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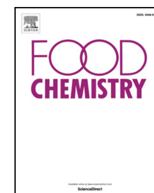

Short communication

High throughput quantitative volatile profiling of melons with silicone rod extraction – thermal desorption – GC–MS for plant breeding line selection

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ABSTRACT

Volatile compounds determine the aroma of fruits, giving their unique flavor characteristics. The aim of many plant breeding projects is to improve the consumers' flavor experience when eating fresh produce. Large scale breeding trials produce thousands of samples which need volatile profiling amongst other phenotypes. Despite this interest, current methods have limitations: sampling unsuitable for field conditions, high cost and the inherent issue of highly variable data, which can hinder interpretation. We introduced a simple and robust sampling methodology based on silicone rod extraction, thermal desorption gas chromatography – mass spectrometry (GC–MS) to address these issues. We used differentiated calibration standards to generate quantitative data for metabolites of varying abundance. The method was used to profile 327 melons with high sensitivity (0.05–10 ng/mL, compound dependent), good reproducibility (7%) and differentiate melon varieties based on their volatile profile. The data were then used for line selection for a desired flavor profile.

1. Introduction

Horticultural breeding has long leaned towards long shelf life for many fruity vegetable crops in order to increase durability for shipment and retail shelf life (Klee & Tieman, 2013). However this focus has often come at the expense of sweetness and aroma, which are some of the most important parameters that determine the quality and consumer preference of fresh fruit (Wyllie, Leach, Wang & Shewfelt, 1995; Blanckenberg, Muller, Theron, & Crouch, 2016). Increasingly, breeding projects are aimed at improving fruit quality traits, including the aroma volatile profile of new varieties (Tieman et al., 2017).

The process of breeding new varieties of fruits and vegetables takes several years, with activities often carried out at multiple geographical locations. Throughout this process, large numbers of samples are generated which need to be phenotyped consistently for taste and flavor. The reliable analysis of volatile metabolites is key to understanding the flavor of produce and is especially important in supporting large scale trials where direct testing methods such as taste panel experiments become non-viable (Scott, 2010). There are several technical challenges specific to this field, which are not supported by established volatile methods. Sampling and sample preservation has to be field-compatible using only basic laboratory facilities to reproducibly sample produce in a high-throughput way. The sample must be suitable for snap freezing in cryogen and shipment overseas (e.g. no glass vials). The volatile assay as a whole has to be cost effective as it is just one of several phenotypic data inputs, used either directly in material selection, or for the development of molecular markers.

Flavor profiling is also challenging because volatile metabolites are present at different abundances and their contribution to the overall flavor experience is not proportional to their relative abundance (Jelen, Majcher & Dziadas, 2012). Thus a method with a broad dynamic range and good sensitivity is preferable, gas chromatography coupled to mass spectrometry (GC–MS) being the most used analytical technique in this area (Condurso et al., 2012; Obando-Ulloa et al., 2008; Vallone et al., 2013; Amaro, Beaulie, Grimm, Stein, & Almeida, 2012; Lubes & Goodarzi, 2017).

Some of the commonly employed volatile sampling methods use dynamic headspace extraction (DHE) (Lignou, Parker, Baxter & Mottram, 2014), solid phase microextraction (SPME) (Obando-Ulloa et al., 2008; Condurso et al., 2012; Song, Gardner, Holland & Beaudry, 1997) and stir bar sorptive extraction (SBSE) (Vallone et al., 2013; Amaro et al., 2012) most often coupled with GC–MS. DHE, SPME and SBSE methods have the advantage of sample pre-concentration prior to the GC–MS analysis. This pre-concentration is achieved by using an

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absorbent phase, typically containing poly(dimethyl siloxane) (PDMS) or Tenax. SPME fibers are available with different coatings, making them suitable for a wide range of analytes, however the thin absorbent film can make it a less sensitive technique than SBSE (Jelen et al., 2012) and more prone to oversaturation. SBSE is a suitable tool for the analysis of low abundance flavor metabolites, however the glass coated magnetic stir bars can be fragile and the repeated re-use of the device raises questions about the stability of the absorbent phase and potential cross-contamination between samples (Camino-Sanchez, Rodriguez-Gomez, Zafra-Gomez, Santos-Fandila, & Vilchez, 2014). These limitations can be overcome by using silicone rods, however this requires some user customization, as silicone rods are not commercially available for analytical use (Allwood et al., 2014; van Pixteren, Paschke & Popp, 2010).

All of these issues have hampered the use of volatile profiling in large scale trials. The presented method based on silicone rod extraction thermal desorption – gas chromatography – mass spectrometry (TD-GC–MS) allows profiling of volatile metabolites of melon (*Cucumis melo*) to support large scale plant breeding trials and molecular marker development through its high throughput, resource efficiency and data quality.

2. Materials and methods

2.1. Materials

Analytical standards were purchased from Sigma (Gillingham, UK), HPLC grade methanol, dichloromethane and pure grade isopropanol from Fisher Scientific. Ultra-pure water was generated using a Millipore water purification system.

Silicone rods, 3 mm OD, translucent, 60° shore A hardness were purchased cut to 20 mm length from Silex Ltd (Bordon, Hampshire, UK). As this material is not intended for analytical use, they were solvent conditioned in bulk (~400 rods at a time) by 24 h Soxhlet extraction in dichloromethane, then thermally conditioned at 275 °C under nitrogen. The conditioned silicone rods were then stored in an airtight container until further use. To avoid sample to sample carry over we chose to work with the rods as single use consumables. The cost of a single rod is approximately 1 US ¢.

Melon used for method development, validation and QC purposes was shop bought in local supermarkets. All QC samples were from the same homogenate, with a new aliquot extracted and run in each sequence. Samples analyzed from the breeding trial were grown at Syngenta's Agadir field station.

The breeding trial: An F6 RIL population of Charentais-type melon (*Cucumis melo*) with varying plant and fruit parameters was grown under irrigated, passive greenhouse conditions in Agadir, Morocco over three seasons covering 2007 and 2008. The trial was an augmented design with repeated checks consisting of a commercial hybrid, Mehari and related parental materials. Each plot consisted of 16 plants, and 3 equally sized, shaped, and commercially mature fruits were selected from each plot for volatile analysis. Tagged fruits were transported to a lab for processing. The data presented in this paper is from 327 samples from the second planting from late 2007.

2.2. Sample homogenization and aliquoting

Approximately 100–250 g chopped fruit was placed into a Waring blender and blended for 1 min. A repetitive pipette (Eppendorf, Stevenage, UK) with a 25 mL tip (tip cut short) was used for the aliquoting; the first aliquot was discarded as recommended by the pipette's manufacturer. 1.5 mL aliquots were dispensed into labelled 4 mL cryovials (Greiner Bio-One, Stonehouse, UK) and after capping, samples were snap frozen in either liquid nitrogen or in an isopropanol – dry ice cryogenic mixture. Samples were stored at −80 °C prior to analysis. Field samples were homogenized and aliquoted at growing sites, then shipped to the laboratory on dry ice.

2.3. ISTD addition, extraction

The frozen samples were removed from the −80 °C freezer and immediately a silicone rod and 20 µL internal standard spike (1,4-dichlorobenzene, 18.75 µg/mL in methanol) were added to each sample, minimizing the amount of time the screw caps were left open to reduce sample loss. The samples were bundled together and shaken for 20 min using a mixer/mill (Spex, 8000 M Mixer/Mill, Stanmore, UK). After the extraction the silicone rod was removed from the sample, rinsed with ultra-pure water, dried on a lint-free tissue and placed into the thermal desorption tube of the TD-GC–MS.

Samples were extracted in sets of 20. The procedure takes about 1.5–2 h per batch of 40 samples, including the required calibration and QC samples.

2.4. Calibration

Calibration samples were prepared by adding 1.5 mL ultra-pure water, 20 µL internal standard spike, 20 µL of the differentiated calibration standard for each level and a silicone rod into a 4 mL cryovial. The calibration standards were then subjected to the same extraction procedure as above.

A differentiated calibration standard can be prepared when the target list of metabolites is established, the process of which has been reported (Kende et al., 2010). First, the typical concentration range of each metabolite was assessed in melon and then a calibration range was assigned. In this method we quantified 41 compounds by external standard calibration and semi-quantified 11 compounds against ethyl-3-hexenoate, where an analytical standard was not available. The 41 quantified compounds were categorized into one of 4 calibration ranges from 100 to 5000 ng/mL based on their natural abundance. The highest concentration mixed standard (L1) was then prepared using 5 mg/mL individual stock solutions to give the appropriate calibration concentration for each analyte when a 20 µL spike was added to 1.5 mL water. The highest concentration mixed standard (L1, 100%) was diluted to give 5 lower concentrations, L2 – 75%, L3 – 50%, L4 – 25%, L5 – 12.5% and L6 – 1%. The LOQ of the method was arbitrarily assigned as L6 of each compound (unless otherwise stated in Table 1). To determine the LOD an additional 7 dilutions were prepared at 0.5, 0.25, 0.125, 0.025, 0.005, 0.001, 0.0001% of the top mixed standard and analyzed in triplicate. The level with signal to noise larger than 3 was selected as the measured LOD for each compound (Table 1).

2.5. TD-GC–MS

Samples were analyzed with a Gerstel – Agilent MPS2 automated TD-GC–MS 7890-5977a system. The thermal desorption unit (TDU) parameters were 30 °C (1.1 min) heated to 250 °C (5 min) at 720 °C/min. The cooled inlet system (CIS) parameters were 30 °C (0.1 min) heated to 275 °C (5 min) at 12 °C/s. The inlet was in solvent vent mode during the thermal desorption step with 40 mL/min flow until 0.01 min, then 6 mL/min for a 4:1 split injection. The column was DB-624 30 m × 0.25 mm × 1.4 µm. Helium was used as the carrier gas, with the temperature gradient of 35 °C (2 min), to 70 °C at 8 °C/min, to 85 °C at 3 °C/min, to 220 °C at 8 °C/min and finally to 275 °C at 25 °C/min. The GC method included back-flushing, by applying an Agilent microfluidic device to clear out any late eluting components by reversing the direction of the carrier gas for the equivalent of 2 column void volumes.

The mass spectrometer was run in full scan mode with 2 min solvent delay scanning from 41 to 350 amu (~5 scans/s) at 230 °C ion source temperature and 150 °C quadrupole temperature in EI (70 eV) mode.

Samples were run in batches of 40 on the GC–MS, with a set of calibration samples (6 levels) and blanks at the start and the end of the sequence, a quality control (QC) sample, with calibration checks (L3)

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