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Multi-trait analysis of post-harvest storage in rocket salad (*Diplotaxis tenuifolia*) links sensorial, volatile and nutritional data

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ABSTRACT

Rocket salad (*Diplotaxis tenuifolia*; wild rocket) is an important component of ready to eat salads providing a distinct peppery flavour and containing nutritionally relevant compounds. Quality deteriorates during post-harvest, in relation to time and storage temperature amongst other factors. Volatile organic compounds (VOCs) are easily measurable from rocket leaves and may provide useful quality indicators for e.g. changes in isothiocyanates derived from nutritionally important glucosinolates. VOC profiles discriminated storage temperatures (0, 5 and 10 °C) and times (over 14 days). More specifically, concentrations of aldehydes and isothiocyanates decreased with time paralleling a fall in vitamin C and a reduction in sensorial quality at the two higher temperatures. Sulphur containing compounds rise at later timepoints and at higher temperatures coincident with an increase in microbial titre, mirroring a further drop in sensorial quality thus indicating their contribution to off-odours.

1. Introduction

Rocket salad is of increasing commercial importance as a fresh cut ready to eat (RTE) salad and is valued for its sensory and nutritional characteristics (Pasini, Verardo, Cerretani, Caboni, & D'Antuono, 2011). Known also as roquette, arugola or rucola it comprises species from two genera in the *Brassicaceae* family: *Eruca sativa* Mill. (cultivated rocket) and *Diplotaxis tenuifolia* (wild rocket). Both species are high in biologically active compounds including ascorbic acid, carotenoids, fibres, polyphenols and glucosinolates (Bell & Wagstaff, 2014; Martínez-Sánchez, Allende, Bennett, Ferreres, & Gil, 2006). Glucosinolates contribute to the pungent flavour of rocket leaves (Bennett et al., 2002) by formation of isothyocyanates (ITCs) through the action of myrosinase on cutting and chewing of leaves (Bennett, Rosa, Mellon, & Kroon, 2006). In *E. sativa* the major glucosinolate appears to be glucoerucin (Nitz & Schnitzler, 2002), which is converted by myrosinase to erucin (4-methylthiobutylisothiocyanate) a compound with strong antioxidant properties (Barillari et al., 2005; Jirovetz, Smith, & Buchbauer, 2002; Miyazawa, Maehara, & Kurose, 2002) but other ITCs were also identified (Miyazawa et al., 2002). Both glucosinolates and ITCs are thought to reduce the risk of carcinogenesis or heart disease (Traka & Mithen, 2009). Volatile organic compounds

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(VOCs) other than ITCs also contribute to the aroma of rocket salad including, in *E. sativa*, sulphur and or nitrogen compounds, esters, alcohols and carbonyl compounds (Blažević & Mastelić, 2008; Miyazawa et al., 2002). Despite its importance as a cut salad there are even fewer reports of VOC profiles in *D. tenuifolia* compared to *E. sativa*.

Storage conditions affect physiological and visual quality of rocket leaves from harvest to consumption (Kader, 2002; Koukounaras, Siomos, & Sfakiotakis, 2007; Watada, Ko, & Minott, 1996). Low temperature is a key factor in quality maintenance, depressing respiration, reducing overall metabolism and extending shelf life (Spinardi & Ferrante, 2012). A temperature of 0 °C is recommended for storage of rocket salad (Cantwell & Kasmire, 2002), however, it is usually shipped and stored at 10 °C and temperature abuse occurs during transport and retail store display (Koukounaras et al., 2007). Moreover, rocket salad in common with most fresh produce has a short shelf life, showing leaf yellowing within 4–8 days even when stored at low (5 °C) temperatures (Ferrante, Incrocci, Maggini, Serra, & Tognoni, 2004) although some reports (Martínez-Sánchez et al., 2006; Nielsen, Bergström, & Borch, 2008) have described shelf life of 14 days for rocket leaves stored at 4 °C. The use of lower temperatures increased shelf life with an extension of 5–6 days at 0 °C compared to 7 °C (Hall, Jobling, & Rogers, 2013). Degradation kinetics indicated that when stored at constant temperature, shelf life is limited by appearance, reaching the limit of marketability after 7.3, 5.8 and 3.7 days, for samples stored at 0, 5 and 15 °C (Amodio, Derossi, Mastrandrea, & Colelli, 2015) respectively. However, leaf yellowing is accompanied by a loss of ascorbic acid (vitamin C) and glucosinolates (Force, O'Hare, Wong, & Irving, 2007). An increase in temperature affected the loss of ascorbic acid more than the release of off-odours, indicating that ascorbic acid levels should limit the shelf life (Amodio et al., 2015). Other nutritionally relevant compounds such as polyphenols, however, increase in vegetables in response to stresses such as wounding and cold (Cisneros-Zevallos, 2003), hence an extension of post-harvest storage may be positive. Polyphenols have been associated with health benefits that in part are related to their antioxidant activity, which may be associated with complex synergistic interactions between different compounds in the produce (Liu, 2004). Sinapic acid and ferulic acid are phenolic acids frequently found in rocket and other plants with potential anti-cancer effects (Liu, 2004) and 7-hydroxycoumarin (also known as umbelliferone) is a potent antioxidant (Kanimozhi, Prasad, Ramachandran, & Pugalendi, 2011). However, information on the impact of post-harvest conditions on levels of these bioactive compounds in rocket salad is lacking, despite their relevance for nutritional value.

Generally, flavour is compromised by periods of storage and off-odours develop towards the end of shelf life (Peneau, Brockhoff, Escher, & Nuessli, 2007). However, there is a lack of information on the effect of storage temperature on VOCs, as well as on concentration of isothiocyanates and on the chemical identity of off-odours in rocket salad.

Relative concentrations of VOCs are easy to measure, and using thermal desorption can be collected with minimal tissue disruption (Spadafora et al., 2015). Therefore monitoring of VOCs could be of use as a marker for sensorial quality and nutritional content. Here, we tested whether changes in VOCs can be linked to physiological markers, phytochemical content and sensorial quality and whether useful markers for changes in nutritional and functional compounds and development of off-odours can be identified. We applied statistical algorithms for ecological and gene expression analyses in a multi-trait analysis of changes in the whole VOC bouquet during post-harvest storage of rocket leaves at three commercially-relevant temperatures over a 14 day period, which is the maximum time considered relevant.

2. Materials and methods

2.1. Plant Material and treatments

Freshly harvested rocket leaves (*Diplotaxis tenuifolia* L.) at commercial maturity, were obtained from a local grower, immediately transported to the RTE salad processing facilities and stored at low temperature (5 °C). Rocket leaves were then inspected for uniformity and visual defects, washed in cold running water, dipped in 100 μg L^{-1} sodium hypochlorite solution for 1 min, and centrifuged to remove the remaining water. Rocket leaves (ca. 100 *g*) were packed in coextruded bioriented polypropylene film bags (Amcor Flexibles Neocel, Palmela, Portugal), heat sealed with a Multivac packaging machine (Gastrovac, Wolfertschwenden, Germany) and stored at 0, 5 and 10 °C for a total storage period of 14 days. The packaging film used permitted an oxygen transmission rate of 1200 cm^{-3} m^{-2} 24 h^{-1} at 23 °C (Amcor Flexibles Neocel, Palmela, Portugal). Three packages were prepared for each evaluation day (0, 2, 6, 9 and 14 days of storage) at each treatment temperature.

2.2. Package atmosphere composition and respiration rate

Oxygen and carbon dioxide concentrations were monitored with a CheckMate II, (PBI Dansensor, Ringsted, Denmark) via a small needle inserted into the package headspace. For respiration rate determination, a closed system method was used: rocket leaves were weighed (ca. 10 g) and placed in 250 mL sealed glass jars, at 4 °C for 3 h. Carbon dioxide production was determined using a CheckMate II, (PBI Dansensor, Ringsted, Denmark) by inserting a small needle into the glass jar headspace through a rubber septum.

2.3. Chlorophyll content

Leaf chlorophyll content was determined using leaf readings from a portable chlorophyll meter (Konica Minolta SPAD-502 Plus; Minolta, Osaka, Japan). The SPAD-502 measurements were made on each analysis day with 15 readings performed for each replicated package from each temperature treatment. The adaxial side of the leaves was always placed toward the emitting window of the instrument and major veins were avoided.

2.4. Phenolic compounds

Total phenolic compound content was determined as described by Ferrante et al. (2004). Fresh leaf tissue (0.1 g) was ground and homogenized with 10 mL methanol. The homogenate was centrifuged (3000 *g*, 15 min). The supernatant was collected (methanol and polyphenols) and 50 μL of Folin–Ciocalteu reagent and 1 mL of 1 N sodium carbonate (Sigma-Aldrich) were added to 50 μL of the methanol extract. The final volume was made up to 2.5 mL with 1.4 mL of deionized water. Samples were allowed to react in the dark. Absorbance readings were taken after 30 min at 765 nm.

Qualitative and quantitative profiles of phenolics were determined by HPLC-DAD (Waters Series 600, Mildford MA, USA) based on the method by Oliveira, Pintado, and Almeida (2012). Separation was performed on a reverse phase Symmetry® C18 column (250 × 4.6 mm I.D., 5 μm particle size and 125 Å pore size) with a guard column containing the same stationary phase (Symmetry® C18). Chromatographic separation of phenolic compounds was carried out with solvent A consisting of formic acid, water and methanol (92.5:5:2.5 V/V), and solvent B consisting of methanol and

water (94:6), under the following conditions: 0–10 min, linear gradient from 0 to 10% solvent B at 0.5 mL min^{-1}, 10–40 min, linear gradient from 10 to 30 % B at 0.65 mL min^{-1} and from 40 to 60 min, linear gradient from 30 to 50 % B 0.75 mL min^{-1}. The solvent system was reset over 10 min from 50 to 0% B at 1 mL min^{-1}. Injection volume was 50 μL. Detection was achieved by a diode array detector (Waters, Massachussets, EUA) at wavelengths ranging from 200 to 600 nm in 2 nm intervals. Absorbance was measured at 280 nm and 320 nm. Retention times and spectra of compounds were analysed by comparison with pure standards (Sigma–Aldrich, Sintra, Portugal) and quantification was performed using calibration curves and expressed as μg g FW^{-1}.

2.5. Antioxidant activity and ascorbic acid content

The ABTS ((2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic) acid) diammonium salt) radical scavenging activity of samples extracted with methanol was measured using the method of Gião et al. (2007). Total antioxidant activity was quantified using a UV mini 1240 spectrophotometer (Shimadzu, Tokyo, Japan), at 734 nm and ascorbic acid was used as a standard to prepare calibration curve in the range of 0.021–0.5 g L^{-1}. Results were expressed as mg ascorbic acid g FW^{-1}.

Ascorbic acid (AA) content was determined by HPLC-DAD (Waters Series 600, Mildford MA, USA). The HPLC analysis of total vitamin C (AA plus DHAA) was achieved after derivatization of DHAA into the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-b]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride (OPDA) according to the procedure of Zapata and Dufour (1992) with some modifications. Fresh samples (3 g) were ground in liquid nitrogen and were homogenized with 6 mL of methanol–water (5:95 V/V) containing citric acid (21.0 g L^{-1}) and EDTA (0.5 g L^{-1}) (Martínez-Sánchez, Allende, Cortes-Galera, & Gil, 2008). Freshly prepared OPDA solution (250 μL) was added to the extracts (750 μL). Samples were allowed to react for 37 min at room temperature in the dark and analysed by HPLC-DAD. Samples of 20 μL were injected into the HPLC-DAD (Waters Series 600, Mildford MA, USA) and separated over a 250 × 4.6 mm I.D., 5 μm particle size and 125 Å pore size Symmetry® C18 column with a guard column containing the same stationary phase (Symmetry® C18). The mobile phase was MeOH/H_2O (5:95 V/V) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate at pH 4.5, with a flow rate of 0.9 mL min^{-1}. DHAA and AA were determined at 348 nm and 261 nm respectively on a photo diode array detector (Waters, Mildford MA, EUA). Retention times and spectra of compounds were analysed by comparison with pure standards (Sigma–Aldrich, Sintra, Portugal) and quantification performed by the calibration curves of AA and DHAA and expressed as μg g FW^{-1}. Three independent analyses were performed in each of the triplicate extracts obtained for each treatment.

2.6. Mesophilic bacteria and total yeast and moulds

Rocket leaf samples (10 g) were homogenized with 0.1% (m/V) peptone water (Sigma, St. Louis, Mo., U.S.A.) for 3 min at 260 rpm in a Stomacher Lab Blender 400 (Sewer Medical, London, U.K.). Serial decimal dilutions were prepared with 0.1% (m/V) peptone water and plated in duplicate on two different media: Plate Count Agar (Merck), for mesophilic cell counts incubated at 30 °C for 24 h; and Potato Dextrose Agar (Merck) for yeasts and moulds incubated at 37 °C for 5 days. All aforementioned media were plated using the Miles and Misra technique (Miles, Misra, & Irwin, 1938) and after incubation colonies where enumerated and CFU mL^{-1} determined.

2.7. Sensory quality

Sensory attributes of RTE rocket were evaluated at the Sensory Analysis Laboratory of CBQF consisting of eight individually partitioned booths equipped with individual computers. On each analysis day, the products were evaluated by a trained eight-member sensory panel, with more than >3 years of sensory evaluation experience. Prior to the beginning of the experiment, training specific to the organoleptic properties of RTE rocket leaves was provided to the evaluators.

The sealed sample bags were coded with random three-digit numbers and presented to the panellist for the evaluation of visual quality, aroma and flavour. The visual quality was evaluated on a rating scale for rocket leaves developed by Amodio et al. (2015) which consists of a 5 point hedonic scale, where 5 = Excellent (Fresh and turgid appearance, bright and uniform green colour); 4 = Good (Slight loss of turgidity and fresh appearance); 3 = Fair (Noticeable loss of turgidity and slight loss of green colour); 2 = Poor (Severe loss of turgidity, wrinkling and yellowing of leaves) and 1 = Very poor (Severe yellowing of leaf blades and wilting, appearance of decay). The aroma was evaluated also on a 5-point scale, where 5 = Excellent (typical of fresh product, no defects); 3 = Acceptable and 1 = Bad (fermentation, degradation smell). A score of 3 was considered the limit of acceptability (Amodio et al., 2015). A 5-point scale was also used to score overall quality.

2.8. VOC sampling and analyses

At each time point 100 g of rocket leaves were inserted into a multi-purpose roasting bag (25 cm × 38 cm, TJM Ltd). The bag was sealed and stored at 20 °C for 1 h to equilibrate the headspace. The leaves were lightly crushed in the bag after sealing it to release the VOCs. Headspace (1000 mL) was collected with an EasyVOC manual pump (Markes International Ltd., Llantrisant, UK) onto SafeLok™ thermodesorption (TD) tubes (Tenax TA & Sulficarb, Markes International Ltd., Llantrisant, UK). Control samples were collected from empty bags on site, retention standards were prepared by loading 1 μL C8-C20 alkane standard (Sigma Aldrich, St. Louis, Mo., U.S.A.) directly onto TD tubes. Each sampling point was collected in triplicate representing three biological replicates.

VOC samples were collected at the Universidade Católica Portuguesa and tubes were transported to Cardiff University by courier. Tubes were desorbed using a TD100 thermodesorption system (Markes International Ltd., Llantrisant, UK) with the following settings for tube desorption: 5 min at 100 °C followed by 5 min at 280 °C, at a trap flow of 40 mL min^{-1} and for trap desorption and transfer: 20 °C s^{-1} to 300 °C, split flow of 5 mL min^{-1} into GC (7890A; Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). VOCs were separated over 60 m, 0.32 mm I.D., 0.5 μm Rx5ms (Restek, High Wycombe, UK) with 2 mL min^{-1} helium as carrier gas under constant flow conditions using the following temperature program: initial temperature 40 °C for 2 min, 5 °C min^{-1} to 240 °C, final hold 5 min. Mass spectra were recorded from m/z 30 to 350 on a time-of-flight mass spectrometer (BenchTOF-dx, Markes International Ltd, Llantrisant, UK).

Data from GC–MS measurements were initially processed using MSD ChemStation software (E.02.01.1177; Agilent Technologies, Inc, Santa Clara, CA, U.S.A.) and deconvoluted and integrated with AMDIS (NIST11) using a custom retention-indexed mass spectral library. Compounds, which were not abundant in all replicates, were excluded from statistical analyses as were compounds abundant in controls. MS spectra from deconvolution were searched against the NIST 2011 library (Software by Stein, version 2.0 g, 2011). Only compounds scoring > 80 % in forward and backward fit were included into custom mass spectral library. Putative

identifications were based on matches of mass spectra (> 80 %) and retention index (RI +/− 15).

2.9. Statistical analyses

Three replicates of each treatment were evaluated on each sampling day. For physiological and biochemical analyses, data were analysed using GraphPad Prism 5 software (GraphPad Software, USA) and SPSS statistics 21 (IBM Corporation, USA), by one-way analysis of variance (ANOVA). All the data are reported as the mean of three replicates ± standard deviation (SD).

VOC data were analysed statistically using R software version 3.1.3 (R core development team 2015) after normalisation of areas and square root transformation to reduce weight of large components. Permutational multivariate analysis of variance (PerMANOVA) and Canonical Analysis of Principal coordinates (CAP) analysis were performed (Anderson & Willis, 2003) using the 'vegan' package (Oksanen et al., 2013) and 'BiodiversityR' package (Kindt & Coe, 2005) in R. Ordination plots were generated for storage day and temperature and a 95 % confidence interval was fitted. Sub-sets of compounds correlated with day, temperature and/or phytochemical content were identified using Weighted Correlation Network Analysis which is a scale-free topology criterion method (WCNA package in R, Langfelder and Horvath (2012)) using a soft threshold power of 6, a deep-split of 3 and module size of 3.

3. Results

3.1. Physiological parameters changed with time and temperature

Storage temperature had a significant effect on rocket respiration rate (Fig. 1a). Carbon dioxide production was significantly lower when rocket leaves were stored at 0 °C compared with 10 °C. Oxygen and carbon dioxide concentrations inside the RTE rocket leaf packages were also significantly affected by the different storage temperatures (Fig 1b and c). Packages stored at the two higher temperatures (5 and 10 °C) showed a significantly faster depletion of O_2 and accumulation of CO_2 levels compared to 0 °C over the first 6 days of storage, as expected. After 9 days CO_2 levels from leaves at the two higher temperatures were similar and were only significantly differentiated from those stored at 0 °C, and by 14 days although differences were still significant, they were smaller.

Chlorophyll concentration was recorded as an indicator of the progression of senescence. There were no significant changes in chlorophyll concentration over the storage period (Fig 1d) either between days of storage or between temperatures of storage.

3.2. Changes in levels of nutritionally-relevant compounds are not all closely linked to temperature or time of storage

During storage, antioxidant capacity was essentially constant (Fig 2a) except for the last time-point where there was a rise in antioxidant capacity in leaves stored at 5 °C. In contrast, the content of vitamin C decreased significantly at all three treatment temperatures (Fig 2b) but this reduction was particularly marked in rocket stored at 10 °C where vitamin C content was lower than that in leaves stored at 0 °C at all time-points. During the first 9 days of storage, the rate of vitamin C depletion was higher when leaves were stored at 10 °C (19.0 μg g^{-1} FW day^{-1}) compared with leaves stored at 5 °C (10.4 μg g^{-1} FW day^{-1}) and 0 °C (7.5 μg g^{-1} - FW day^{-1}). Concentrations in leaves stored at 10 °C and 5 °C were similar until 9 days of storage after which leaves at 5 °C retained significantly more vitamin C than those stored at 10 °C.

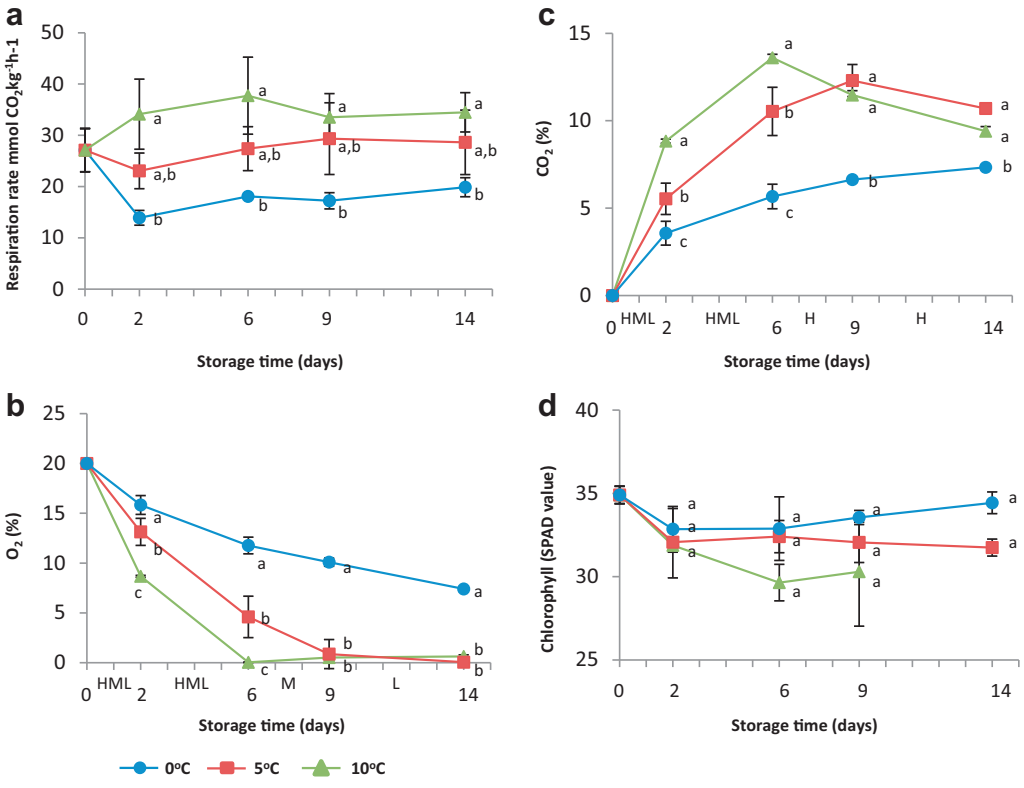

Fig. 1. Physiological and biochemical parameters: (a) respiration rate, (b) O_2, (c) CO_2, (d) chlorophyll in rocket leaves stored at 0 °C, (●), 5 °C (■) and 10 °C (▲) during 14 days of storage (mean ± S.D.; n = 3). Lowercase letters above symbols indicate statistically different values between the different temperatures at each time point, upper case letters (H = 10 °C, M = 5 °C and L = 0 °C) indicate significant differences between each time point or to the fresh cut control for each temperature using ANOVA and Tukey's range test ($P < 0.05$).

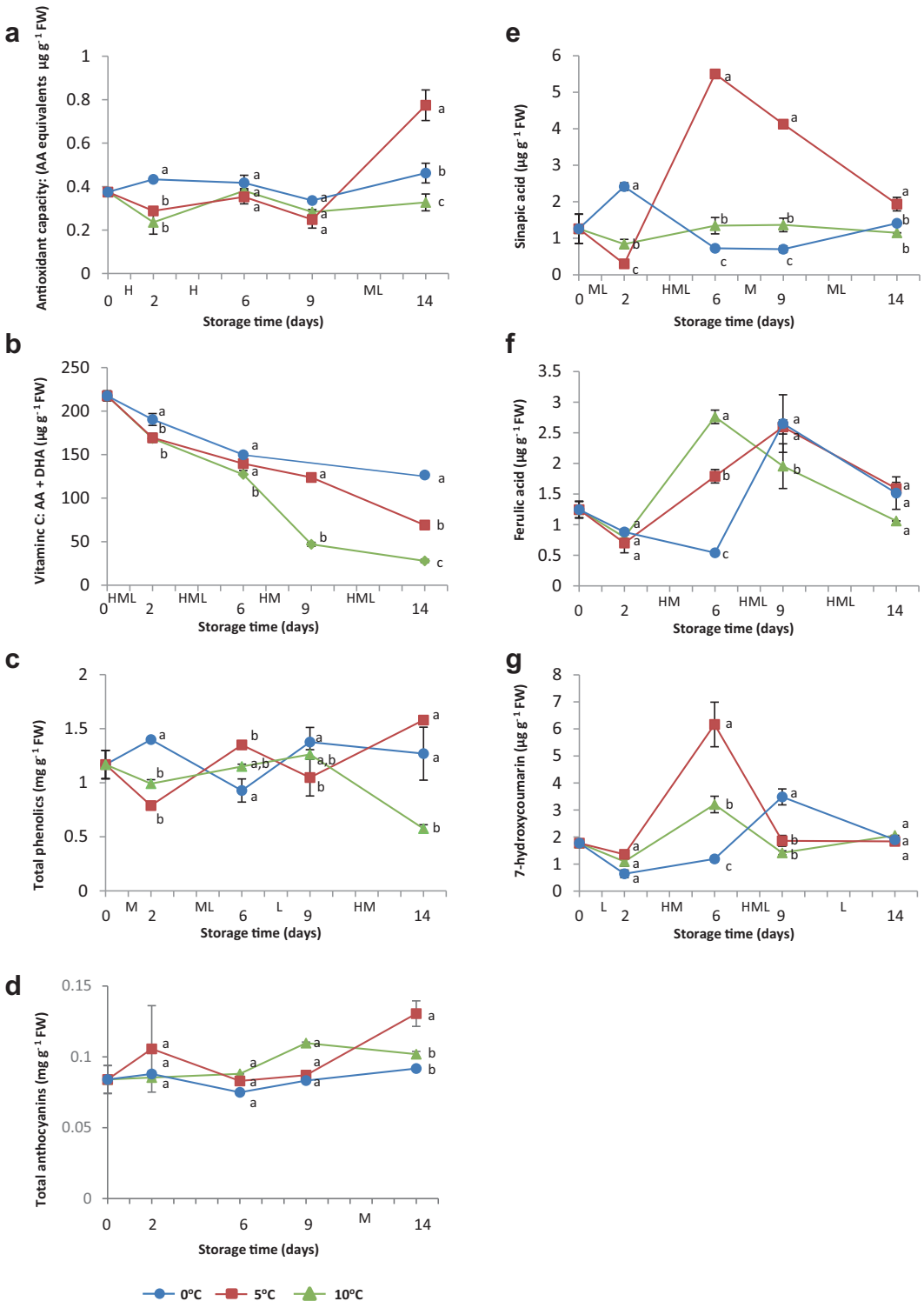

Fig. 2. Phytochemical content and antioxidant activity: changes in (a) antioxidant capacity (ascorbic acid equivalents) (b) vitamin C (ascorbic acid + dehydroascorbic acid), (c) total phenolics, (d) total anthocyanins, (e) sinapic acid, (f) ferulic acid and (g) 7-hydroxycoumarin contents in rocket leaves stored at 0 °C, (●), 5 °C (■) and 10 °C (▲) during 14 days of storage (mean ± S.D.; n = 3). Lower case letters above symbols indicate statistically different values between the different temperatures at each time point, upper case letters (H = 10 °C, M = 5 °C and L = 0 °C) indicate significant differences between each time point or to the fresh cut control for each temperature only, using ANOVA and Tukey's range test ($P < 0.05$).

Changes in total phenolics were more complex (Fig 2c). Although there were significant differences between temperature treatments at several time-points, overall levels fluctuated. Nevertheless, at the last time-point the concentration in leaves held at 10 °C was significantly lower than that in leaves stored at the two lower temperatures. Analyses of individual phenolics revealed some interesting patterns: both ferulic acid (Fig 2f) and 7-hydroxycoumarin (Fig 2g) peaked at 6–9 days of storage at all

three temperatures reaching concentrations that were significantly higher than fresh-cut. In contrast sinapic acid (Fig 2e) showed maximum content earlier, at 2–6 days at the two lower temperatures, but remained relatively constant at 10 °C. Total anthocyanins by contrast remained very constant and only changed with storage at 5 °C, which showed a significant increase from 9 to 14 days of storage and at day 14 significantly higher levels than rocket stored at both 0 and 10 °C at day 14 (Fig 2d).

3.3. Microbial monitoring during storage

Low numbers of mesophilic bacteria and fungi were present at day 0, and there was only a slight increase in CFUs at any of the three temperatures until day 6 (Fig 3a). Fungal counts increased particularly at higher storage temperatures, and showed high variability at last stage of storage. In the case of mesophilic bacteria, counts at day 14 were significantly greater at 10 °C compared to the two lower temperatures (Fig 3a).

3.4. Sensorial analyses provide evidence of greater deterioration of attributes at 10 °C compared to 5 °C and 0 °C

There was a significant drop in aroma (Fig 3b), visual quality (Fig 3c) and freshness (Fig 3d) between day 2 and day 6 in leaves stored at 10 °C. Sensorial scores for leaves stored at 5 and 0 °C showed slight but not statistically significant changes by comparison over this time interval. By day 9 of storage leaves showed significant differences in sensorial scores, with the scores being highest at 0 °C intermediate at 5 °C and lowest at 10 °C, as expected. In the case of leaves stored at 0 °C, all three quality characters scored remained remarkably constant throughout the storage period (Fig 3b-d).

3.5. Analysis of volatile organic compounds discriminates rocket leaves on the basis of time and temperature of storage

A total of 43 compounds were identified from all the rocket salad samples across all three temperatures of storage, and all time points by comparison to custom libraries derived from NIST 11 (Suppl. Table 1). The major compound class was aldehydes (8), followed by sulphur compounds (6), ketones (4), furane (4), esters (5), alcohols (3), isothiocyanate (3), nitrogenous compounds (3), alkanes (2), alkenes (2), terpenes (2), and 1 unidentified compound.

VOC profiles were significantly different amongst days of storage (PerMANOVA, $P < 0.001$, $R^2 = 0.430$), temperature of storage ($P < 0.000$, $R^2 = 0.185$), and individual samples ($P < 0.001$, $R^2 = 0.31$). Overall PerMANOVA accounted for 92.5% of variation of the data set. Linear discrimination plots from CAP showed a clear separation of days with a percentage of correct classification of 94.87% at $P < 0.005$ (Fig 4a) and clear separation of profiles of fresh leaves from those stored at any of the three temperatures (percentage of correct classification of 97.43% at $P < 0.005$). However, leaves stored at 5 and 10 °C could not be distinguished from each other on the basis of their VOC profiles (Fig. 4b).

CAP was repeated with days of storage and temperature combined into a single sample category (=13 samples) to assess discrimination across time and temperature. The analysis classified correctly 87.17% of samples ($P < 0.005$, n = 3) and most samples were discriminated in the resulting discriminant plot (Fig 4c). Fresh leaves grouped with day 2 of storage at 10 and 5 °C. Also, day 2 at 5 °C grouped with day 2 of storage at 0 °C The rest were discriminated from each other. Overall, there appears to be a tendency for the later time points to cluster in the negative portion of LD1, while the earlier time points cluster on the positive side.

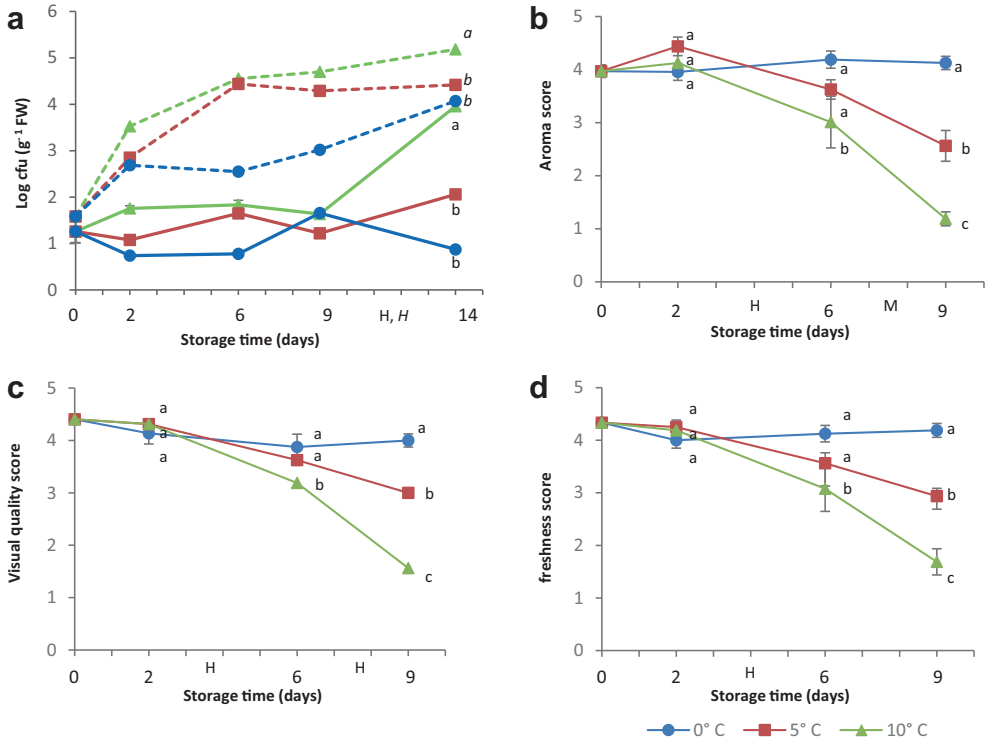

Fig. 3. Microbial growth and Sensorial analyses: changes in (a) microbial growth: bacterial (solid line) fungal (dashed line); n = 3, (b) aroma, (c) visual quality, (d) freshness; n = 8–35, in rocket salad stored at 0 °C, (●), 5 °C (■) and 10 °C (▲) during 9–14 days of storage (mean ± S.E.). Lowercase letters indicate statistically different values between the different temperatures at each time point, upper case letters (H = 10 °C, M = 5 °C and L = 0 °C) indicate significant differences between each time point or the fresh cut control, using ANOVA and Tukey's range test ($P < 0.05$). Only significant differences at each time point are shown for microbial growth; *italics* for fungal growth.

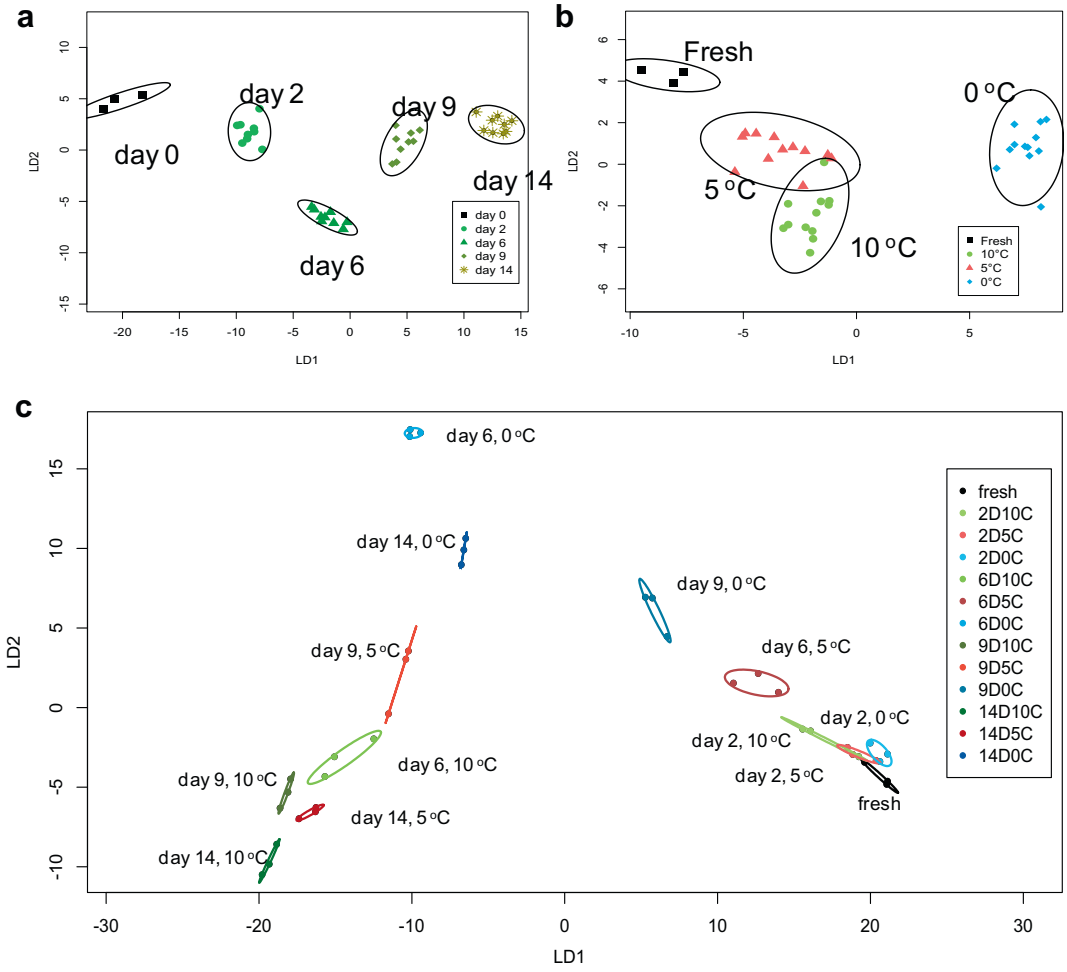

Fig. 4. Canonical Analysis of Principal coordinates based on all VOCs from rocket salad using TD-GC-TOF-MS. A CAP model was produced for rocket salad samples stored (a) for 0, 2, 6, 9 and 14 days, (b) at 0, 5 or 10 °C and (c) combined time (0, 2, 6, 9 and 14 d) and temperature (0, 5 or 10 °C) into a single sample category. The plots use the first two linear discriminants (LD); each ellipse represents the 95% confidence interval. Percentage of correct classifications was 94.87% ($P < 0.005$, n = 6) for days of storage (a), 97.43% ($P < 0.005$, n = 12) for temperature of storage (b) and 87.17% ($P < 0.005$, n = 3) for combined days of storage and temperature.

3.6. Correlation analysis of physiological and biochemical parameters

WCNA was used to correlate the physiological state of the rocket leaves, their VOC bouquet and their phytochemical content with time and temperature of storage. The analysis clustered the parameters into a total of seven modules (Fig. 5a; Suppl. Table 1). Six of the modules showed statistically significant positive (brown and green) and negative (blue, red, turquoise and yellow) correlations with day of storage, of which the green, turquoise and yellow modules showed relatively high correlation ($R^2 = 0.6, -0.83, -0.74$ resp.). Four of the modules were statistically significantly positively (red and blue) and negatively (brown and yellow) correlated with temperature of storage. From these modules individual VOCs (Suppl. Table 2), physiological and phytochemical characters that were significantly correlated with days of storage or temperature respectively were used to create two heat maps one for days (Fig. 5b) and another for temperature of storage (Fig. 5c).

Twenty-one VOCs were negatively correlated with days of storage, they were dominated by aldehydes (7), followed by ITCs (3) and esters (3). Days were also negatively correlated with ascorbic acid content and O_2 concentration inside the packages. Dimethyl sulphide, dimethyl sulfoxide and dimethyl disulphide (Suppl. Table 1, C26, C27, C29) dominated the modules that were positively correlated with days of storage. CO_2 was also positively correlated with days of storage (Fig 5b, Suppl. Table 2). Of particular interest is also the close correlation between levels of vitamin C and C5 and C6 aldehydes, 4-Oxohex-2-enal, 2,4-hexadienal, E/Z-2-hexenal, E/Z-3-hexenal and E/Z-2-pentenal (Suppl. Table 1, (C1, C8, C9, C10, C12, C15 and C16).

Four VOCs were higher at 0 °C and decreased with increasing temperature: 5-nonanone oxime (C20), pyrrolidine-1-dithiocarboxylic acid 2-oxo-pentyl ester (C40), 3-heptene (C2), and n-hexyl isothiocyanate (C33) (Fig. 5c). In contrast, the relative amount of four aldehydes 2,4-hexadienal, E and Z-2-hexenal, E/Z-3-hexenal (C8, C9, C10 and C16) increased with temperature.

4. Discussion

The high perishability of rocket is a consequence of its high respiration rate (Koukounaras et al., 2007) and this is further modulated by the temperature of storage, indicating that storage temperature is a key factor in the shelf life of rocket leaves. In our experiments, respiration rate of rocket leaves stored at 10 °C was 2-fold greater than those stored at 0 °C. Results presented here are in agreement with Martínez-Sánchez et al. (2008) who reported a 2 and 4-fold increase in respiration rate when temperature increased from 1 to 12 °C. The initial rise in CO_2 followed by a plateau at later time points and the inverse relationship between O_2 and CO_2 levels is also in agreement with previous work (Koukounaras et al., 2007; Martínez-Sánchez et al., 2006). The rel-

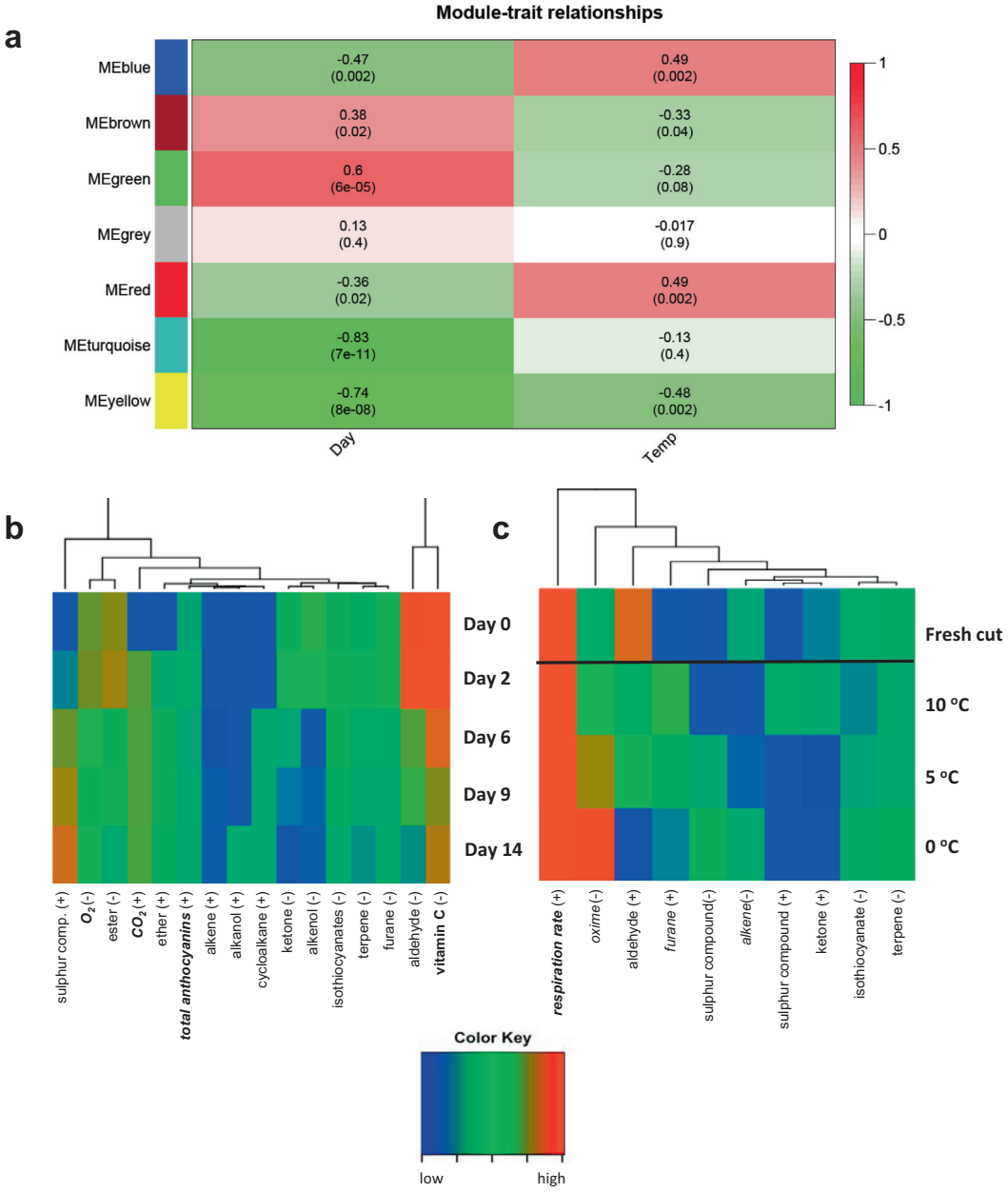

Fig. 5. Multi-trait correlation analysis of physiological indicators, nutritionally relevant phytochemicals and VOCs in rocket salad stored at three different temperatures (0, 5 and 10 °C) over a 14 day storage period. (a) WCNA modules: the score and significance (*P* values in brackets) are according to a Pearson analysis. (b) and (c) heat maps of multi-trait correlation analysis based on (b) days of storage and (c) temperature for physiological indicators (***bold italics***) nutritionally relevant phytochemicals (**bold**) and VOCs. Blue indicates a low content, green intermediate and red a high content for each character.

atively constant chlorophyll levels throughout the storage period, indicated that senescence was effectively delayed at all three temperatures. This is consistent with a reduction in respiration rate at lower temperatures since senescence is an energy requiring process (Lim, Kim, & Nam, 2007). Another factor affecting senescence progression is the level of O_2: by 6 days at the two higher temperatures O_2 had dropped to levels < 5 % and this inhibits natural senescence; this effect was previously observed (Løkke, Fast Seefeldt, & Edelenbos, 2012) leading to loss of Mg from the chlorophyll resulting in an olive-brown colour instead of yellowing (Toivonen & Brummell, 2008). Total concentration of anthocyanins also did not change during the storage period indicating that there was no clear stress-induction as might have been predicted (Cisneros-Zevallos, 2003), although levels of anthocyanins are generally low in rocket leaves.

The reduction of vitamin C during storage may be related to the wound response from the initial injury of the leaves during harvest and transport that in many species results in a reduction of vitamin C (Suza et al., 2010). However, Koukounaras et al. (2007) did not find a correlation between degree of wounding and vitamin C levels in rocket leaves. The effects may instead be due to detached leaf dark-induced senescence. The loss of vitamin C, but without significant loss of antioxidant capacity and relatively constant chlorophyll is consistent with studies on spinach and Arabidopsis that indicate that at this very early stage of dark-induced senescence ethylene signaling may play a major role (Gergoff, Chaves, & Guillermo Bartoli, 2010). This is likely a consequence of the hormone imbalance caused by detachment from the plant.

However, the relatively better retention of vitamin C at 0 °C was not accompanied by increases in total phenolics or individual phe-

nolic compounds with nutritional value. For example, 7-hydroxycoumarin levels was at its maximum concentration after 6–9 days of storage when there had already been a significant reduction in vitamin C.

The fall in visual quality score with storage time at 5 °C is consistent with previous studies (Martínez-Sánchez et al., 2006). The retention of all three quality scores at 0 °C compared to the two higher temperatures indicates a potential threshold effect between 0 and 5 °C that has a strong influence on sensorial quality. The change in scores at days 6–9 also coincides with the increase in microbial load, which may be affecting both appearance and VOCs.

The multitrait analysis showed correlations between changes in VOCs and other biochemical and physiological parameters and identified some of the VOCs as useful quality markers. The parallel fall in seven aldehyde VOCs and vitamin C suggests that these VOCs might be useful as markers for changes in vitamin C during storage. In addition, the change in four of the aldehydes with temperature could represent an indicator of a breach in the cold supply chain. The loss of ITCs at higher temperatures fits with the loss of their precursors, glucosinolates at 4 °C or at 15 °C (Force et al., 2007) indicating that measurement of ITCs may be a useful marker.

The rise in three sulphur-containing compounds (C27, C26 and C29) at later time-points parallels the drop in sensorial quality including a fall in aroma score and the increase in microbial growth (Fig 3) indicating that they may contribute to off–odours. Although dimethyl sulfoxide (DMSO, C27) is almost odourless with a faint smell of garlic, dimethyl sulphide (DMS, C26) has a distinctive smell described as 'rotten cabbage' and has a very low odour threshold. It has also been associated with the odour of cooked cauliflower (Engel, Baty, Le Corre, Souchon, & Martin, 2002). Furthermore, DMSO can break down to form DMS (Glindemann, Novak, & Witherspoon, 2006). Dimethyl disulphide (DMDS, C29) is also reported as having a very unpleasant odour with a low odour threshold (Zinder & Brock, 1978). Two of these three compounds, DMS and DMDS were previously identified in a study of off-odours produced by *E. sativa* (Nielsen et al., 2008) who also found higher levels in leaves stored at 8 °C compared to 4 °C. Furthermore, these two sulphides increased when the leaves were inoculated with *Pseudomonas* spp. and *Xanthomonas* spp. bacteria and at low oxygen levels below 1%. This low level of O_2 was reached in our study here of *D. tenuifolia* when leaves were stored at 10 °C for $\geqslant$ 6 days and at 5 °C for $\geqslant$ 9 days, which fits with the rise in bacterial counts and the differential decrease in aroma score noted by the sensorial analyses at 9 days. However, Nielsen et al. (2008) also noted that *Pseudomonas* spp. and *Xanthomonas* spp. do not normally grow well at low oxygen levels, so the DMS and DMDS may also derive from anaerobic metabolism within the leaves or the presence of other microorganisms.

5. Conclusions

VOC bouquets recorded with TD-GC–MS-TOF discriminated well between days of storage and temperature of storage and may be a useful and rapid method of detecting breaches to the cold chain or for assessing shelf life. Analysis of functional phenolics revealed changes related to both temperature and time of storage suggesting optimal conditions for maximal intake. Furthermore, a multi-trait analysis revealed that specific VOCs could be used as useful markers to indicate nutritional deterioration of *D. tenuifolia* bagged salad leaves, which would only be otherwise detected by relatively laborious biochemical assays. Specifically a group of aldehydes correlated closely with a drop in vitamin C content and the fall in ITCs correlated well with previous studies showing changes in the nutritionally-relevant glucosinolates.

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Conflict of interest

The authors declare no conflict of interest

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.107.

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