Project title:	Understanding the causes of crumbly fruit in red raspberry
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- Progress is being made in improving our understanding of the development of crumbly fruit in red raspberry.

Background and expected deliverables

The raspberry fruit is an aggregation of multiple fertilized ovaries (drupelets). The number of styles per flower is an indication of the number of drupelets per berry with the smallest fruits having around 60 drupelets (Jennings, 1988). A berry consists of a high number of fertilized ovaries and is quite complex. It cannot be assumed that all the carpels will be pollinated at the same time and that all the ovules are fertilized simultaneously.

This Ph.D. project intends to define the trigger(s) of the crumbly fruit condition and the chosen approach is to induce the “crumbliness” by mechanically damaging the flower before the pollination, to artificially reduce the number of fertilised ovaries and force the plants to grow misshapen fruits.

In the first year of the project, a hypothesis of a hormonal coordinating process regulating and synchronizing the growth of all the fertilized ovaries was formulated and a series of trials, designed to interfere with this hormonic regulating system were completed. Crumbly fruit was successfully induced by mechanically damaging the flower receptacle before pollination.

In the second year of this Ph.D. study, an analytical method was developed to profile the hormones involved in this process. A better description of the work done to achieve this is described in this report.

Summary of project and main conclusions

The hypothesis of a hormonal process controlling the fruit development, in which the receptacle acts as a leading regulatory hub, was tested. Samples of receptacle and drupelets at two different stages, green and red fruit, from both control and crumbly induced material, were analysed with the developed analytical method described in the Science Section of this report. An absolute quantification of eighteen endogenous plant hormones was performed

with this developed method. The selected eighteen phytohormones were representatives of six of the main plant hormones groups (i.e. abscisic acid, auxins, cytokinins, gibberellins, jasmonates and salicylates). The challenging choice, from a technical prospective, to create a method for the simultaneous detection/quantification of so many different compounds was brought about by the lack of literature data about hormonic fruit development models. This made the use of a large range of different plant hormones indispensable; in theory none of the phytohormone groups could be excluded by the hypothetical list of potential candidates involved in the fruit development process.

During the first year of the project, crumbly induction experiments were carried out in three different environments: growth room, glasshouse and field. In the second year, the same experiments were replicated only in controlled growth rooms where the stable and optimal growing conditions reduced the potential effect of the environment on the crumbly induction experiments, while increasing the chance that the crumbly condition is induced by the applied treatment.

At the time of writing, the metabolomic analysis was not yet complete, but the forthcoming results should help better explain the roles of phytohormones in the fruit development process of both normal and artificially uneven raspberries, helping to identify the main hormones involved in the process.

A clear overview of the molecular processes behind the development of crumbly fruit in red raspberry could potentially allow the development of a chemical hormone treatment. This would be applied by growers at the onset of the crumbly symptoms (i.e. spraying of specific hormones involved in the fruit development). This might help to recover the fruit's proper shape and reduce the loss of marketable fruit.

Financial benefits

- This study is examining the scientific reasons for the development of crumbly fruit. It is unlikely to deliver any immediate financial benefit to growers.

Action points for growers

- There are no direct action points for growers resulting from this work at present. However, in light of recent evidence of crumbly fruit developing over time, the

understanding of the trigger(s) of crumbly fruit disorder will enable the development of more robust genotypes leading to improved raspberry varieties, fine-tuning of potential agrochemical treatments and a new standard testing method for Plant Health Certification.

SCIENCE SECTION

Introduction

Project SF 167 aims to understand what triggers the conditions known as crumbly fruit, which occurs to differing degrees in different raspberry cultivars and indicates a partial failure in the physiological processes in fruit development. Little is known about the cause of crumbly fruit in raspberries. A hormonal coordinating process regulating and synchronizing the growth of fertilized ovaries was hypothesised. In general, plants have evolved many different strategies to cope with the challenging environmental conditions which they come across. Plants cannot move to escape stress factors whether abiotic or biotic, and for this reason, over time, they have evolved a series of traits that allow them, for instance, to regenerate damaged organs and tissues and/or to re-direct growth in response to external stimuli (Vanneste and Friml 2009). Small endogenous signalling molecules, called phytohormones (plant hormones), are responsible for the control and coordination of the physiological processes that plants activate to react to external environmental factors, as well as to regulate their growth. Such compounds act at very low concentrations locally, at or near the site of synthesis or even in distant tissues (Santner, Calderon-Villalobos, and Estelle 2009). In general, plant hormones act through extensive crosstalk between themselves and/or other signalling pathways and the results of these interactions are additive, synergistic or antagonistic actions that determine specific and complex physiological outcomes (Vanneste and Friml 2009; Pan, Welti, and Wang 2008).

Phytohormones, according to their structure and function, are divided into eight classes: abscisic acid (**ABA**), auxins (**AUX$_s$**), cytokinins (**CK$_s$**), gibberellins (**GA$_s$**), jasmonates (**JA$_s$**), salicylates (**SA$_s$**), brassinosteroids (**BR$_s$**) and ethylene (Santner, Calderon-Villalobos, and Estelle 2009). Although each class of phytohormones is linked with specific and typical biological effects (e.g. SA for plant defence, GA$_s$ for organ elongation and flowering time, CK$_s$ for germination, etc.), as stated before, the biological processes are regulated by complex networks involving different hormonal signals (Cao et al. 2016). In plants, hormone interactions can occur at least at two different levels, hormone distribution (i.e. the opposing action of **AUX$_s$** and **CK$_s$** during lateral root initiation) and gene expression (i.e. **AUX$_s$** and **BR$_s$** repress the same genes suggesting coordination between the two signalling pathways) (Santner, Calderon-Villalobos, and Estelle 2009). The activity of plant hormones depends on their availability which is in turn affected by their metabolism, localisation, transport and signal transduction and the modulation at any of these levels. There are myriads of possible combinations which can determine different physiological processes (Simura et al. 2018).

Jasmonic acid (JA) is synthesized via the octadecanoid pathway, is conjugated to isoleucine to form JA-Ile or converted to methyl jasmonate (MeJA), its levels increase rapidly in response

to a wide range of stimuli, herbivory or mechanical wounding and in general abiotic stresses; increased hormones levels result in the upregulation of plant defence genes (Santner, Calderon-Villalobos, and Estelle 2009). Studies conducted on plant mutants with alteration in the genes involved in biosynthesis, perception and signalling of JA were unable to produce stamens with elongated filaments and viable pollen; only exogenous treatments with JA restored the stamen development of these mutants suggesting that JA play an important role for stamen development and consequent plant fertility (Ozga et al. 2017). MeJA and JA are plant regulators of different processes such as seed germination, flower and fruit development; MeJA, can easily diffuse through the membranes and consequently could be the perfect signal transducer for intra and intercellular mediating jasmonate-responsive plant responses (Seo et al. 2001). In plants the biosynthesis of hormones methyl esters is catalysed by carboxyl methyltransferase that transfers a methyl group from S-adenosyl-L-Met to the carboxyl group of small molecules such salicylic acid, benzoic acid, jasmonic acid and Indole-3-acetic acid (Zubieta et al. 2003). Methylation can be considered as an alternative regulating mechanism to conjugation with amino acids or sugars for modulating the activity of the free acid (i.e. BA, CA, JA, IAA and SA); a potential advantage of methylation over conjugation is the physical property of the final compounds. The methylated phytohormones are non-polar compounds that can diffuse passively through the membranes whereas polar compounds such as conjugated plant hormones require an active transport system. Methylated plant hormones could be then ideal candidate for intra and intercellular transport of hormones, allowing plants to easily move hormones, short and long distance, in a specific site (i.e. tissue or organ) where they can conduct their regulating action (Qin et al. 2005).

Analytical methods designed to simultaneously analyse multiple classes of phytohormones, are lacking. The best compromise currently is to work out the complexity behind the regulation of physiological processes mediated by plant hormones, allowing and facilitating the study of the hormone networks and functions (Pan, Welti, and Wang 2008).

In the last two-three decades, phytohormones have been extensively studied and many protocols have been examined to develop the analytical methods to detect hormones content in plants. In particular great technological progress has been achieved with immunoassays (Weiler 1984) and hyphenated techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) (Muller, Duchting, and Weiler 2002; Kowalczyk and Sandberg 2001) and Liquid Chromatography–Mass Spectrometry (LC-MS) (Simura et al. 2018; Trapp et al. 2014; Pan, Welti, and Wang 2008; Cao et al. 2016). The immunological detection methods are characterized by high sensitivity and because they do not require multi step extraction procedures, no loss of material is experienced with the analysis but obviously they are very specific, requiring a method for each hormone to be detected; for this reason they are

unsuitable and too laborious for simultaneous detections of multiple classes of phytohormones (Trapp et al. 2014). The GC-MS methods have been extensively used in the past and are still the most employed analytical system for the simultaneous detection of multiple hormones but the derivatization steps that must be applied to enhance compounds volatility, to improve their stability and facilitate GC separation and MS ionisation (Du, Ruan, and Liu 2012) make the analysis laborious and the high temperatures reached in the GC injector could easily degrade and transform the target analytes, compromising the method reliability (Cao et al. 2016).

LC-MS, especially in the last decade, has become a valid alternative technique to trace plant hormones as it can overcome the drawbacks of the GC-MS and higher sensitivity and the lower detection limits that can be reached with these systems (Cao et al. 2016). There are many different LC-MS techniques available but for the most accurate quantification, the liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS), although it cannot screen for unknown compounds, is the technique to be chosen for the detection of target compounds because it has greater sensitivity, repeatability and a wider dynamic range (Cao et al. 2016).

The right analytical instrument is only a small part of a complete separation based analytical process. Sample preparation is crucial and can account for the 80% of the total time of analysis. All the following steps of identification, confirmation and quantification can be greatly affect by the extraction process and are crucial in the analysis of trace compounds such as phytohormones (Du, Ruan, and Liu 2012). In general sample preparation involves many procedures: sampling, freeze drying, comminution (i.e. grinding with mortar and pestle), homogenization, extraction from the matrix and purification. This last procedure being very important, especially for complex plant extraction, where, whichever the instrument selected, it is not recommended to load the crude plant extract directly through the column to avoid its damage and fouling (Du, Ruan, and Liu 2012) .

Many methods have been developed and at the same time many commercial products are available for the removal of sample matrices and the enrichment of sample (Cao et al. 2016). Liquid-liquid extraction (LLE) exploits the difference in solubility of the target compounds between two liquid phases; for example separating analytes from an aqueous solution to a non-polar or less polar solvent (i.e. hexane, diethyl ether, dichloromethane/isopropanol, etc.). Solid phase extraction (SPE), on the other hand, exploits the interaction (i.e. absorption, hydrogen bonding, polar and non-polar interactions, cation and anion exchange and size exclusion) between the analytes and the sorbent material. Studying and understanding the best mechanism of interaction helps to choose the most suitable and performing sorbent material increasing the purification efficiency (Du, Ruan, and Liu 2012). Salycylates are generally recognised as hormones mainly involved plant defence response (Santner et al.,

2009) but their role in fruit growth cannot be completely excluded. In particular cinnamic acid, an intermediate in the biosynthetic pathway of salicylic acid, seems to be an important inhibitor of auxins transport (Steenackers et al. 2017) and again its role in the regulation of fruit growth, even though indirect, cannot be excluded.

It is well established that plant hormones are signalling molecules that participate in the regulation of fruit development processes. Fruit growth is a complex process where the key stage is pollination/fertilization which triggers cell division and then cell enlargement for both ovary (pericarp) and ovule (seed) development. Phytohormones action and their interactions affect fruit development in different ways, for instance the biosynthesis of a hormone might be affected by another hormone or the hormonal signal transduction pathway might be affected by an analogous component(s) of a different hormone signal transduction pathway. In other cases, different classes of hormones, affecting the growth of different cells or tissues, must act in concert to guarantee the normal fruit development or a class of hormones begins a process but to complete it, another class of hormones is required (Ozga and Reinecke 2003).

Many plant hormone groups (auxins, cytokinins, gibberellins, salicylates, jasmonates and ABA) are involved in the fruit development process, but a clear model of its hormonal regulation is not currently available. Fruit growth can be divided into two main phases: before and after pollination/fertilization. During the first phase, at the very beginning, the production of new cells is the leading factor responsible for the development of flower and fruit primordia. Low levels of auxins and their conjugates as well as of gibberellins maintain temporally inactive flower and fruit primordia (Obroucheva 2014). Growth is slowed and senescence occurs unless events triggered by pollination/fertilization, such as cell division and cell enlargement both in the ovary (fruit pericarp) and in the ovules (seeds), occur to promote the fruit growth (Ozga and Reinecke 2003). For a review of current knowledge on the stages in raspberry development to fruiting see Graham and Simpson (2018).

In tomato, the inactive state of the ovules is supported by high concentration of ethylene and ABA (Vriezen et al. 2008; Obroucheva 2014); after pollination/fertilization, in the fertilized ovules of tomato ovaries, two main events occur, the reduction of ethylene and ABA and the increase of the auxin content. In particular the main event leading to fruit set is the increase of auxin levels because they induce the biosynthesis of gibberellins; both these two classes of phytohormones exert active growth of fruit but while auxins influence cell division, the gibberellins are responsible for cell extension (Serrani et al. 2008). Cytokinins too regulate cell division in both pericarp and seed, for instance, during the first 10 days of fruit growth in lupine, cytokinins levels increase in the pedicel due to intense import through phloem and xylem vessels; such accumulation might be related to downregulation, induced by GAs, of

genes encoding enzymes involved in cytokinins conjugation such as *zeatin glucosyl transferase* and *zeatin xylosyl transferase* (Vriezen et al. 2008).

GAs synthesized in the growing fruit induce the developmental programme responsible for cell growth in the immature fruits; GAs seem to downregulate the expression of genes involved in the synthesis of ethylene and of the components of its signal transduction pathway and at the same time they also attenuate ABA synthesis.

Regarding the hormonal regulation of fruit ripening in non-climacteric crop such as raspberry, ABA seems to be the only phytohormone directly involved in this stage. In strawberry, for instance, treatments with 0.5 mM ABA turned white mature berry into deep red berries in a week while untreated berries did not reach even pink colour in the same time period. The main role of ABA in fruit ripening might be confirmed by upregulation of genes encoding ABA precursors such as xanthoxine as well as ABA receptor such as PYR1. Ethylene in strawberry seems to be only weakly involved during fruit reddening as the upregulation of genes codifying ethylene receptors. Auxins, while deeply involved during fruit growth and maturation, start to decrease during white fruit causing the suppression of genes encoding *endo-β-glucanase*, *pectate lyase* and *pectin methyltransferase* as well as of genes involved in the conversions of flavonoids and in the synthesis of aromatic compounds (Harpster, Brummell, and Dunsmuir 1998).

According to the current models, the fruit development is regulated by the interplay of different hormones and the combined action of auxins, gibberellins, cytokinins seem to play the main role in fruit set and their levels increase substantially; this has been already validated by exogenous applications of these three hormones which cause parthenocarpic fruit in tomato (Kumar, Khurana, and Sharma 2014). ABA levels decrease at fruit set as a consequence of downregulation of genes involved in ABA biosynthesis (NCED1 and NSY) and of upregulation of a gene (CYP707A) involved in its degradation (Vriezen et al. 2008). The development of fruit and seed is intimately connected and synchronised with the last being a source of hormones, especially auxins, gibberellins and cytokinins, which are then transported in the surrounding tissues where they are involved in stimulating the growth and even determine the final size of the fruit (Ozga and Reinecke 2003). At maturation, when fruit prepares to undergo ripening, auxins and cytokinins seem to be involved and, in a tomato *ripening inhibitor (rin)* mutant, the levels of both these two hormones at maturation stage where higher than those of wild-type fruits (Davey and Van Staden 1978). In strawberry the removal of achenes, the source of auxins from the surrounding tissues, causes rapid ripening of the receptacle unless exogenous applications of auxins stall the process (Given, Venis, and Gierson 1988). Auxins seem to play the major role at fruit maturation, observations in strawberry and mango suggest that reduction of auxins represents a prerequisite to commence ripening (Zaharah et al. 2012; Given, Venis, and Gierson 1988) but experiments

with a cytokinin-deficient mutant in *Arabidopsis thaliana* showed unsynchronized ripening phenotype (Kumar, Khurana, and Sharma 2014) while observations of cytokinins decrease before ripening initiation suggest that cytokinins too might play a role in fruit maturation (Bottcher, Boss, and Davies 2011).

Fruit ripening involves the activation of several genetic and biochemical pathways leading in a coordinated and synchronised fashion, to colour changes, conversion of complex carbohydrates to sugars, accumulation of flavour and aroma compounds and change in the cell wall dynamics that cause either dehiscence or softening. In non-climacteric fruit ABA seems to have the strongest role during fruit ripening, its content increase and experiments in tomato, aiming to postpone ABA accumulation, cause a delay in fruit ripening (Zhang, Yuan, and Leng 2009).

In the second year, the project focused on the hypothesis of a hormonal coordinating process regulating and synchronizing the growth of all the fertilized ovaries was formulated and a series of trials. In this work, a simple analytical method was developed to detect 18 phytohormones representing the six most important classes of plant hormones: abscisic acid (**ABA**), salicylates (benzoic acid **BA**, cinnamic acid **CA**, salicylic acid **SA**, methyl benzoate **MeBA**, methyl cinnamate **MeCA** and methyl salicylate **MeSA**), jasmonates (jasmonic acid **JA**, methyl jasmonate **MeJA** and 13-epi-12-oxo-phytodienoic acid **OPDA**), auxins (indole-3-acetic acid **IAA**, indole-3-butyric acid **IBA**, Indole-3-carboxylic acid **ICA** and methyl indole-3-acetate **MeIAA**), gibberellins (gibberellic acid A_1 **GA_1**, gibberellic acid A_3 **GA_3**, gibberellic acid A_4 **GA_4**) and cytokinin (**zeatin**). A method was developed to allow analysis of the raspberry fruits extract at two different stages, green berry and red berry both in the receptacle and in the drupelets to try to verify the hypothesis that fruit development is coordinated and regulated by the receptacle, acting as a leading hub, synchronizing the growth of the many fertilized ovaries that will constitute the fruit drupelet. Samples of crumbly induced fruit (flowers mechanically damaged) and control (normal developed fruits) have been collected, drupelets and receptacle for each berry have been separated with the aim of verify which phytohormones are primarily involved in the fruit development and what are, if any, the differences between a crumbly fruit and a normal fruit (control) and how, if the hypothesis is correct, the receptacle fulfils its function of raspberry fruit regulator.

Materials and methods

Chemicals and materials

The 18 authentic standards of phytohormones were purchased from three different suppliers. SigmaAldrich Co. Ltd. (Gillingham, UK): IAA, IBA, ICA, MeIAA, SA, CA, BA, MeBA, MeCA, MeSA, GA_3, GA_4, and MeJA. OPDA and JA were purchased from Cayman Chemicals (Cambridge Bioscience, Cambridge, UK) while GA_1 was purchased from OlchemIm Ltd (Olomouc, Czech Republic). All the organic solvents used for the extraction (methanol, acetonitrile, 2-propanol, HCl and dichloromethane) were analytical grade and were purchased from Fisher-Scientific (Loughborough, UK). The chromatographic separation was performed using: formic acid, methanol and water HPLC grade (these last two of J. T. Baker quality) were purchased from Fisher-Scientific Ltd (Loughborough, UK). Deionised water, used for all the aqueous solutions, was obtained using ELGA DV25 system (High Wycombe, UK). Isotopically labelled standards (trans cinnamic-d_7 acid, indole-3-acetic-2,2-d_2 and Jasmonic-d_5-acid) were purchased from SigmaAldrich Co. Ltd. (Gillingham, UK) while ($[^2H_2]GA_1$, $[^2H_2]GA_3$, $[^2H_5]$MeIAA, $[^2H_6]$-trans zeatin, $[^2H_6]$(+)-cis,trans-ABA and $[^2H_4]$SA) were purchased from OlchemIm Ltd. (Olomouc, Czech Republic).

Apparatus

Chemical analysis of plant material (receptacle and drupelets) was performed on an Agilent 1260 high performance liquid chromatography (HPLC) system consisting of quaternary pump, a Diode Array Detector (DAD), a Temperature Control Device and a solvent Thermostat module (Agilent Infinity 1290) coupled to an Agilent 6460A Triple Quadrupole Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA). The sample extract (5 µL) was injected onto a 150 x 2 mm (5µm) Gemini RP C18 (110 Å) column fitted with a Gemini C_{18} 4 x 2 mm Security guard cartridge (Phenomenex, Cheshire, UK). Samples were eluted at a flow rate of 0.3 ml min^{-1}, using a gradient separation with two mobile phases A = 0.1% formic acid in deionized water and B = 0.1% formic acid in methanol. The elution gradient lasted 29 min in total and was as follows: A/B 95/5 (v/v) hold for 2 min, ramped up to 35% B in 1 min, followed by further ramping up from 35% to 55% in 15.5 min and further ramped up from 55% up to 100% in 3 min and hold for 2 min. Within 0.5 min the gradient was returned to the initial composition of 5% and held for 5 min (Table 1).

Table 1: Chromatographic elution gradient

Time (min)	A%	B%	Flow (mL min^{-1})
0	95	5	0.3
2	95	5	0.3
3	65	35	0.3
18.5	45	55	0.3
21.5	0	100	0.3
23.5	0	100	0.3
24	95	5	0.3
29	95	5	0.3

Mass detection was carried out in negative or positive ion mode depending on the phytohormone (Figure 1, Table 2) using a jet stream electrospray ionization (ESI) interface coupled to the triple quadrupole system (Agilent, USA). For ESI, the gas temperature, gas flow, nebulizer pressure, sheath gas temperature, sheath gas flow, capillary cap and nozzle voltage were optimized with the help of Agilent Source Optimizer Software and set to 350°C, 10 L min^{-1}, 30 psi, 4000°C, 11 L min^{-1} and 3500 V (both ion modes). Collision energies and fragmentor voltages for transition states of the 17 phytohormones were optimized using Agilent Optimizer Software. Hereby the most sensitive transitions (i.e. transitions with the highest intensity of the product ions were chosen to build the final multiple reactions monitoring (MRM) method.

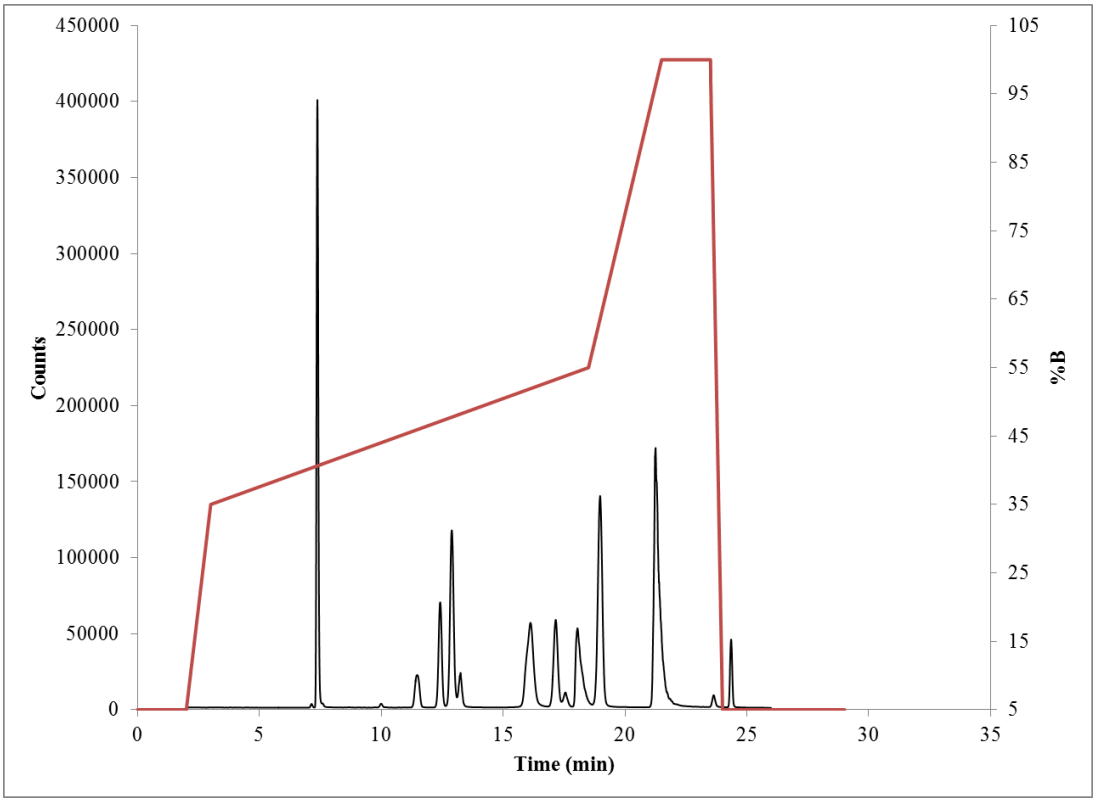


Figure 1: Chromatogram of the standards mix containing the 18 targets compounds; elution gradient (red line)

Table 2: Phytohormones, their respective precursor ion and product ion with optimized fragmentor voltage and collision energy

Compound Name	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy	Polarity	tR (min)
d-GA_1 [$^2H_2$]	351.2	333.2	10	105	4	Positive	11.68
d-GA_1 [$^2H_2$]	351.2	305.2	10	105	4	Positive	11.68
GA_1	349.2	331.1	10	95	0	Positive	11.89
GA_1	349.2	285.1	10	95	16	Positive	11.89
d-GA_3 [$^2H_2$]	347.1	241.2	10	150	12	Negative	11.2
d-GA_3 [$^2H_2$]	347.1	143.1	10	150	32	Negative	11.2
GA_3	345.1	239.2	10	155	12	Negative	11.44
GA_3	345.1	143.1	10	155	24	Negative	11.44
GA_4	331.1	287.2	10	220	16	Negative	24.28
GA_4	331.1	257.1	10	220	20	Negative	24.28
OPDA	291.2	247.2	10	175	12	Negative	25.43
OPDA	291.2	165.1	10	175	16	Negative	25.49
d-ABA [$^2H_6$]	269.2	225.2	10	190	8	Negative	15.65
d-ABA [$^2H_6$]	269.2	159.1	10	190	8	Negative	15.65
ABA	263.1	219.1	10	95	8	Negative	16.04
ABA	263.1	153.1	10	95	4	Negative	16.04
MeJA	225.1	151.1	10	85	8	Positive	23.95
MeJA	225.1	133.1	10	85	12	Positive	23.95
d-Zeatin [$^2H_5$]	225.1	137	10	100	16	Positive	7.2
d-Zeatin [$^2H_5$]	225.1	135.9	10	100	16	Positive	7.2
Zeatin	220.1	202.1	10	90	8	Positive	7.32
Zeatin	220.1	136	10	90	16	Positive	7.32
d-JA [$^2H_5$]	214.2	62.1	10	130	8	Negative	18.95
d-JA [$^2H_5$]	214.2	42.1	10	130	48	Negative	18.95
Jasmonic Acid	209.1	109	10	113	16	Negative	19.36
Jasmonic Acid	209.1	59.1	10	113	8	Negative	19.36
IBA	204.1	186.1	10	90	8	Positive	18.95
IBA	204.1	130.1	10	90	28	Positive	18.95
d-MeIAA [$^2H_5$]	195.1	135	10	90	16	Positive	16.6
d-MeIAA [$^2H_5$]	195.1	134.1	10	90	12	Positive	16.6
MeIAA	190.1	130	10	75	12	Positive	17.14
MeIAA	190.1	77.1	10	75	50	Positive	17.14
d-IAA [$^2H_2$]	178.1	132	10	90	12	Positive	12.6
d-IAA [$^2H_2$]	178.1	78.1	10	90	48	Positive	12.6
IAA	176.1	130.1	10	85	12	Positive	12.89
IAA	176.1	77.1	10	85	50	Positive	12.89
MeCA	163.1	131	10	70	8	Positive	23.62
MeCA	163.1	103.1	10	70	20	Positive	23.62
ICA	162.1	144	10	80	12	Positive	12.47
ICA	162.1	118.1	10	80	12	Positive	12.47
d-CA	154.1	110.1	10	115	12	Negative	16.97
CA	147	103.1	10	100	8	Negative	17.55
MeSA	153.1	121	10	85	12	Positive	21.48
MeSA	153.1	65.1	10	85	32	Positive	21.48
MeBA	137.1	100	10	85	4	Positive	18.41
MeBA	137.1	77.1	10	85	28	Positive	18.41
d-SA [$^2H_4$]	141.1	97	10	90	16	Negative	15.1
d-SA [$^2H_4$]	141.1	69.1	10	90	32	Negative	15.1
SA	137	93.1	10	75	16	Negative	15.49
SA	137	65.1	10	75	32	Negative	15.49
BA	121	77.1	10	105	8	Negative	12.98

As shown in Table 2, 33 transitions were part of the MRM mode, each with a dwell time of 10 ms and a delay time of 3.5 ms, leading to a total cycle time of 445.5 ms and thus 2.5 Hz (2.5 cycles per second). Peak areas of the 18 phytohormones and of the nine isotopically labelled internal standards were integrated with Agilent MassHunter Quantitative Software (Agilent, USA; version B.07.00).

Plant material

Glen Ample long canes were purchased from EU plants Ltd. (Abingdon, UK). Plants were grown in controlled environment, in a Nijssen plant growth room (Leiden, The Netherlands), with the following parameters set for the first two weeks: minimum temperature 10°C, maximum temperature 14°C, relative humidity 70% and daylight length 16 hours (from 7:00 AM to 11:00 PM); such conditions helped the plants to acclimatize. After the two weeks of acclimatization, all the environmental parameters remained unchanged except the maximum temperature was increased by two degrees, from 16 to 18°C. The first flowers, immediately after anthesis, were collected and dried at room temperature for two days to help the pollen to mature. The dried flowers were closed in Petri dishes and stored at 0.1°C. They were used as a pollen reservoir for the crumbly induction experiments. Fruits originated by the crumbly fruit induction experiments (previously described in this work), both damage of the receptacle and control, were collected at two different stages, green and red berry and immediately frozen in liquid nitrogen. They were then stored at -80°C to be used for the hormones profiling analysis.

Phytohormones extraction

Three different extraction protocols were tested: two liquid-liquid-extractions (LLE) and a solid-phase-extraction (SPE).

MeOH:H_2O method

For the optimization, the composition of the solution was evaluated by performing the extraction with four different methanol: water (CH_3OH:H_2O) ratios (v:v 8:2, 7:3, 6:4 and 1:1). Plant material was finely ground with mortar and pestle adding liquid nitrogen regularly to keep the sample as frozen as possible to avoid thawing. 100 mg of frozen plant material was transferred in a 1.5 mL Eppendorf tube and 1 mL of extraction buffer was added; the experiment was repeated to test the four different extraction buffers. Samples were loaded onto a shaker, VIBRAX® VXR basic (IKA® England Ltd, UK) and shaken at 100 revolutions per minute (rpm) for 30 min in cold room (-20°C) and then centrifuged at 16,000 g for 5 min

at 4°C; the pellet was re-extracted, following the same procedures, while the supernatant was dried in speed vacuum at 30°C and reconstituted in 100 μL of pure CH_3OH. The reconstituted samples were centrifuged for 10 min at 16,000 g at 4°C, the supernatant was transferred into insert-equipped vials (Fisher-Scientific (Loughborough, UK) and analysed.

2-propanol:H_2O:HCl method

Plant material was finely ground with a mortar and pestle adding liquid nitrogen regularly to keep the sample as frozen as possible to avoid thawing. 300 mg of frozen plant material was transferred to a 15 mL tube and 3 mL of extraction solvent 2-propanol:water:hydrochloric acid (C_3H_8O:H_2O:HCl) (v:v:v 2:1:0.002) was added, keeping the sample to solvent ratio at 1:10 (mg μL^{-1}). The sample was loaded onto a shaker, Multi Reax (Heidolph Instruments GmbH & CO. KG, Germany) and shaken (100 rpm) for 30 min in cold room (-20°C) and then centrifuged at 16,000 g for 10 minutes at 4°C. The supernatant was saved and 3 mL of dichloromethane (CH_2Cl_2) were added to each sample, again loaded onto a shaker and shaken (100 rpm) for 30 min in cold room (-20°C) followed by 10 minutes of centrifugation at 16,000 g and 4°C. The two formed phases (lower and upper) were each pipetted into a new 2 mL tube and dried in speed vacuum at 30°C; the two samples were reconstituted in 300 μL of pure CH_3OH and centrifuged for 10 min at 16,000 g at 4°C; the supernatant was analysed.

SPE Oasis® HBL extraction method

Plant material (50 mg, drupelets and receptacle from both green and red stages) was weighted into 2 mL Eppendorf tube and was frozen into liquid nitrogen to help its homogenization. 1 mL of extraction solution, containing a mixture of isotopically labelled internal standards, was added to the tube together with 2 stainless steel beads (3 mm of diameter). The extraction solution, ice-cold 50% aqueous acetonitrile, was spiked with nine isotopically labelled standards (trans cinnamic-d_7 acid, indole-3-acetic-2,2-d_2, jasmonic-d_5-acid, [$^2H_2$]GA_1, [$^2H_2$]GA_3, [$^2H_5$]MeIAA, [$^2H_6$]trans-Zeatin, [$^2H_6$](+)-cis,trans-ABA and [$^2H_4$]SA), in amounts ranging from 0.18 to 0.91 ppm (parts per million) in order to have at least one internal standard for each of the six phytohormones groups; the best compromise to allow an absolute quantification of the endogenous plant hormones in the samples analysed. The Eppendorf tubes, were loaded onto a vibration mill, model MM 301 (Retsch GmbH & Co. KG, Haan, Germany) with an operating frequency of 30 Hz for 1 minute and homogenized. The mixture was first sonicated for 3 minutes in a cold room (4 °C) and then extracted using a benchtop laboratory rotator at 15 rpm for 30 min in cold room (4 °C). The extraction was followed by centrifugation at 16,600 g at 4 °C for 10 min. The supernatant was saved and transferred to a new 2 mL Eppendorf tube for further purification. Samples were purified using

Oasis® HLB reverse-phase, polymer-based, solid phase extraction (RP-SPE) cartridge (1 cc/30 mg) from Waters Ltd (Elstree, UK). The solid phase extraction protocol consists of four steps, first of all the cartridge was conditioned/activated with 1 mL of pure methanol followed by 1 mL of distilled water. The conditioning/activation was followed by the column equilibration step with 1 mL of 50% aqueous acetonitrile (v:v) and then by the sample loading. The flow through was collected in a 7 mL amber glass vial (SigmaAldrich Co. Ltd, UK) together with the 30% aqueous acetonitrile solution (v:v) used to rinse the cartridge. Samples were dried in speed vacuum at 30°C and stored at -80 °C; before performing the analysis the samples were reconstituted with 40 µL of 30% aqueous acetonitrile (v:v) and analysed with the LC-MS/MS system.

Preparation of standards solutions

For each target phytohormone, a stock solution was prepared at mg/mL in, pure methanol, 50% aqueous methanol or pure distilled water, depending on the compound solubility (details in Table 3).

Table 3: Phytohormones stock solutions and their concentration in the authentic standards mix solution

Phytohormones	[ppm] stock solutions	solvent	volume of stock (mL)	dilution factor	standards mix final [ppm]
GA_3	1000	50:50 v:v H_2O:MeOH	0.5	14	71
ABA	1000	50:50 v:v H_2O:MeOH	0.5	14	71
SA	1000	50:50 v:v H_2O:MeOH	0.5	14	71
ICA	1000	50:50 v:v H_2O:MeOH	1	7	143
CA	1000	50:50 v:v H_2O:MeOH	1	7	143
BA	1000	50:50 v:v H_2O:MeOH	1	7	143
MeCA	1000	50:50 v:v H_2O:MeOH	0.25	28	36
MeIAA	1000	50:50 v:v H_2O:MeOH	0.25	28	36
IBA	1000	50:50 v:v H_2O:MeOH	0.05	140	7
IAA	1000	50:50 v:v H_2O:MeOH	0.05	140	7
OPDA	1000	50:50 v:v H_2O:MeOH	0.05	140	7
zeatin	500	50:50 v:v H_2O:MeOH	0.05	140	4
MeSA	625	100:100 v:v H_2O	0.8	9	71
MeJA	1030	50:50 v:v H_2O:MeOH	0.02	350	3
JA	3000	50:50 v:v H_2O:MeOH	0.333	21	143
GA_4	5000	50:50 v:v H_2O:MeOH	0.2	35	143
MeBA	2100	100:100 v:v H_2O	0.24	29	72
GA_1	1000	100:100 v:v CH_3OH	0.5	14	71
		Total Volume (mL)	7		

A standards mix solution was prepared diluting the stock solution in CH_3OH:H_2O (50:50) and the final concentration of each compound was empirically optimized by analysing

progressively decreasing standards mix concentrations. The compounds that better ionized are better detected by the instrument and are obviously required in lower concentration. Table 3 shows the final concentration of each target plant hormone in the final standards mix. For the nine isotopically labelled standards, the mix solution was prepared by diluting the stock solutions at different concentrations depending on the range of the calibration curve. The final concentration for each of the deuterated standard was: roughly 0.9 ppm for d-MeIAA, d-ABA, d-CA, d-JA, d-IAA and d-SA while roughly 0.18 ppm for d-GA_3, d-GA_1 and d-Zeatin.

Method Validation

Limit of detection and limit of quantification

Terms such as sensitivity, Limit of Detection (LoD) and the Limit of Quantification (LoQ) are used to indicate the smallest concentration of a target compounds that can be reliably measured by an analytical procedure (Armbruster and Pry, 2008). In practice, an assay is not able to measure the analyte concentration down to zero. There must be always a sufficient analyte concentration that produces a signal that can be reliably distinguished from the 'noise' that is the signal produced in absence of the analyte (Armbruster and Pry, 2008). For the analytical methods based on HPLC (High Pressure Liquid Chromatography) the LoD is expressed in terms of signal-to-noise levels and it was calculated as three times the standard deviation of the lower value of the calibration curve divided by the slope of the curve noise level while the LoQ was calculated by multiplying the standard deviation of the lower value of the calibration curve by ten and then dividing by the slope of the curve.

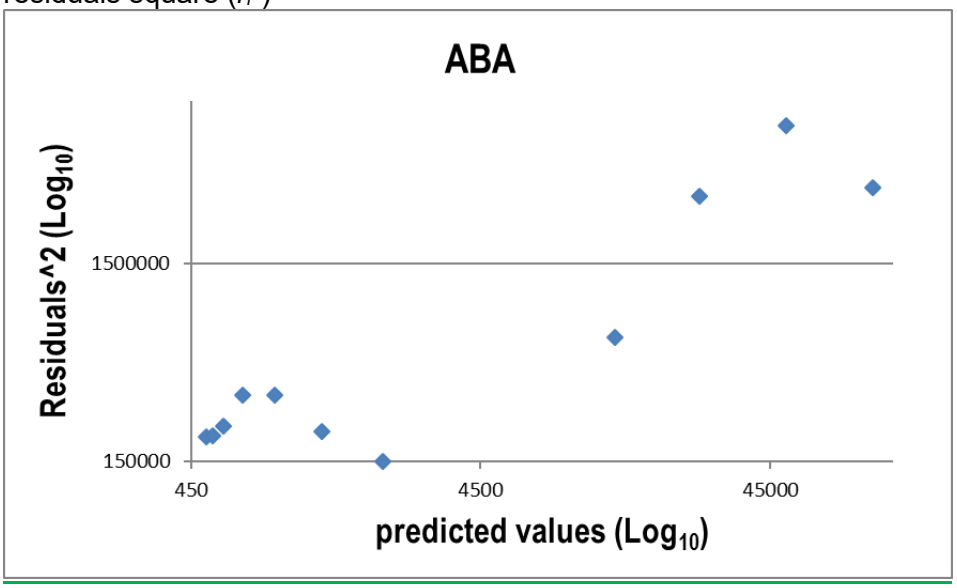
Calibration curve and linearity

The calibration curves were obtained with 12 serial dilutions. The standards mix solution, containing all the eighteen target phytohormones, was first diluted 1:8 and this solution was the starting point for 11 (1:2) serial dilutions that produced the 12 different concentrations solutions used to create the calibration curves. For each dilution the analysis was repeated three times. Data were processed using a Microsoft Excel spreadsheet to calculate the average peak area and the standard deviation. The regression parameters were obtained by introducing the respective formulas into the worksheet; the procedure was repeated for all the 18 compounds of interest. The intervals that best fitted the linear regression model were chosen; for those compounds with better ionising properties and subsequent better detection, a broader linear dynamic range was found (i.e. ABA, SA and IAA).

The linear regression models are the most commonly used analysis to predict the concentration of analytes in unknown samples. Usually in the case of linearity ranges larger than two orders of magnitude, the variance of each point might be different and the larger

deviations present at higher concentration influence (weight) the regression line more than those of the smaller concentrations (Almeida, Castel-Branco, and Falcao 2002). In many cases, an increase of variance as a function of the concentration occurs and the instrument errors, instead of being always uniform, correlates with the independent variable (analyte concentration) and so variance and standard deviation are larger at higher concentration and the accuracy in the lower end of the range is impaired; this phenomenon is called heteroscedasticity and must be addressed in order to produce stable curves. Four different statistical tests have been used to check for heteroscedasticity; two graphical tests and two more formal tests: Breusch-Pagan and Abridged-White. In equations with intercepts the correlation coefficient between residuals (r_i) and predicted values (Y_i) is always zero so any graph of r_i versus Y_i reflects a random scatter of points about a line with zero slope, then systematic patterns are indication of inadequate model or a changing variance. Another graphic way to detect heteroscedasticity is the plot of squared residuals (r_i^2) versus Y_i this because the square residual reflects the contribution of a given response to the error sum of squares which is an estimate of the variance σ^2. In case the squared residuals are varying in a systematic way, they increase or decrease with predicted values, a plot of (r_i^2) versus (Y_i) should detect such trends, see Figure 2, (Gunst and Mason, 1980).

Figure 2: Heteroscedasticity graphical test, linear regression predicted values (Y_i) versus residuals square (r_i^2)



In order to check whether heteroscedasticity (error terms with unequal variances) is present or not in our model, a regression analysis is run by selecting the analyte peak area as dependent variable and its concentration as independent variable. The p-value is checked and if lower than 0.01, the regression is significant to 99% level and by looking at the residuals the heteroscedasticity can be assessed. A plot of residuals versus predicted values is produced and if the values are scattered randomly about a line of zero slope then the

regression model is adequate while a systematic distribution of the values the opposite situation can be stated and the null hypothesis (homoscedasticity) can be rejected and the alternative one (heteroscedasticity) holds. The other graphical test foresees the creation of a new variable, "residuals^2", by simply squaring the residuals whose values are plotted against the predicted values and again a systematic distribution of (r_i^2) is an indication of heteroscedasticity (Gunst and Mason, 1980). The graphic tests give just an Indication while the Breusch-Pagan and the Abridged-White tests give stronger evidence of potential inadequate regression model with error terms characterized by unequal variance. In the Breusch-Pagan test, the square residuals (dependent variable) are regressed against the analyte concentrations (independent variable) and in case the p-value of the regression analysis is lower than 0.01 the null hypothesis (homoscedasticity) is rejected meaning that error terms are heteroscedastic. To further confirm the result a second statistical test can be run, the Abridged-White foresees the regression of the residuals square r_i^2 against the predicted square (Y_1^2), a new variable simply calculated by squaring the predicted values Y_1, and again if the p-value is lower than 0.01 the null hypothesis (homoscedasticity) is rejected while the alternative one holds.

In case of assessed heteroscedasticity, a measure must be adopted to address it and the best solution is the selection of a correct weighting factor, for grouped data with 3 replicates at each concentration, is to weight by 1/variance of each group, scaled so that the weights sum to the number of points. For LC-MS/MS the best weighing factor is $1/x^2$ as reported in (Gu et al. 2014) .

Quality controls

The accuracy and precision of the analytical method was assessed with quality control (QC) trials, consisting in spiking the plant material with three different levels of phytohormones (high, medium and low). Plant material (50 mg of receptacle and drupelets both green and red stages) was spiked with 100 µL of high, medium and low concentrated authentic standards mix solutions of all the 18 target phytohormones (Table 4).

Table 4: Concentration in ppb of each target compounds in the quality control experiments

Phytohormones	HQC*	MQC*	LQC*
GA_3	2000	250	15
ABA	2000	250	10
SA	2000	250	30
ICA	20000	500	30
CA	3000	500	40
BA	2000	250	10
MeCA	15000	600	30
MeIAA	3000	250	10
IBA	2500	300	25
IAA	1000	250	20
OPDA	2000000	125000	4500
zeatin	3000	400	30
MeSA	15000	500	30
MeJA	1000	100	2
JA	2000	250	15
GA_4	20000	1500	200
MeBA	7000	600	80
GA_1	3000	500	30
***concentration in ppb**			

Results

Although the development of the analytical method to hormone profile raspberry fruit is not completed yet, some considerable progress has been achieved to support the choice of the most performing extraction system. The first two methods, MeOH:H_2O method and 2-proanol:H_2O:HCl method, we considered and used as reference were both using liquid-liquid phase extraction, methanol:H_2O (Trapp et al. 2014) and methanol:H2O:HCl (Pan, Welti, and Wang 2008); although faster and less laborious, they were not as performant as the solid-phase extraction method, SPE Oasis® HBL extraction method (Simura et al. 2018). To validate the methods a calibration curves for these methods were obtained with 12 serial dilutions. The linear regression models are the most commonly used analysis to predict the concentration of analytes in unknown samples (Table 5).

Table 5: Calibration curves, dynamic linear range and correlation coefficient for each of the 18 target phytohormones of interest

Phytohormones	Equation	R^2	LoD	LoQ	curve range (ppb)
ABA	y = 50.495x + 483.93*	0.9984	1.04	3.46	0.49 - 2000
BA	y = 1.5651x + 12.34	0.9984	54.28	180.93	0.49 - 2000
CA	y = 2.859x - 180.69*	0.9952	12.67	42.24	15.63 - 4000
GA_4	y = 1.7177x + 634.43*	0.9947	19.00	63.34	14.65 - 30000
IAA	y = 143.58x - 1074.3*	0.9992	1.17	3.89	0.98 - 1000
IBA	y = 164.91x - 6575	0.9985	1.25	4.17	2.93 - 3000
ICA	y = 28.074x + 10529*	0.994	8.68	28.94	7.32 - 30000
JA	y = 38.191x + 58.93*	0.9999	5.64	18.82	1.95 - 2000
MeBA	y = 0.2122x - 36.679	0.9965	84.85	282.83	62.5 - 8000
MeCA	y = 14.035x + 168.97	0.9996	8.84	29.46	9.77 - 20000
MeIAA	y = 377.88x + 8373.6*	0.9981	1.35	4.52	0.98 - 4000
MeJA	y = 201.63x + 477.28*	0.9998	0.48	1.59	0.24 - 1000
MeSA	y = 1.0311x + 21.973	0.9999	51.59	171.98	7.32 - 30000
SA	y = 63.159x + 4598.9*	0.9947	2.70	9.01	3.91 - 2000
Zeatin	y = 375.89x + 14786*	0.9889	0.39	1.28	0.73 - 3000
OPDA	y = 0.0022x - 8.1224	0.9999	2504.79	8349.30	3906.25 - 2000000
GA_3	y = 8.7118x - 2.0286	0.9999	0.99	3.29	0.49 - 2000
GA_1	y = 18.812x + 140.03	0.9998	1.73	5.76	3.91 - 4000

*a $1/x^2$ weighting factor applied

The regression analysis allows to predict values for the dependent variable (**y**) when values of the independent variables (x_n) are known or fixed; it is expressed by the equation **y = a + bx** where (**y**) is the dependent variable (it is measured with error), (**x**) the independent variable (it is known without error) while (**a**) and (**b**) are respectively the y-intercept and the slope of the regression equation. In general for each value of (**x**), there is a subpopulation of y-values normally distributed and the means of all the subpopulations of (**y**) lie on the same straight line indicating that all the y-values have same variance (Almeida, Castel-Branco, and Falcao 2002).

Table 6: Comparison of the three different extraction protocols tested (left) and of the three different drying systems tested for the solid phase extraction (right); all data from receptacle (green berry) samples

Samples extraction protocols*				Samples drying procedures**					
SPE - C_2H_3N	CH_3OH	IPA:H_20		Speed vacuum	N_2 stream	Freeze drying	LoD	LoQ	curve range (ppb)
907.11	**214.50**	**56.40**	**ABA**	**779**	**599**	**585**	1.04	3.46	0.49 - 2000
39			**BA**	39	24	25	54.28	180.93	0.49 - 2000
16		73	**CA**	**68.80**	**68.80**	**67.40**	12.67	42.24	15.63 - 4000
51			**GA_4**	51	46	83	19.00	63.34	14.65 - 30000
379	56	119	**IAA**	**10.12**	**9.16**	**10.18**	1.17	3.89	0.98 - 1000
1.69			**IBA**	**70.59**	**44.62**	**53.91**	1.25	4.17	2.93 - 3000
1511	810	208	**ICA**	1511	933	1473	8.68	28.94	7.32 - 30000
344	433	213	**JA**	7.46	16.31	**26.08**	5.64	18.82	1.95 - 2000
23			**MeBA**	82.93	71.81	81.54	84.85	282.83	62.5 - 8000
191			**MeCA**	191	52	89	8.84	29.46	9.77 - 20000
1349			**MeIAA**	1349	1767	3847	1.35	4.52	0.98 - 4000
870	1615		**MeJA**	**41.08**	**38.47**	**1.95**	0.48	1.59	0.24 - 1000
24			**MeSA**	24	14	13	51.59	171.98	7.32 - 30000
332.68		370	**SA**	**989.49**	**806.17**	**926.20**	2.70	9.01	3.91 - 2000
1512	768	133.67	**Zeatin**	1512	1658	3201	0.39	1.28	0.73 - 3000
58.86	195	29.3	**OPDA**	**30446.55**	**16660.18**	**17919.27**	2504.79	8349.30	3906.25 - 2000000

*samples dried in speed vacuum

**Samples processed with Solid Phase Extraction (SPE)

red character = peak area

blue character (bold) = concentration (ppb)

red character (underline) = concentration in (ppb) but under limit of detection and quantification

blue character (underline) = concentration (ppb) over the LoD but under the LoQ

In Table 6, the three left columns are reported the results of the comparison of the three different extraction methods tested during the development of this analytical method. In blue and bold characters is reported the quantification of the detected endogenous phytohormones extracted and analysed from receptacle sample of green berries. All the samples (left side of the table, were dried using the same technique (speed vacuum) and in the case of SPE, three target compounds, ABA, IBA and SA were detected while with the other two extraction methods only ABA and in lower amounts, respectively circa 4.5 times for the methanolic extraction and 16 times for the isopropanol one; so despite its longer procedure, the solid-phase extraction (SPE), was the method chosen as it allowed better quantification.

The other important result achieved was the identification of the most performing drying system. After extraction and purification, samples must be dried and then resolubilised in much lower volume to increase the concentration of the target analytes. The three different methods were tested, speed vacuum, nitrogen stream and freeze drying. The main concern was for the methylated compounds which being aromatic tend to be extremely volatile and lost during the drying step. The speed vacuum was performed by setting the temperature at its lowest (30ºC) and despite this potential limit, it was the most performant drying system. Table 6, right side, shows the comparison of the analysis of the three drying systems and

although the freeze drying allowed the detection of one more compound (Jasmonic acid) compared to the other two drying systems, the speed vacuum gave always better results in terms of analytes detection and quantification. For these reasons and even for its much higher feasibility, speed vacuum was adopted for the analytical method of our study. The drying process with nitrogen stream takes too long because, while the evaporation of the 0.8 mL of acetonitrile is fast, the remaining 1.2 mL of water require too much time to dry, around 3 hours, so the complete multi-samples processing lasts days; this may have caused the worse performances compared to the other two drying systems as the compounds easily degrade in aqueous solutions.

The results so far seems are promising; the analysis of the endogenous phytohormones done during the method development was performed without the use of internal standards (isotopically labelled compounds) which acting as carriers of the endogenous compounds should help improve the recoveries. The metabolomic analysis will help unveil part of the mechanisms behind the hormonal regulation of fruit development and transcriptomic studies; in parallel, microarray and qRT-PCR, should help to get a better overview of the process. In addition, gene expression data across ripening stages can be used to look at the regulation of hormones across development; such data sets are available in the James Hutton Institute and so far, they have not been analysed.

Discussion

One of the main challenges for future work remains the complete understanding of the hormonal circuits underlying the whole fruit development process. The development of an analytical method designed to detect the principal six groups of plant hormones, represent a valid starting point to help discover part of the metabolic pathways that regulates the fruit growth in raspberry wild-type fruits and in treated ones where the crumbly fruit condition has been induced by mechanical damaging the flowers before pollination. The majority of data available in the scientific literature indicate the involvement of different hormones in the regulation of fruit development and although the concerted action of auxins and/or gibberellins and/or cytokinins, through their biosynthesis and/or signalling pathway, seems to play a major role, on the basis of the high complexity of the whole process, the involvement of other phytohormones cannot be excluded a priori. In raspberry crumbly fruit condition, where berry abnormalities occur, different hormonal pathways might be involved in the regulation of these misshapen fruits and then a larger spectrum of different plant hormones must be taken into account to better understand the whole process. An analytical method designed to detect 18 different plant hormones, covering six groups represents a good starting point to help understand the hormonal molecular circuits behind the fruit development.

A hypothesis of a hormonal coordinating process regulating and synchronizing the growth of all the fertilized ovaries was formulated. An analytical method to profile the hormones involved in process of was developed. Therefore, three different methods, MeOH:H2O, 2-propanol:H2O:HCl and SPE Oasis® HBL extraction method, were compared. Furthermore, three different drying methods, speed vacuum, nitrogen and freeze-drying, were tested. The SPE Oasis® HBL extraction method combined with speed vacuum as the drying method was the best performing method. The first two methods, MeOH:H2O method and 2-proanol:H2O:HCl method, we considered and used as reference were both using liquid-liquid phase extraction, methanol:H2O (Trapp et al. 2014) and methanol:H2O:HCl (Pan, Welti, and Wang 2008); although faster and less laborious, they were not as performant as the solid-phase extraction method (Simura et al. 2018). This probably is because the relative impure samples obtained with liquid-liquid extraction can cause interference and perhaps ion suppression reducing the quality of the signal for the target analytes.

The hypothesis describes that fruit regulating hormones are synthesized in the receptacle and then moves to the surrounding drupelets where they coordinate and synchronize the growth of the fruit. According to this hypothesis and with the ease of which methylated compounds are transported through the cells, they might play an important role in regulating the fruit growth and high levels of them might be found in the receptacle where they are synthesized.

Conclusions

An analytical method, SPE Oasis® HBL extraction method combined with speed vacuum as the drying method, has been developed and the results from trials with spare samples of receptacle and drupelets showed that many compounds can be detected and at least four of them, Zeatin, SA, MeSA, IAA and OPDA can be quantified. A first tranche of 100 samples from the growth room experiments, will be analysed in the second part of October and if the results are interesting the remaining 200 samples will be analysed. Based on the results of the analysis, a potential chemical treatment to cure crumbly fruits at the beginning of their onset could be proposed and eventually tested in spring on fruits where the “crumbliness” will be induced by mechanical damaging.

References

Almeida, A. M., M. M. Castel-Branco, and A. C. Falcao. 2002. 'Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods', *Journal of*

Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 774: 215-22.

Bottcher, C., P. K. Boss, and C. Davies. 2011. 'Acyl substrate preferences of an IAA-amido synthetase account for variations in grape (Vitis vinifera L.) berry ripening caused by different auxinic compounds indicating the importance of auxin conjugation in plant development', *J Exp Bot*, 62: 4267-80.

Cao, Z. Y., L. H. Sun, R. X. Mou, L. P. Zhang, X. Y. Lin, Z. W. Zhu, and M. X. Chen. 2016. 'Profiling of phytohormones and their major metabolites in rice using binary solid-phase extraction and liquid chromatography-triple quadrupole mass spectrometry', *Journal of Chromatography A*, 1451: 67-74.

Du, F. Y., G. H. Ruan, and H. W. Liu. 2012. 'Analytical methods for tracing plant hormones', *Analytical and Bioanalytical Chemistry*, 403: 55-74.

Given, N. K., M. A. Venis, and D. Gierson. 1988. 'Hormonal regulation of ripening in the strawberry, a non-climacteric fruit', *Planta*, 174: 402-6.

Gu, H. D., G. W. Liu, J. Wang, A. F. Aubry, and M. E. Arnold. 2014. 'Selecting the Correct Weighting Factors for Linear and Quadratic Calibration Curves with Least-Squares Regression Algorithm in Bioanalytical LC-MS/MS Assays and Impacts of Using Incorrect Weighting Factors on Curve Stability, Data Quality, and Assay Performance', *Analytical Chemistry*, 86: 8959-66.

Harpster, M. H., D. A. Brummell, and P. Dunsmuir. 1998. 'Expression analysis of a ripening-specific, auxin-repressed endo-1, 4-beta-glucanase gene in strawberry', *Plant Physiol*, 118: 1307-16.

Kowalczyk, M., and G. Sandberg. 2001. 'Quantitative analysis of indole-3-acetic acid metabolites in Arabidopsis', *Plant Physiology*, 127: 1845-53.

Kumar, R., A. Khurana, and A. K. Sharma. 2014. 'Role of plant hormones and their interplay in development and ripening of fleshy fruits', *Journal of Experimental Botany*, 65: 4561-75.

Muller, A., P. Duchting, and E. W. Weiler. 2002. 'A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to Arabidopsis thaliana', *Planta*, 216: 44-56.

Obroucheva, N. V. 2014. 'Hormonal regulation during plant fruit development', *Russian Journal of Developmental Biology*, 45: 11-21.

Ozga, J. A., H. Kaur, R. P. Savada, and D. M. Reinecke. 2017. 'Hormonal regulation of reproductive growth under normal and heat-stress conditions in legume and other model crop species', *Journal of Experimental Botany*, 68: 1885-94.

Ozga, J. A., and D. M. Reinecke. 2003. 'Hormonal interactions in fruit development', *Journal of Plant Growth Regulation*, 22: 73-81.

Pan, X. Q., R. Welti, and X. M. Wang. 2008. 'Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry', *Phytochemistry*, 69: 1773-81.

Qin, G., H. Gu, Y. Zhao, Z. Ma, G. Shi, Y. Yang, E. Pichersky, H. Chen, M. Liu, Z. Chen, and L. J. Qu. 2005. 'An indole-3-acetic acid carboxyl methyltransferase regulates Arabidopsis leaf development', *Plant Cell*, 17: 2693-704.

Santner, A., L. I. A. Calderon-Villalobos, and M. Estelle. 2009. 'Plant hormones are versatile chemical regulators of plant growth', *Nature Chemical Biology*, 5: 301-07.

Seo, H. S., J. T. Song, J. J. Cheong, Y. H. Lee, Y. W. Lee, I. Hwang, J. S. Lee, and Y. D. Choi. 2001. 'Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses', *Proceedings of the National Academy of Sciences of the United States of America*, 98: 4788-93.

Serrani, J. C., O. Ruiz-Rivero, M. Fos, and J. L. Garcia-Martinez. 2008. 'Auxin-induced fruit-set in tomato is mediated in part by gibberellins', *Plant Journal*, 56: 922-34.

Simura, J., I. Antoniadi, J. Siroka, D. Tarkowska, M. Strnad, K. Ljung, and O. Novak. 2018. 'Plant Hormonomics: Multiple Phytohormone Profiling by Targeted Metabolomics', *Plant Physiol*, 177: 476-89.

Steenackers, W., P. Klima, M. Quareshy, I. Cesarino, R. P. Kumpf, S. Corneillie, P. Araujo, T. Viaene, G. Goeminne, M. K. Nowack, K. Ljung, J. Friml, J. J. Blakeslee, O. Novak, E. Zazimalova, R. Napier, W. Boerjan, and B. Vanholme. 2017. 'cis-Cinnamic Acid Is a Novel, Natural Auxin Efflux Inhibitor That Promotes Lateral Root Formation', *Plant Physiology*, 173: 552-65.

Trapp, M. A., G. D. De Souza, E. Rodrigues, W. Boland, and A. Mithofer. 2014. 'Validated method for phytohormone quantification in plants', *Frontiers in Plant Science*, 5.

Vanneste, S., and J. Friml. 2009. 'Auxin: a trigger for change in plant development', *Cell*, 136: 1005-16.

Vriezen, W. H., R. Feron, F. Maretto, J. Keijman, and C. Mariani. 2008. 'Changes in tomato ovary transcriptome demonstrate complex hormonal regulation of fruit set', *New Phytol*, 177: 60-76.

Weiler, E. W. 1984. 'Immunoassay of Plant-Growth Regulators', *Annual Review of Plant Physiology and Plant Molecular Biology*, 35: 85-95.

Zaharah, S. S., Z. Singh, G. M. Symons, and J. B. Reid. 2012. 'Role of Brassinosteroids, Ethylene, Abscisic Acid, and Indole-3-Acetic Acid in Mango Fruit Ripening', *Journal of Plant Growth Regulation*, 31: 363-72.

Zhang, M., B. Yuan, and P. Leng. 2009. 'The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit', *J Exp Bot*, 60: 1579-88.

Zubieta, C., J. R. Ross, P. Koscheski, Y. Yang, E. Pichersky, and J. P. Noel. 2003. 'Structural basis for substrate recognition in the salicylic acid carboxyl methyltransferase family', *Plant Cell*, 15: 1704-16.

