1665						
11.64	822.486						
			9.63	344.2430	C18H19NO5	[M+H]1+	330.133601
11.8	866.4905	851,722,723	10.09	866.4885	C45H73NO16	[M-H2O+H]1+	866.489651
11.86	207.7				$C_{11}H_{12}O_5$	[M+H]1+	
11.88	312.123						
11.92	165.05				$C_9H_8O_3$	[M+H]1+	
12	344.2431	102,123,184	10.31	344.2429	C18H30O5	[M+NH4]1+	344.243151
12.15	638.3084	456,293,474					
12.3	368.3636	323.216,126					
12.4	448.218	269,431,233					
12.48	1009.1461	991,986					
12.65	1051.5706	539,417,928					
13.15	859.4236	707,723,398					
			10.45	330.1334	C18H19NO5	[M+H]1+	330.133601
13.56	868.5075			868.6904	C45H73NO15	[M+H]1+	
13.8	852.5123	707,798,709		852.6932	C45H73NO14	[M+H]1+	
			10.63	638.3066			
14.24	938.5135	895,835,735	12.26	938.5097	C46H77NO17	[M+Na]1+	938.508375
14.47	344.1492	207,175,147					
14.68	314.1388	177,145,117					
			12.45	430.3312	C27H43NO3	[M+H]1+	430.331571
14.72	1059.2008		12.58	1059.1973	C24H22O13	[2M+Na]1+	1059.20131
14.91	584.3586	184,412,567					

The comparative outputs from a potatoe extract prepared according to the DEVELONUTRI SOP (1st annual report) and analysed on Thermo LTQ-FTMS (Orbitrap) and LTQ-ICR-MS. (Continued)

Potato							
ORBITRAP			FTMS				
		MS2	RetentionTime	M/Z	Formula	adduct	mass
15.13	1072.228		13.04	1072.2245			
15.35	1077.2244		13.17	1077.4701			
15.85	550.3149						
			13.59	786.2843	C14H24O12	[2M+NH4]1+	786.287386
			14.17	141.3362			
			14.44	346.2586			
16.51	918.416	902,547,546	14.58	918.4139			
16.74	552.3302	184,535,412					
17.28	348.2745		15.34	348.2742	C18H34O5	[M+NH4]1+	348.274451
17.59	532.3194	516,499,184					
18.84	536.3354	449,375,481					
			16.21	625.2540			
19.19	607.2658	396,295,397	16.44	607.2643			
19.52	552.3303	535,184,443					
19.9	534.1396	184,516,517					
20.66	595.2885	578,577,371					
20.92	214.0894						
			16.66	536.3341	C26H50NO8P	[M+H]1+	536.334684
			16.85	348.2742			
			18.34	362.2899			
			18.56	362.2898			
			19.29	612.3138	C34H47NO10	[M-H2O+H]1+	612.316711
21.79	694.4022	609,660,635	19.36	694.4003			
21.97	597.3044	616,581,536					
			19.43	439.1914	C24H32O4S	[M+Na]1+	439.191355
22.26	476.2776	337,460,306	19.84	476.2768	C23H42NO7P	[M+H]1+	476.277169
22.47	518.3253	184,501,185	20.11	518.3236	C26H48NO7P	[M+H]1+	518.324119
23.18	598.3264	564,356,582					
23.33	696.4175	628,640,389	20.79	696.4159			
			21.06	432.2378			
23.85	478.2936	337,460,306	21.33	478.2925	C23H46NO8P	[M-H2O+H]1+	478.292819
24.57	520.3404	184,503,185	21.7	520.3393	C26H50NO7P	[M+H]1+	520.339769
25.72	454.2931						
25.99	496.3402	184,479,283					
26.31	522.3558	184,506,185					
			22.2	532.3476			
26.79	534.364	499,517,355	24.05	534.3632			

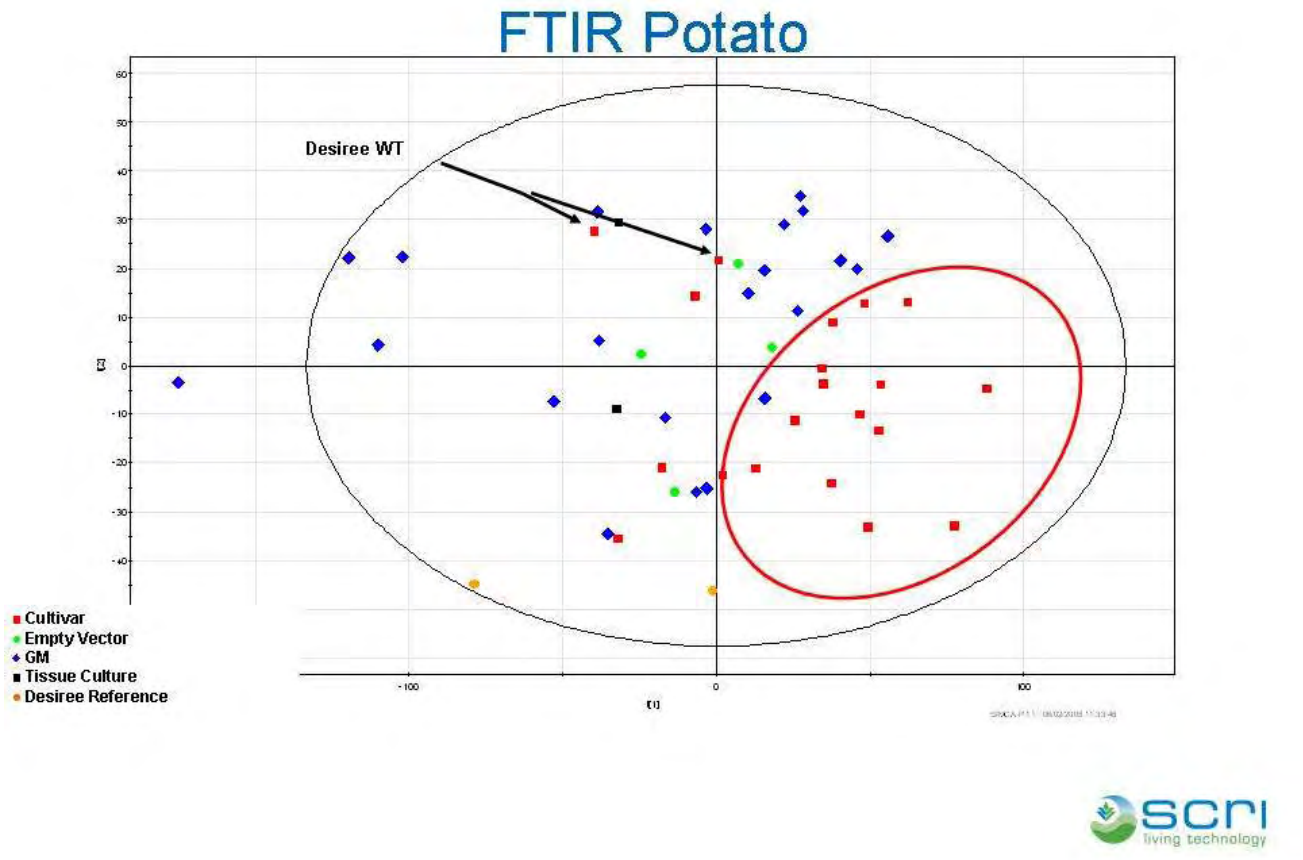
FT-IR. **P1,** P6 & P8.

FT-IR was explored as a facile method for profiling by P1,6 and 8. Simplistic inclusion of the crop powders into KBr as solid-in-solid suspicions seemed to work well for all wavelengths (here wavenumbers) giving a Beers law direct relationship between amount and wavenumber response



DRIFT Correlation between dilution factor in KBR and the absorbance of freeze dried potato powder at 1200cm^{-1}.

Tis was exploited for a range of components within the crops and more fully analysed in year 3. The use of untargeted FT-IR did highlight that is was a very rapid method to distinguish samples based on composition as highlighted below



PCA of DRIFT data derived from freeze-dried powders of potato. The potatoes were a selection of cultivars (NOFORISK, 2nd annual report) and a specific GM potato (down-regulated SGT-1 leading to modified glycoalkaloid balance) plus the associated tissue culture vector only and wild type controls..

FT-IR demonstrated that the majority of the cultivars segregated away from the GM material however the parent wild type was within the GM group. This result confirms and reflects the segregations seen for the associated GC- and LC-MS analyses on these samples (as part of NOFORISK: http://www.scri.ac.uk/research/ppfq/foodquality/foodsafety/noforisk).

WP 4 Biological material

Central to the progress of the project is the provision of biological materials. This work package is concerned with providing biological material for the activities of other WPs dedicated to the development of the metabolic profile methods and to organise the experiments for demonstration activities. The crops for preliminary analysis will be derived from a range of agricultural regimes and locations across Europe and South Africa. These will include GM material for each crop. To capture added value biological material will be gift aided from existing EU projects. Practical demonstration material will be provided via the SMEs. Sampling will occur at all stages of the post-harvest food chain.. The materials will be fed into WP's 1-3 across the project duration.

Tomato cultivars and transgenic lines

The following varieties as representative of the tomato germplasm: Ailsa craig, Money maker, M82, Kumato, Roma and Rodade. Furthermore, two GM varieties that can be considered prototypes for the GM material formally sold in Europe have been acquired. The GM materials are in the Alisa craig background, and the modification represents down-regulation either by antisense RNA technology or by homology induced gene silencing of the poly-galacturonase (PG) gene.

The sites considered for the tomato growing were P2, P3 and P11. P2 and P3 sowed the tomato seeds in the glasshouse while P11 in the field (no transgenic material was grown in this site due to the restrictions in GM commercialization and field sowing in South Africa); P2 has not cultivated the varieties Roma and Rodade.

The variation in sowing time is an inherent consequence of the different geographical regions (South Africa, Southern Italy, UK). Growing conditions reflected the standard agricultural practices of each location. For each variety six plants were grown from which a minimum of six fruits were harvested. The yield in fruit of four to six plants was freeze-dried (as whole fruit) at each site and sent to P2 leader for milling, aliquoting and distribution to other partners.

The UK greenhouse trial (P2) was carried out in May 2008, while fruit material from P11 was freeze dried and sent to P2 in September 2008. The fruit material from P3 was sent in January 2009 to P2 where it has been milled into a fine powder and distributed among project partners for analysis.

Potato cultivars and transgenic lines

The selection of the potato cultivars was carried out in coordination with other national and EU projects dealing with this crop where P1 was involved. Six cultivars have been selected to represent the different end uses: Boiling (Desiree and Estima); French Fries (Pentland Dell and Maris Piper); Potato chips/crisps (Lady Rosetta and Saturna). The field trial was described in the first annual report: for each cultivar three biological replicates were harvested.

Furthermore, P1 generated a significant amount of the potato cultivar Desiree, which had been down-regulated with respect to the gene Solanosyl glucosyl transferase-1 (SGT-1), from a parallel FP6 project (NOFORISK) that gave permission to use this material for DEVELONUTRI. The material had been grown under cover (tygan) according to national (Scottish) GM plant restrictions. This growth environment is a hybrid between glasshouse and field conditions.

Due to 2008 being an abnormally wet year potato harvest was delayed until 1st October 2008. After harvesting the potato tubers were chopped (following the validated opposite eights approach) then frozen under liquid nitrogen and stored at-20 ºC while waiting to be freeze dried. Upon freeze drying, potato tubers w milled to < 2mm and stored in airtight containers at -20 ºC. The potato samples will be sent out soon to participating labs from **P1**.

Durum wheat cultivars

Since durum wheat is traditionally grown in Southern Europe and mainly in Italy, the cultivars chosen are among the most adapted to the Mediterranean environment and their site selection was based on the data from last 15 years Italian durum wheat national trials. The following cultivars have been selected: Creso, Simeto, Svevo, Saragolla, Sfinge and PR22D89. These genotypes are a good representation of the most popular, high yielding/high quality durum wheat cultivars available. The six cultivars were sown in 3 locations across Italy: Fiorenzuola D'Arda (northern Italy), Foggia (southern Italy) and Catania (Sicily). One location is the experimental farm of P5, where the same cultivars have been also grown under an organic farming system (side-by-side with the standard farming system). Growing conditions were according to the standard agricultural practices in each location. In early summer and from every location ~1kg of seed/cultivar for each of the three biological replicates were harvested and sent to P5. The samples were milled and freeze dried centrally at P5 and distributed to the consortium partners.

An addition to the project planned material, a Recombinant Imbred Line (RIL) population (~100 lines) derived from the cross Latino x Primadur, segregating for carotenoid content, has been grown in two locations by P5 and seeds have been milled and will be subjected to metabolic determination to establish the genetic bases of carotenoid content in durum wheat.

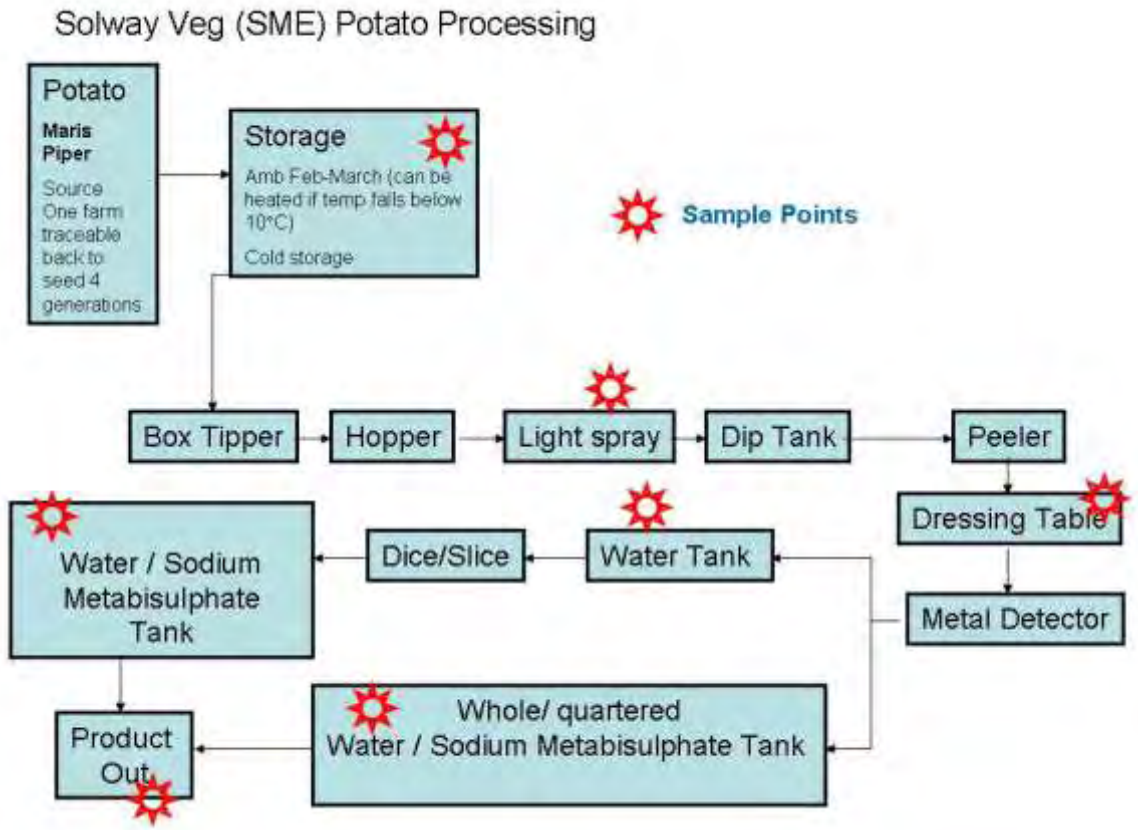
Bread wheat cultivars and transgenic lines

Six cultivars have been chosen as representative of a number of spring and winter bread wheat genotypes most adapted to European conditions: three Italian wheat cultivars (Bilanca, Blasco and Bokaro); two UK wheat cultivars (Glasgow and Mascot) and one Swiss wheat cultivar, Zinal. Growing conditions were according to the standard agricultural practices for each location (P5-South Italy, P4-Swiss and P1-Scotland). The cultivars were harvested between June and October 2008 (depending on different geographical regions) and about 1kg of seed/cultivar for each of the three biological replicates (two replicates only in Switzerland and Scotland) were sent and stored in Zurich by P4 for milling. All samples of bread wheat have been milled (to pass a 0.5mm sieve) by P13 and distributed to the analytical project partners.

The genetically modified wheat material was cultivated by P4. A field test in Reckenholz (Switzerland) had been set up and approved (in collaboration with the National Research Program 59 from Swiss National Foundation). In the second half of March 2008 two spring bread wheat varieties were sown. The first GM wheat was a Frisal wheat line expressing two proteins with antifungal activities: chitinase and ß-1,3-glucanase. These GM plants contain the bar gene as a selection marker, providing herbicide tolerance against Basta. The second transgenic wheat was a Bobwhite wheat expressing the allele *Pm3b* coding for resistance against powdery mildew. These GM plants contain phospho-mannose isomerase as a selection marker gene. In June 2008, before the plants began flowering, a group of some 30 activists entered the field and destroyed part of the plants by cutting them down with scythes. This hampered the statistical analysis by reducing the number of samples and, in addition, provided injuries thereby facilitating infections by *Fusarium graminearum* (the pathogen causing fusarium head blight). Therefore, part of this material was not grown according to regular agronomic standards and we have not been able to harvest sufficient material from all repetitions. Seeds were harvested from the GM-field trial in July 2008.

Identification of a "core sample set"
During the two project meetings in 2008 a large discussion developed on the strategy to analyze the materials according to the aims of the project. Due to the large number of samples (overall about 1000 samples were prepared including replicates), the time required for analyses and the difficulties that we expect during the analysis of such big data set, it was decided to select a subset of samples. This core sample set was selected among all samples available with the aim of including the largest amount of variability within a feasible number of samples. The following table (Table 16) summarizes the core sample set

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 (below) 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 samples is ongoing and is expected to be complete by mid March 2009 following which the material will be distributed to the appropriate partners for analysis.



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

Details of the core sample set for analysis in year 3

Crop	Cultivar/GM	Growing location/conditions	Total samples (including 2 tech reps)	Partner responsible for sample preparation
Bread wheat	Bobwhite/GM marker phosphomannose synthhase + Pm3b	CH, summer 2008- field testing approved (3 samples)	62	P4
Bread wheat	Bobwhite (sister line - control)	CH, summer 2008- field testing approved (3 samples)		
Bread wheat	Frisal /GM marker bar + chitinase and ß-1,3-glucanase	CH, summer 2008- field testing approved (2 samples)		
Bread wheat	Frisal (sister line - control)	CH, summer 2008- field testing approved (1 samples)		
Bread wheat	BILANCIA	Italy, Swiss, Scotland autumn 2007-summer 2008		
Bread wheat	ZINAL	Italy, Swiss, Scotland autumn 2007-summer 2008		
Bread wheat	MASCOT	Italy, Swiss, Scotland autumn 2007-summer 2008		
Durum wheat	CRESO	Foggia (Southern Italy) standard field, Foggia organic farming, Fiorenzuola D'Arda (Northern Italy) standard field	54	P5
Durum wheat	SIMETO	Foggia (Southern Italy) standard field, Foggia organic farming, Fiorenzuola D'Arda (Northern Italy) standard field		
Durum wheat	PR22D89	Foggia (Southern Italy) standard field, Foggia organic farming, Fiorenzuola D'Arda (Northern Italy) standard field		
Tomato	Ailsa craig	UK and Italy (Greenhouse), South Africa (field)	120	P2
Tomato	Money maker	UK and Italy (Greenhouse), South Africa (field)		
Tomato	M82	UK and Italy (Greenhouse), South Africa (field)		
Tomato	Kumato	UK and Italy (Greenhouse), South Africa (field)		
Tomato	Rodade	Italy (Greenhouse), South Africa (field)		
Tomato	Roma	Italy (Greenhouse), South Africa (field)		
Tomato GM	PG-antisense	UK and Italy (Greenhouse)		
Tomato GM	PG-sense	UK and Italy (Greenhouse)		
Potato	Desiree (Boiler potato)	Scotland	42	P1
Potato	Estima (Boiler potato)	Scotland		
Potato	Pentland Dell (French fries)	Scotland		
Potato	Maris Piper (French fries)	Scotland		
Potato	Saturna (Crisper/Potato chips)	Scotland		

Potato	Lady Rosetta (Crisper/Potato chips)	Scotland		
Potato GM	GM – SGT	Scotland Gifted FP6 NOFORISK		

TOTAL 278

Bread wheat processing
In order to confine the number of samples and in order to choose sampling probes which actually might differ in their metabolic and mineral content, P13 has supplied P4 with four samples from the milling process: (1) starting material from the silo after removal of stones etc. (2) after moistening and a 24 hours stand before removal of the bran, (3) white flour 400 and (4) half white flour 720, all from the same starting material. The two samples (1) and (2) have been milled according to the standard method (Brabender Quadrumat Junior 0.230mm). The process samples 3 and 4 will follow soon for analysis.

Durum wheat processing
P14 has supplied P5 with samples from three different point of pasta production: (1) semolina (starting material), (2) after extrusion and (3) dried pasta (end-product). Five different types of pasta have been selected for sampling: whole grain organic pasta, pasta with 12% protein content, pasta with 13% protein content, pasta with 12% protein content + vitamins and pasta with 13% protein + vitamins. Samples were transported back to P5, freeze dried, milled and distributed to consortium partners in preparation for analysis.

Tomato processing
No SMEs working on tomato products are involved in the DEVELONUTRI consortium, therefore partners are looking for a external company willing to provide the material from their processing lines. Nevertheless, due to the seasonal working period in tomato factory (mainly concentrated during summer) a final decision on the tomato processing sample set will be taken in the next months.

Table 17. A list of the samples provided by SMEs taken at distinct, representative points throughout the food transformation process.

Sample	Processing Steps	Total samples (inc. 2 technical replicates)
Whole grain organic pasta **12% protein pasta** **13% protein pasta** **12% protein pasta + vitamins** **13% protein pasta + vitamins**	- semolina - after extrusion - dried pasta	30
Potato (cv. Maris Piper) **(2 biological reps x point)**	- product in - light sprey - dressing table - water tank -water/sodium metabisulphate tank1 -water/sodium metabisulphate tank2 - product out	28
Bread wheat milled	- starting material - after moistening (before removal of the bran) - white flour 400 -half white flour 720	8
Tomato processing	…	…