Research Article

Metabolomic approach to identifying bioactive compounds in berries: Advances toward fruit nutritional enhancement

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Plant polyphenolics continue to be the focus of attention with regard to their putative impact on human health. An increasing and ageing human population means that the focus on nutrition and nutritional enhancement or optimisation of our foodstuffs is paramount. Using the raspberry as a model, we have shown how modern metabolic profiling approaches can be used to identify the changes in the level of beneficial polyphenolics in fruit breeding segregating populations and how the level of these components is determined by genetic and/or environmental control. Interestingly, the vitamin C content appeared to be significantly influenced by environment (growth conditions) whilst the content of the polyphenols such as cyanidin, pelargonidin and quercetin glycosides appeared much more tightly regulated, suggesting a rigorous genetic control. Preliminary metabolic profiling showed that the fruit polyphenolic profiles divided into two gross groups segregating on the basis of relative levels of cyanidin-3-sophoroside and cyanidin-3-rutinoside, compounds implicated as conferring human health benefits.

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1 Introduction

The evidence supporting the beneficial health effects of fruit is both accruing [1–5] and historical [6, 7]. Subdivisions of fruit, the berries, are increasingly becoming the focus of studies regarding their proposed ability to prevent or ameliorate the problems of degenerative diseases [8–10]. It should be noted that here we are dealing with berries as understood by the public (raspberries, strawberries, blackcurrants etc), and not the classical definition of berries, "fruit which have more than one seed within the fruit and that seed is not compartmentalised". According to the classical definition, strawberries are not truly berries while melons and tomatoes are. Similarly, raspberries have a seed in each druplet (juice sac) and are not truly berries. Here, we will deal with public's accepted understanding of berries, as this will encompass the most popular, and as we hope to show, potentially nutritional fruit.

The phytochemical basis of the nutritional benefits derived from fruit can largely be divided into two classes: small (<2 kDa) and large (>2 kDa) molecular weight (Mwt) components. The large, predominantly polysaccharide, components have been elegantly dealt with by McDougall *et al.* [11] and will not be dealt with here. With regard to the small Mwt, soluble components the health benefits are believed to be attributable to are vitamin C (Vit. C) [12, 13], soluble fibre [14, 15] and the chemically diverse polyphenols [16–19].

The mono-, oligo- and polyphenols found in fruit, often constituting a chemically and structurally diverse group of phytochemicals (phenols) *in vivo*, generally can be attributed to several distinct base structural units upon which there is a range of additional chemical moieties, methyl groups, ether-linked phenols, mono etc. These encompass the anthocya-

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Abbreviations: DIMS, direct infusion MS; **PCA**, principal component analysis; **TEAC**, Trolox equivalent antioxidant capacity; **Vit. C**, vitamin C

nins, flavanols, flavanals, flavanones, isoflavones, caffeic acid, phenolic acids, catechins and ellagitannins.

Increasingly over the last decade, there has been a groundswell of reports attributing beneficial biological activity to the fruit phenolics [8, 10, 16–19]. The vast majority of studies have focussed on well-defined *in vitro* systems employing mammalian cell model systems, such as HeLa, Caco2, HT29, Hep G2, etc to study absorption, anti-cancer, metabolism effects etc. However, the direct translation of the benefits reported in these *in vitro* studies to *in vivo* results has lagged behind and is only now gathering pace. For example there are several intervention studies published, highlighting or attributing their beneficial effects (albeit sometimes marginal) with regard to markers of colon [20] and oesophageal [21] cancer, cardiovascular disease (CVD) [22], visual acuity [23] etc to the polyphenolic components in fruit. In addition, there are several major intervention trials either ongoing or planned with the focus on fruit such as strawberry (cholesterol-lowering), pomegranate (prostate cancer), blueberry (inflammation) (clinical trial identifiers NCT00345722, NCT00336934, NCT00303238, respectively) and blackcurrant (CVD) (Anon 2007, Cardiovascular function and intake of soft fruit: Effects of qualitative and quantitative variation in berry antioxidant status. http://www.chss.org.uk/pdf/research/2007/heart_research_jan_07.pdf).

The accretion of both *in vitro* and *in vivo* data has meant that the nutritional enhancement of foods, and the raw materials, in this case fruit, has shifted focus away from simply Vit. C and micronutrients towards the polyphenolics. This causes something of a problem for any breeding effort since, as has been previously highlighted, the polyphenolics are chemically diverse and their analysis, under normal approaches, requires a considerable effort via traditional, targeted analysis, which has been adopted in some fruit-breeding programmes such as grape, blackcurrant etc. The emergence in the last 5–10 years of metabolomics, in particular direct infusion, LC and GC-MS-based approaches [24–26], has meant that previous analytical restrictions with regard to chemical compounds under scrutiny are not necessarily applicable, at least at the qualitative level. In addition, a significantly greater breadth of the metabolite pool, in this case the polyphenolic pool (phenolome) can be captured and reported upon in much detail and in much greater depth than was previously possible.

Metabolomics, the simultaneous reporting upon the multiple distinct metabolites at specific time points and the interpretation of the metabolite changes within a biological frame of reference, is fairly unusual for an analytical technique, as it has been driven in the first instance by plant science ([24] and references therein). It is within this field, in conjunction with matched proteomics and transcriptomics that advances continue to be made.

It is only in the last few years that mammalian-based sciences, in particular disease and pharmaceutical studies, have adopted these approaches and applied them to their systems in a very successful manner. For example, Griffin *et al.* [27, 28] recently reviewed the application of NMR-based metabolomics to xenobiotic toxicity, and disease diagnosis and drug safety, whilst the MS-based analytical approaches are gaining credence with reports focussed on conditions such as hepatitis [29] and cancer [30], and non-specifically via the establishment of the human metabolome database (www.metabolomics.ca).

In this report, we aim to show how we have applied state-of-the-art analytical approaches to get a significantly greater degree of understanding and detail from our phytochemical studies and how this can help in existing and future nutrition and health related studies. We hope to show how these studies can impinge and inform upon decisions in the plant breeding process to help steer future plant breeding and the potential nutritional enhancement of plant food products.

2 Materials and methods

A widely segregating raspberry (*Rubus idaeus*) population was generated from a cross between the European cv. Glen Moy and the North American cv. Latham, described in some detail by Graham *et al.* [31]. This population is ideal for studying polyphenolics segregating, as it does for a wide range of parameters including fruit quality attributes. It should be noted that the raspberry populations used here were grown in two distinct environments (H field and B field) over many years for the purposes of plant pathogen testing. H field is a low-input site where the crop was not sprayed with fungicide etc. and little cane management was carried out. This site is also somewhat shaded from any direct sunlight being planted between windbreaks. Conversely, B field was classified as a high-health site where the plants were sprayed with fungicide, regularly fertilised etc., planted on ridges and in direct sunlight. This variation in agronomic practices, sunlight and soil conditions has meant that the resultant plants and the fruit produced varied with respect to comparative phenotypes such as yield, height, root sucker morphology etc. Here, we will focus only on 1 year's data, 2005. The entire segregating population of 300 individuals and both parents was cloned by the propagation of root material and planted at two field locations in randomised complete block trials with three replicates and two plant plots at both locations (H field and B field). For analytical purposes and for standardisation of fruit collection, selected fruit ripening at the extremes of the season were removed from the analysis and only 96 samples which were ripe within a narrow window (2 weeks) were used.

Fruit juices and optimised extract generation were carried out as follows Berries were removed from the freezer and allowed to thaw overnight (20°C), after which 20 g of fruit was homogenized in a hand-held tissue grinder for

1 min. The resultant pulp was decanted into centrifuge bottles and stored on ice until sufficient samples had been generated to fill a rotor. Samples were then centrifuged for 20 min at 3500 × *g* and the supernatant was filtered through a Whatman No 1 paper. The resultant supernatant was aliquoted into Eppendorf tubes and frozen at –80°C. Prior to analysis, an aliquot was defrosted. No repeated freezing was allowed. For the optimised extracts, the same procedure was used but ACN containing 0.1% acetic acid was added in a 1:1 (fruit weight/solvent) ratio. This extraction method has been devised in house and yields the maximum diversity of phytochemicals from a broad range of fruit (data not shown). The determination of Trolox equivalent antioxidant capacity (TEAC) and total phenols, anthocyanins and Vit. C was performed exactly as described by Deighton *et al.* [9]. All reference compounds were obtained for the assays from Aldrich/Sigma (UK) or Extrasynthese (France).

Using the metabolomic approach, *i.e.* no targeted ion monitoring, direct infusion MS (DIMS) was carried out on LCQ-Deca (ThermoFinnigan) controlled by the XCALIBUR software (version 1.4, ThermoFinnigan). Mode of ionisation was ESI in negative and positive ion scanning from *m/z* 80–2000. The sample (juice or extract) was injected into the running LC-MS mobile phase and then directly into the ESI source. The flow from the LC system runs through the system at 200 µL/min of acidified 50% ACN. Data acquisition lasted 1 min and was followed by three blanks (solvent only) to ensure no carry over between samples. The following parameters were recorded: capillary temperature: 275°C, capillary voltage: 16 kV, spray-voltage: 5 kV, tube lens: –5 V, sheath gas: 70 arbitrary units and auxiliary gas: 15 arbitrary units. The autosampler (Surveyor AS, ThermoFinnigan) tray temperature control was set at 4°C. The comparative standards used for structural confirmation of the compounds discussed in Section 3 were obtained from Aldrich/Sigma or Extrasynthese, or were generated in house and identified in previous reports ([8–10] and reference therein). Structural confirmation was done using MS^n fragmentation pattern comparison with the standards.

Statistical analysis and data mining of the DIMS data were performed using a principal component analysis (PCA) approach. Prior to PCA, each DIMS spectrum was background subtracted and the peaks attributed to solvent impurities (in the optimised extract spectra) discounted. The mass spectrum for each sample direct infusion underwent normalisation to remove any run-to-run MS variability. This was achieved by dividing each peak intensity by the total intensity across the mass spectral range. This dataset was then exported to MS-Excel and analysed using the statistical package Genstat® (Hemel Hempstead, UK).

3 Results and discussion

The predominant compounds known to be bioactive within *Rubus* species (here raspberry) are Vit. C and the broad class of chemical moieties that are the (poly)phenols. In complement to the metabolic profiling analyses, the gross analyses of these components were undertaken to see whether any variation had occurred within the segregating cross and could be exploited downstream in a breeding programme. Within this cross, there was a large variation in Vit. C levels (Fig. 1), which is known to be strongly influenced by environmental factors with a distinct shift to levels higher than the parents in the cross, grown in the B field environment, the high-health site. This is important because the ability to tease apart the relative contributions of the genetic and environmental influence on end product quality, here Vit. C, polyphenolics etc, is paramount if we are to optimise and maintain the nutritional relevance of our foodstuffs.

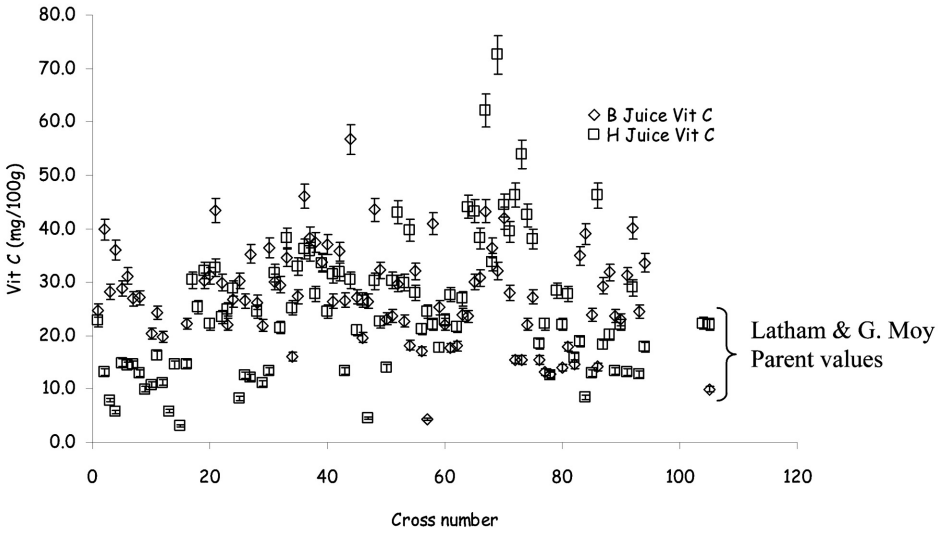

Figure 1. The Vit. C contents of juices derived from the *Rubus idaeus* segregating cross grown in two distinct environments, H and B field.

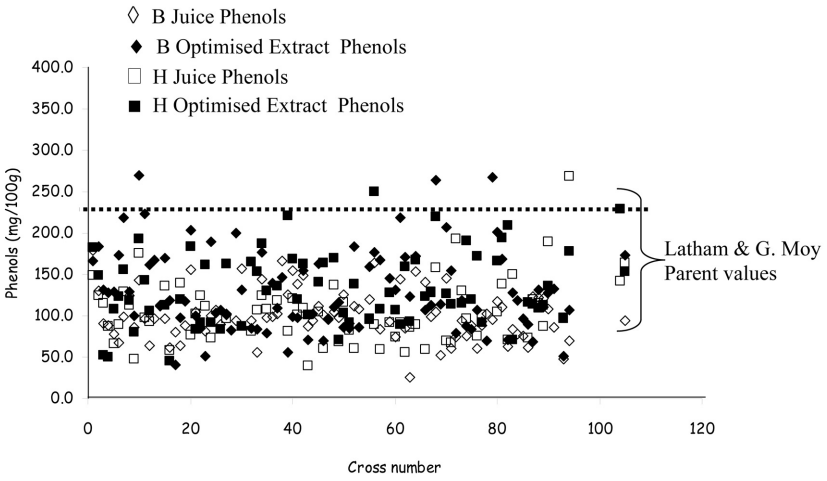

Figure 2. The phenol contents of juices and optimised extracts derived from the *Rubus idaeus* segregating cross grown in two distinct environments, H and B field. The line (……) represents the upper limit of the parental phenol content values. The error for each phenol content measurement is routinely ±2 mg/100 g.

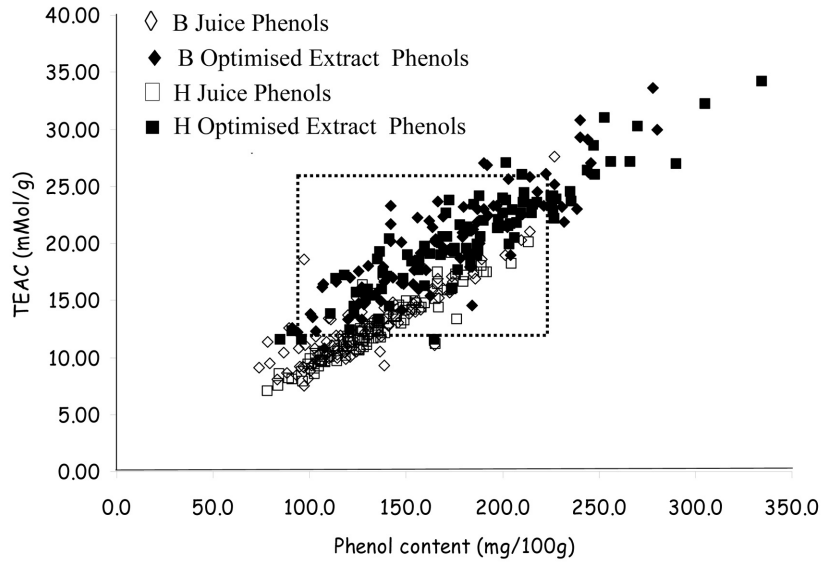

Figure 3. The relationship between the phenol contents and the antioxidant capacities of juices and optimised extracts derived from the *Rubus idaeus* segregating cross grown in two distinct environments, H and B field. The range of parental values is highlighted by the dashed box.

The analogous measurements of phenol contents yielded two main findings. First, the phenol levels in the optimised extracts were, without exception, higher than those found in the corresponding juice. This has a significant bearing on juice versus whole fruit consumption particularly if the polyphenols continue to be shown to be beneficial to human health [17–19, 21–23]. Secondly, a much greater genetic regulation was apparent with regard to the total phenol content values with the majority of the values falling between those of the parents regardless of environment. However, several of the progeny from the crosses did breach this threshold and should be regarded as lines to be followed for development and testing for production of food and/or bioactivity.

Often the bioactivity of the berry-derived polyphenolic components goes hand-in-glove with the *in vitro* antioxidant activity (measured here as TEAC) and this latter parameter is now commonly measured when looking at bioactivities [8–11]. Within the raspberry segregating cross, clearly there were direct relationships (Fig. 2) between the phenol content in the juice and in the optimised extracts (R^2 ~0.9 and 0.75 for the juice and optimise extracts, respectively). Interestingly the environment (H or B field) had minimal impact on the R^2 value of the phenol *vs*. TEAC relationships.

These data show (Fig. 3) that when one consumes the whole fruit then the chemistries evident are clearly different from those of the juice with the optimised extracts dominating the upper phenol and TEAC quartile. Although a significant proportion of the phenols in the optimised extracts appear to be cell wall/pulp associated, as reflected in their elevated phenol contents in comparison to the juice values, they may become solubilized or bioavailable *in vivo* following digestion and the subsequent pH shift associated with transit through the gastrointestinal tract [8].

However, these measurements, although incredibly informative with regard to phenol load *per se*, add nothing with regard to the specific chemistries involved and it is here

that the metabolomic or metabolic profiling approach added value.

Here, we used the DIMS approach wherein the extract is infused into the mass spectrometer and the intensity of all the ions recorded. This approach allows selected mass fragmentation to be performed, thereby yielding structural information. DIMS analysis for multiple samples produces massive datasets and this means that multi-variate statistical analysis, in this case principal component analysis (PCA), must be used. This approach allows the datasets (mass ions and fragments) to be collated and the relative differences between the samples within the selected analytical set to be determined and represented visually. Equally as importantly, the data can then be interrogated to determine which metabolites are driving this separation. This PCA is shown for the segregating crosses in B and H fields (Figs. 4.1 and 5.1, respectively). In addition, the total metabolite segregations have been interrogated to illustrate the metabolites driving this segregation (Figs. 4.2 and 5.2).

It is clear immediately that the environmental effect on the phytochemistry of the cross progeny is significant, as the segregation of the lines in the PCA plots for each environment (B and H field; Figs. 4.1 and 5.1, respectively) is at first glance very different. However, closer examination shows that the progeny separated similarly with respect to positive or negative values in the PCA scores one and two but that their actual position away from the zero point varied. This suggests that the basic underlying metabolite distribution remains similar (phytochemical type), and is therefore predominantly determined via genetic control but that the environment is impinging upon the level of specific phytochemicals.

Interrogation of the PCA plots to determine which compounds are driving these segregations is, from a nutritional viewpoint, quite exciting, as the dominant features are the polyphenols. A host of polyphenols were characterised following comparison to standards but the most evident amongst these were the following: cyanidin 3-glucoside, cyanidin 3-sophoroside, cyanidin 3-glucosylrutinoside cyanidin 3-rutinoside, pelargonidin 3-sophoroside, pelargonidin 3-glucosylrutinoside and quercetin acetylrutinoside. The majority of these compounds have been implicated as putatively beneficial to human health and have long had hopes pinned on them for using them in prophylaxis or the therapy of many diseases [32–34]. In addition these, and the flavonoids in general, have been proposed to impact upon biological pathways in pathologies, such as biotransformation of carcinogens, DNA damage, cell proliferation, apoptosis and inflammation, and as such their daily intake could help increase the human baseline of health or ability to counter (or retard) the onset of disease [35–38].

Interestingly, despite the relatively different segregation of the lines with respect to all the metabolites the dominant ones, the polyphenols identified above, display remarkably similar segregating patterns in the scores in both environments (Figs. 4.2 and 5.2). The most obvious feature is that of the segregation between the cyanidin-3-sophoroside (A) and cyanidin-3-rutinoside (C) groups, which are cleanly segregated according to score 2. This is very informative and useful as it allows rapidly identifying (screen for) plant progeny relatively elevated in these compounds, thereby potentially allowing targeted breeding, *e.g.* cyanidin-3-rutinoside-enhanced raspberries. This could be achieved by

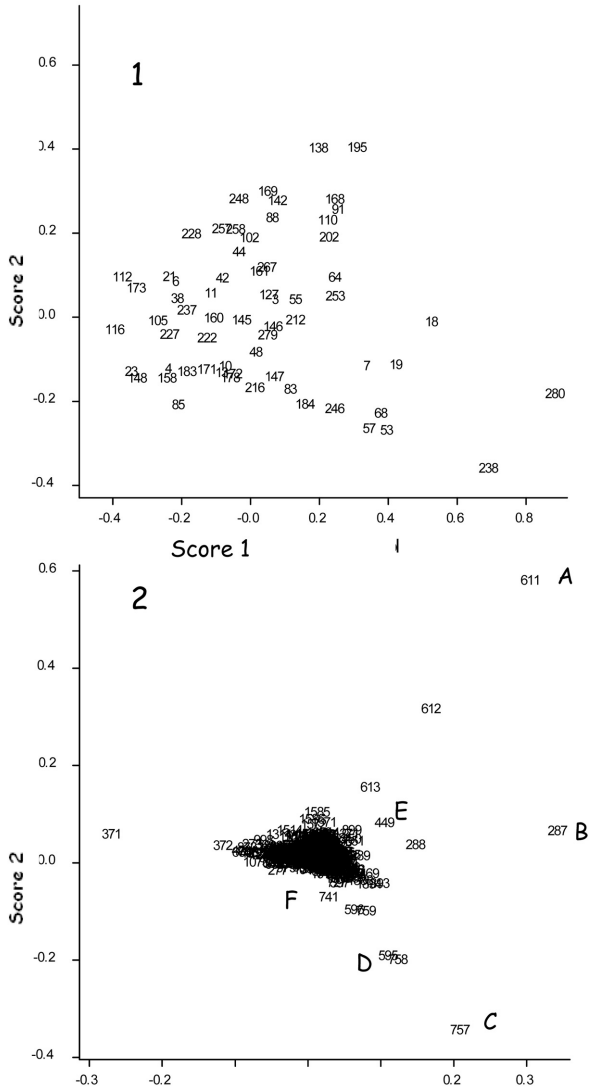

Figure 4. (1) PCA score plot (1 × 2) generated from direct infusion MS metabolites of the *Rubus* segregating cross in the environment B field. The numbers refer to the specific progeny of the cross within the specific environment; SCRI field experimental notation. (2) The same score plot but interrogated on the basis of the ions dominating and creating this segregation: (A) cyanidin 3-sophoroside (*m/z* 611), (B) *m/z* 287 (cyanidin), (C) cyanidin 3-glucosylrutinoside (*m/z* 757), (D) cyanidin 3-rutinoside (*m/z* 595) or pelargonidin 3-sophoroside (*m/z* 595), (E) cyanidin 3-glucoside (*m/z* 449), (F) pelargonidin 3-glucosylrutinoside (*m/z* 741), (G) quercetin acetylrutinoside (*m/z* 651).

analysis and isolation of the selected progeny and then crossing with similarly elevated lines, thereby ultimately yielding a fruit (foodstuff) with a tailored phytochemical composition. Similar approaches can (and will) be undertaken with other key components such as the pelargonidin high/low progeny and those with modified ellagitannin and quercetin levels [8–10].

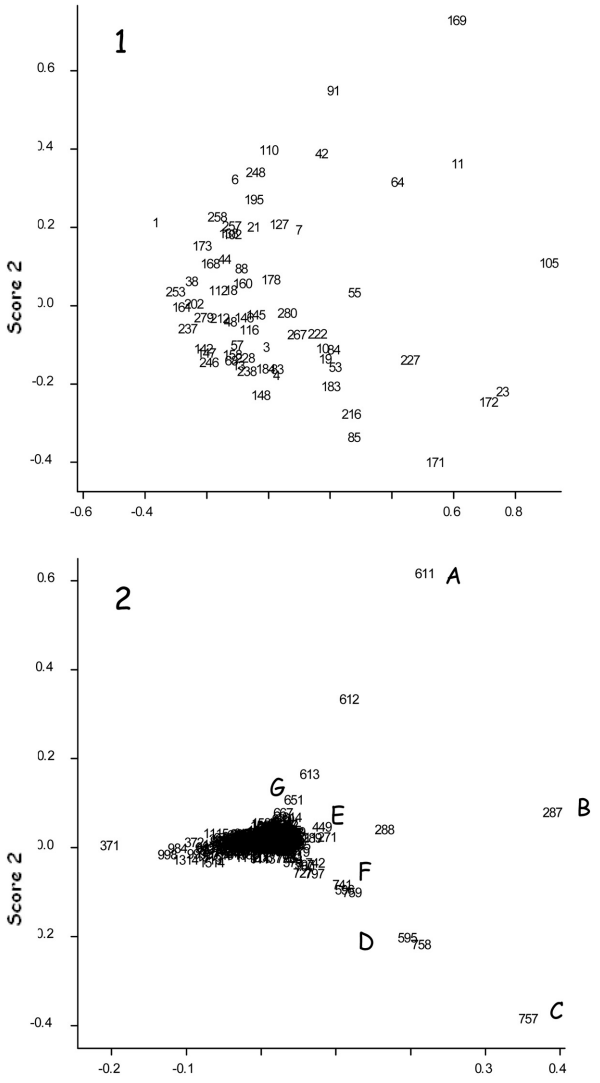

Figure 5. (1) PCA score plot (1 × 2) generated from direct infusion MS metabolites of the *Rubus* segregating cross in the environment H field. The numbers refer to the specific progeny of the cross within the specific environment; SCRI field experimental notation. (2) The same score plot but interrogated on the basis of the ions dominating and creating this segregation (A) cyanidin 3-sophoroside (*m/z* 611), (B) *m/z* 287 (cyanidin), (C) cyanidin 3-glucosylrutinoside (*m/z* 757), (D) cyanidin 3-rutinoside (*m/z* 595) or pelargonidin 3-sophoroside (*m/z* 595), (E) cyanidin 3-glucoside (*m/z* 449), (F) pelargonidin 3-glucosylrutinoside (*m/z* 741), (G) quercetin acetylrutinoside (*m/z* 651).

4 Concluding remarks

In summary, the metabolic profiling approaches are highly relevant to the interface between plant breeding for food and human nutrition. If we are to nutritionally enhance plant-derived food in an appreciable timescale and within the current economic conditions, every opportunity must be taken to ensure that we are capturing the optimum level of information regarding known and putative nutritionally relevant compounds. We have shown here, albeit briefly, that the technology is now within our grasp and that nutritional enhancement using standard breeding approaches is entirely feasible. The next step would be to develop marker-assisted breeding approaches based on mapping robust data derived from well-replicated field trials over different environments.

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