Running title: Quantification of volatiles in muskmelon and watermelon aroma

Quantification of prominent volatile compounds responsible for muskmelon and watermelon aroma by purge and trap extraction followed by gas chromatography-mass spectrometry determination.

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Abstract

A dynamic headspace purge and trap (DHS-P&T) methodology for the determination and quantification of 61 volatile compounds responsible for muskmelon and watermelon aroma has been developed and validated. The methodology is based on the application of purge and trap extraction followed by gas chromatography coupled to (ion trap) mass spectrometry detection. For this purpose two different P&T sorbent cartridges have been evaluated. The influence of different extraction factors (sample weight, extraction time, and purge flow) over extraction efficiency has been studied and optimized using response surface methodology. Precision, expressed as repeatability, has been evaluated by analysing six replicates of real samples, showing relative standard deviations between 3 and 27%. Linearity has been studied in the range of 10 to 6130 ng mL^{-1} depending on the compound response, showing coefficients of correlation between 0.995 and 0.999. Detection limits ranged between 0.1 and 274 ng g^{-1}. The methodology developed is well suited for analysis of large number of muskmelon and watermelon samples in plant breeding programs.

Keywords: GC-MS; P&T; organoleptic quality, *Cucurbitaceae*, response surface methodology.

1. Introduction

In recent years, there has been increasing concern among consumers regarding fruit and vegetable organoleptic quality. In developed countries, an appreciable percentage of consumers are willing to pay a premium for high organoleptic quality products, mainly for fruits and vegetables. Considering that muskmelons and watermelons are important horticultural crops around the world with an increasing demand, the determination of the organoleptic quality characteristics is becoming highly important. These determinations are relevant as quality controls in the production/storage/distribution system and in research programmes, especially in the breeding sector in which obtaining new cultivars with better flavour characteristics is an increasingly important objective.

Muskmelon and watermelon flavour is directly related to their sugar content, mainly sucrose, which can reach 97% of the total soluble solids content (Kultur, Harrison, & Staub, 2001; Yau, Rosnah, Noraziah, Chin, & Osman, 2010). Additionally, other minor components, such as organic acids and especially volatile compounds, also contribute to define flavour nuances, and they can also modulate the perception of sweetness (Monforte & Alvarez, 2006; Yamaguchi, Hughes, Yabumoto, & Jennings, 1977). This influence of aroma on the overall flavour perception adds credence to the recommendation that aroma should be evaluated along with the soluble solids content. It has been observed that high levels of soluble solids do not always have correspondingly high organoleptic quality values (Lester et al., 1992; Yabumoto, Yamaguchi, & Jennings, 1978). Moreover, the high variability of the aromatic characteristics of muskmelon (Beaulieu, 2006a; Horvat & Senter, 1987; Wyllie, Leach, Wang, & Shewfelt, 1995) and watermelon (Kim, Lee, & Kim, 1999; Yajima, Sakakibara, Ide, Yanai, & Hayashi, 1985) makes this an attribute to be considered as a quality trait with increasing importance.

Evaluation of organoleptic quality is difficult. A commonly used determination of this complex quality attribute of fruits and vegetables is the evaluation of sensory characteristics by a taste panel. Nevertheless, evaluations of sensory attributes are often not readily interpreted and the number of

samples that can be evaluated in a single session is low. Olfactometry combined with triangle tests would be a good approach to simplify these evaluations and uses a more speedy method but, despite its simple data interpretation, is rather tedious and needs huge sample numbers. In this context, there has been great interest in the substitution of these tedious and expensive evaluations by an objective quantification of compounds that correlates with the sensory evaluation.

A high number of compounds with different degrees of volatility including esters, alcohols, and carbonyl compounds (especially those containing a nine-carbon straight chain) are the major chemical determinants of muskmelon fruit quality as perceived by consumers (Beaulieu & Lancaster, 2007; Gonda et al., 2010). Until now, around 300 volatile compounds have been identified in muskmelon and watermelon samples (Beaulieu & Grimm, 2001; Beaulieu & Lancaster, 2007; Beaulieu & Lea, 2006; Beaulieu, 2006a, 2006b; Homatidou, Karvouni, Dourtoglou, & Poulos, 1992; Itrat, 2006; Jordán, Shaw, & Goodner, 2001; Kourkoutas, Elmore, & Mottram, 2006; Liu et al., 2012; Obando-Ulloa & Moreno, 2008; Obando-Ulloa & Ruiz, 2010; Perry, Wang, & Lin, 2009; Saftner, Abbott, Lester, & Vinyard, 2006; Verzera, Dima, Tripodi, & Ziino, 2011). Due to the important role of volatile components in organoleptic quality of foods and beverages, these compounds have been widely studied and analytical methodologies for their determination in fruit matrices are continuously being developed, including both GC (Beaulieu, Ingram, Lea, & Bett-Garber, 2004; Beaulieu & Lancaster, 2007; Beaulieu & Lea, 2006; Gonda et al., 2010; Kemp, 1975; Kourkoutas et al., 2006; Liu et al., 2012) and GC-O (Jordán et al., 2001; Lignou, Parker, Baxter, & Mottram, 2014) methods.

A direct headspace injection could be a simple, cheap and fast option for the analysis of aromatic volatile compounds in vegetal samples. However, many volatile analytes in the vapour phase of this kind of sample are often present in too low concentrations (Beltran et al., 2006). Beaulieu & Grimm (2001) and Beaulieu (2006b) extensively analyzed melon aroma via solid phase microextraction (SPME), reporting and verifying 25 of the aforementioned compounds for the first time in cantaloupe melons. Since then, several studies reported SPME as a good semi-quantitative

methodology for the determination of volatile organic compounds (VOCs) in several vegetal samples including muskmelon (Kourkoutas et al., 2006; Verzera et al., 2011). This simple methodology presents some advantages such as a good compound pre-concentration in vapour and liquid phases, a solventless sample preparation method, short extraction times, and the possibility of using different sorbent materials depending on the nature of target analytes. However, this methology presents some disadvantages in the quantification process: the amount of analytes transferred from a sample to the SPME fiber is highly dependent on the sample matrix, adsorption capability, and other several extraction related parameters (Bicchi, Cordero, Liberto, Rubiolo, & Sgorbini, 2004).

On the other hand, headspace purge and trap technique dynamically captures the volatile sample fraction in a solid adsorbent which can be recovered by subsequent thermal or solvent desorption. According to Contarini & Povolo (2002) the purge and trap technique can be more suited to the determination of compounds with lower molecular weight than SPME. The main advantages of the former are the possibility to extract several samples at the same time together with the use of calibration curves in solvent for quantification. In SPME, calibration has to be performed in spiked sample matrices, as obtaining blank samples to prepare calibration curves is a problem in some cases. In addition, although it is well known that a SPME fiber can be repeatedly used (more than 100 times in automated devices) calibration curves should be done now and then to ensure quantitation, especially for very complex samples. Therefore, the P&T technique allows quantitation of the volatiles of interest, which is an advantage compared to traditional qualitative SPME methods. Likewise, when a large number of samples needs to be analysed, the P&T methodology has two important advantages that will facilitate its use in plant breeding program contexts. Firstly, the relatively low cost and amount of solvents involved and, secondly, the capability of extracting several samples simultaneously (depending on the laboratory configuration). Consequently the aim of the present study has been the development and validation of a dynamic headspace purge and trap methodology for volatile compounds extraction followed by gas chromatography coupled to mass spectrometry (GC-MS) determination (Buttery et al., 1982; Homatidou et al., 1992; Kemp,

1975; Verzera et al., 2011) that might be effectively applied to the analysis of a large number of muskmelon and watermelon samples.

2. Material and Methods

2.1. Target compounds selected

An extensive literature review showed that around 300 volatile organic compounds have been described to be present in muskmelon and watermelon samples (Supplementary information, **Table S1**). However, there are considerable differences in concentration, effect on muskmelon-like or watermelon-like aroma, or feasibility to be detected by humans due to their odour thresholds. In most of the reviewed studies, mainly in those where the authors compared muskmelon varieties and maturity stages, the number of relevant compounds was around or below ninety (Amaro, Beaulieu, Grimm, Stein, & Almeida, 2012; Beaulieu & Grimm, 2001; Obando-Ulloa & Moreno, 2008; Verzera et al., 2011).

The compounds studied in this work were selected following two criteria: the number of papers in which they were cited and the concentration levels reported. In addition, chromatographic behaviour of the selected compounds in the GC-MS system used was also considered. **Table 1** shows the 61 volatile compounds analysed in the present work.

2.2. Samples

Cultivars of five muskmelon types and one of watermelon commonly produced in Spain were cultivated open air in a spring-summer cycle at Jaume I University with common growing conditions. The plant materials were selected according to their economic importance in the Mediterranean basin and in order to study different aromatic fruit profiles. Fruits from “Piel de sapo” muskmelon type (*Cucumis melo* L. var *saccharinus* Naud.), the most economically important type in Spain, were used in the optimization of the volatile organic compounds (VOCs) extraction. “Charentais” muskmelon type (*Cucumis melo* var. *cantalupensis* Naud.) was used in the validation method

because it has an aromatic profile with the highest number of volatile compounds. Fruits of "Tendral" type (*Cucumis melo* L. var *inodorus* Naud.), "Charentais" type (*Cucumis melo* L. var *cantalupensis* Naud.), "Galia" type (*Cucumis melo* L. var *reticulatus* Ser.), "Amarillo" and "Piel de sapo" types (*Cucumis melo* L. var *saccharinus* Naud.) and watermelon (*Citrullus vulgaris* Schrad) were used for the real sample quantitation analysis.

Fruits were harvested at commercially optimum maturity states by checking several morphological attributes and basic organoleptic properties such as soluble solid content (minimum 12 ºBrix for "Charentais" and "Galia" types and minimum 10 ºBrix for the other types). After sampling, fruits were ground in a blender Thermomix® TM31 (Vorwerk España M.S.L., Madrid, Spain) in the case of the "Piel de Sapo" used for optimization of the method for enough time to obtain a homogenous sample; the other melon types were processed in an 1100W blender until they were completely homogenized. In both cases samples were immediately stored at -80°C until analysis.

2.3. Chemicals and supplies

Reference standards of volatile compounds studied (**Table 1**) were supplied by Supelco (Sigma-Aldrich and Fluka; Barcelona, Spain) as pure compounds (90 - 99.5% purity). Standard stock solutions (500 mg L^{-1}) of the individual volatile standards were prepared in acetone, from which seven mixed standard solutions (around 50 mg L^{-1}) were obtained, by 10-fold volume dilution in acetone. Calibration solutions were prepared from working solutions by consecutive volume dilutions with n-hexane to different final concentrations, according to the detector response for each compound (more concentrated when response was lower). All standard solutions were stored at –18 °C in sealed glass vials (without leaving any headspace) to avoid analyte losses and to ensure reproducibility, as recommended in many references (Kozłowska, Polkowska, Namieśnik, & Przyjazny, 2006). GC grade solvents were obtained from Scharlab (Barcelona, Spain).

Supelclean™ ENVI-Carb™ 120-400 mesh, 500mg and TENAX TA® 60-80 mesh, 500mg SPE Tubes 6 mL (Supelco, Barcelona, Spain) were used as traps.

2.4. Optimized extraction procedure

The extraction was carried out by a dynamic headspace system (DHS) using a purge & trap home-made device (Beltran et al., 2006) using commercial (500mg) SPE cartridges as trap. Before analysis, trap cartridges (EnviCarb SPE 500 mg in the optimized method) were conditioned with 5 mL of diethyl ether (Et_2O), followed by 5 mL of n-hexane and finally vacuum dried for 10 minutes. A sample amount of 30 g of homogenized fruit was weighed into a 150 mL flask closed with a glass cap with two connection tubes; the inlet tube was connected to a dry nitrogen gas (N_2) source and the outlet tube to the trap. The extraction time was adjusted to 49 minutes using a nitrogen flow rate of 1.6 L min^{-1}. Samples were stirred at 300rpm using a magnetic stir bar and heated at 40ºC.

After extraction, each cartridge was eluted with 5 mL of diethyl ether/hexane (1:1) followed by 5 mL of diethyl ether directly into a graduated glass tube. The extract was evaporated under a gentle nitrogen stream at a controlled temperature of 35°C to a final volume of 0.5 mL. The final extract was divided in two aliquots in vials with 200 µL inserts, sealed and stored in a freezer at -20°C until their analysis by GC-MS (ion trap).

Optimized recommended procedure was developed by applying a multivariate response surface optimization of the factors considered most important and influencing extraction efficiency. Three factors at three different levels were considered (A: sample weight (g); B: extraction time (minutes); C: gas flow (% max. scale of 1.6L min^{-1}). Sample weight was studied in the range from 5 to 30 g (levels 5, 17.5 and 30), extraction time from 15 to 60 minutes (levels 15, 37.5 and 60) and gas flow from 0.16 to 1.6 mL min^{-1} (levels 10, 55 and 100 % max scale). The studied parameter ranges were selected according to previous experience with a similar method developed for tomato samples (Beltran et al., 2006). A Box-Behnken design (Anderson & Whitcomb, 2005) consisting in 17 experiments (ran in three blocks) was obtained and results obtained after extraction and GC-MS determination were analyzed using Design Expert Software (Version 9.0.2, Stat-Ease, Inc.,

Minneapolis, USA). Optimization experiments were carried out using, as indicated above, a "Piel de sapo" muskmelon sample.

As response variables, only the twelve most prominent compounds in this muskmelon type were used: (E,Z)-2,6-nonadienal, (Z)-3-hexen-1-ol, (Z)-3-nonen-1-ol acetate, (Z)-6-nonenal, (E)-2-nonenal, (E,Z)-2,6-nonadien-1-ol, (Z)-3-nonen-1-ol, (Z)-6-nonen-1-ol, 1-hexanol, 1-nonanol, benzaldehyde and nonanal. A quadratic regression model was used to adjust experimental data and fittings to the data were checked with ANOVA. The optimal extraction conditions were determined using a weighted desirability function together with a simplex algorithm with variable size (Anderson & Whitcomb, 2005). This desirability function finds the combination of extraction factors which better satisfies the objective fixed for each VOC studied (maximization of extraction) when considering all the compounds simultaneously. The verification of the validity and adequacy of the predictive extraction model was checked by the application of the optimum extraction conditions selected to six replicate samples and comparing model predictions with experimentally obtained values using a two sided t-test (α= 0.01).

2.5. GC-MS determination

A Varian CP-3800 gas chromatograph coupled to a mass spectrometry detector (Saturn 4000, Varian) was used for the determination of volatile compounds in muskmelon and watermelon samples. The analytes were separated on a 30 m×0.25 mm Supelcowax 10 (0.25 µm film thickness) capillary column, using helium at a constant flow of 1 mL min^{-1} as carrier gas.

The temperature program was: 40 °C for 5 min, then increased to 160 °C at 4 °C min^{-1}, and finally increased to 250 °C at 30 °C min^{-1}, with a final isothermal stage of 1 min (total chromatographic analysis time 39 min). Injection of 1 µL of sample in the splitless mode (injection port temperature 220 °C) was performed using a Varian 8400 autosampler equipped with a 10 µL syringe. MS (ion trap) determinations were performed in full scan mode (m/z scan range of 50 – 200 Da) using electron impact ionization (70 eV) in positive mode and external ionization configuration. GC-MS

interface, ion trap and manifold temperatures were set at 275°C, 190°C and 60°C, respectively. Retention index for all studied compounds have been calculated by using a standard containing n-alkanes (C7-C30) on Supelcowax 10 capillary column and following the formula given by Kovats (1958).

Quantitation was carried out by using external standard calibration curves obtained by using peak areas from the corresponding extracted ion chromatograms for the selected quantitation ion (Q) for each compound (**Table 1**). Compounds with concentration exceeding linearity range were quantified by diluting extracts with n-hexane until proper concentration.

2.6. Validation

Method validation was carried out by applying a strategy considering linearity, precision (instrumental, intra-day, and inter-day), selectivity, and detection limits, using "Charentais" muskmelon samples. The validation parameters applied were:

Linearity: GC-MS injection of three replicates of the complete calibration curve with seven concentration levels, defined according to expected real sample contents for each compound.

Selectivity: a procedure blank (complete extraction procedure applied over an empty flask) was injected in the GC-MS system.

Instrumental precision: a single sample extract was injected 6 consecutive times.

The intra–day repeatability was determined by extracting and analyzing 6 replicates of the same sample under identical experimental conditions on the same day.

Inter-day reproducibility was performed by analysing 12 replicates of the same sample on three different days in a period of 5 days (6 replicates on day 1, 3 replicates on day 3 and 3 replicates on day 5).

Limits of detection (LOD) were calculated as the lowest concentration corresponding to a peak with a signal/noise ratio of 3.

3. Results and Discussion

3.1. GC-MS determination

Table 1 shows retention time, retention index and quantification m/z ions for each studied compound, obtained after injection of solvent standard solutions prepared in n-hexane. The quantification ions were selected according to their abundance in the mass spectrum and their signal-to-noise ratio. As an example **Figure 1** shows the chromatograms obtained for (a) a standard mixture of the 61 compounds selected and (b) a "Charentais" muskmelon sample extract.

3.2. Extraction optimization

Once the GC-MS conditions for the selected compounds were obtained the extraction conditions were optimized. Optimization was divided in two steps: firstly effectiveness of the sorbent type used as trap was studied. Secondly, a more accurate procedure based in Response Surface Method optimization was applied to obtain the exact extraction conditions that favoured overall extraction of all studied compounds.

In the first step, two commercial adsorbents (500 mg), ENVI-Carb and TENAX were evaluated applying the purge and trap procedure with initial fixed conditions (sample weight 30 g, extraction temperature 40 °C, extraction time 30 minutes, purge gas flow 1.6 L min^{-1}) according to Beltran et al. (2006). After extraction, each cartridge was eluted with three different solvents of increasing polarity which were collected separately: **Fraction 1**, elution with 5 mL hexane - diethyl ether (1:1) mixture, **Fraction 2** elution with 5 mL diethyl ether and **Fraction 3** consisting of 5 mL ethyl acetate. Each fraction collected was concentrated to 0.5 mL final volume (adding 0.5 mL of hexane prior to N_2 stream evaporation) and injected in the GC-MS system In order to get the maximum information from these experiments, two "Piel de sapo" homogenized muskmelon samples were spiked with a mixture of 61 target compounds giving a final nominal sample concentration of around 50 ng g^{-1} for each compound. Chromatograms obtained were integrated (using Q ion extracted chromatograms) and the peak areas for each compound in both adsorbents compared. Results indicated that almost

all volatile compounds were eluted in the first fraction (**Fraction 1**), whereas in **Fraction 2** (5 mL of diethyl ether) only the most retained compounds were still present corresponding to less than 10% of peak areas obtained in **Fraction 1** (except for 1-pentanol in Tenax cartridge that showed a peak area of around 45% of the peak area in **Fraction 1**). In **Fraction 3** (5 mL ethyl acetate) none of the compounds studied was detected. Accordingly, selected elution conditions were 5 mL hexane - diethyl ether (1:1) mixture followed by 5 mL diethyl ether, collected together and evaporated to a final volume of 0.5 mL.

In relation to the effectiveness of the adsorbents results showed that all target analytes were detected in both cases (Tenax and EnviCarb sorbent extracts). In addition, peak areas obtained for more than 65% of compounds were of the same order (approximated as the sum areas for both elution fractions). Differences found were especially relevant for 2-methylpropyl acetate and methyl-2-methyl butyrate that had signals in EnviCarb of around 40% and 20%, respectively compared to those obtained in Tenax (values for 2-methylpropyl acetate and methyl-2-methyl butyrate were 82650 and 42335 in Tenax sorbent, and 34010 and 8550 for EnviCarb). Although the performance of Tenax sorbent is a little better than EnviCarb, both sorbents allow extraction of all considered compounds, and were considered suitable according the aims of the developed method.

To complete the first stage of the extraction optimization, a breakthrough volume test was performed by using two cartridges connected sequentially to retain analytes (trap). Eluting the two cartridges separately as indicated before, the results showed that only 8 compounds reached the second EnviCarb cartridge and always with reduced signals. With Tenax cartridges, the number of compounds reaching the second cartridge increased to 16.

In conclusion, these results, coupled to the fact that EnviCarb cartridges are much less expensive than those of Tenax (about one third of the price, which is an important factor because in plant breeding programs it is necessary to make hundreds of samples for a complete evaluation of the segregant generations) and their availability (Tenax cartridges are manufactured on demand and

sometimes it takes too long, up to 8 weeks) led to the EnviCarb cartridge being the option selected to continue the work.

The next step in method development was optimization of "purge and trap" conditions. Taking into account the vast diversity of compounds and their physicochemical characteristics, it is really difficult to find which combination of factors maximizes the response (in terms of peak area for all compounds) with the smallest number of simple experiments possible. Therefore, the use of an experimental response surface multivariate design was considered with 3 main factors in the extraction process. These factors were sample weight, extraction time, and nitrogen purge flow. Sample weight was studied in the range of 5 to 30 g, extraction time from 15 to 60 minutes and purge flow from 0.16 to 1.6 mL min^{-1}. Seventeen extraction experiments were performed using a sample of "Piel de sapo" muskmelon type in a Box-Behnken design (in three blocks). Extracts obtained were analysed by GC-MS and the peak areas for twelve compounds selected according to peak areas and representativeness of the main VOCs groups (aldehydes, alcohols and esters) were obtained. The responses (corresponding to the quantitation ion chromatograms in each case) were used to determine the best mathematical models explaining the variability of the results. All the response surface models developed to explain the influence of the studied factors on extraction efficiency were statistically significant ($p < 0.01$). The factors (sample weight, extraction time and gas flow) and interactions that influence the extractions performed for each studied volatile compound are summarized in **Table 2** (column 2, model adjust group of parameters). For these models, regression coefficients of determination showed that, in general, the modelled response covers the point cloud of the experimental results well or very well (columns 3 and 4 in **Table 2**) and unexplained point cloud curvatures which might diminish their usefulness do not exist (as shown by the inexistence of significance between "lack of fit" of residues to the pure error of the model, column 5 in **Table 2**). Moreover, 10 of the 12 calculated models showed high or very high precision as shown by the signal-to-noise ratio parameter (column 6, **Table 2**). Consequently, we consider that the models can explain the experimental results obtained in a complete way.

The developed models showed that factor A (sample weight) and factor B (extraction time) were the main factors influencing extraction for (Z)-3-hexen-1-ol, (E,Z)-2,6-nonadien-1-ol, (Z)-3-nonen-1-ol, benzaldehyde, (Z)-6-nonen-1-ol, and 1-hexanol. Factors A and C (gas flow (% max. scale; 1.6 L min^{-1})) were relevant in the extraction of nonanal, (Z)-3-hexen-1-ol, and (Z)-6-nonenal; and factors B and C were important for the rest of the compounds. The magnitude and influence of each factor on each compound extraction can be seen better in the individual graphic representation of the models (**Figure 2**). In these plots, it is easy to see the conditions that maximize each volatile compound extraction and that the maximums of all these models were obtained at different values of the studied factors. So, the determination of a common optimum combination was extremely difficult, since the maximum values of all these models did not coincide. To solve this problem, a weighted desirability function (Anderson & Whitcomb, 2005) targeted to maximize the extraction for each compound was applied. Desirability results indicated that the optimum joint extraction conditions corresponded to 30 g sample weight, 49 minutes of extraction time and a gas flow of 1.6 L min^{-1} (marked as solid black lines in the factors plane of each subplot in **Figure 2**). With the calculated optimal levels for the extraction parameters we can obtain the extraction values expected for each compound (listed numerically as the "predicted mean" column in **Table 2**).

To check the reliability of the optimized model conditions, an assay was performed by extracting six replicates of a "Piel de sapo" sample with the optimized conditions and subsequent GC-MS determination. Predicted extraction means from the models were used to construct confidence prediction intervals (columns 8 and 10 in **Table 2)**. Actual confirmation values obtained (column 9 in **Table 2**) generally fall within these confidence intervals and, consequently, there are not significant differences between predicted and observed extraction values. Only benzaldehyde and (Z)-3-hexen-1-ol acetate were exceptions. In the case of benzaldehyde, the result was an anomalous value because other samples of "Piel de sapo" muskmelon analysed under the optimal extraction conditions showed benzaldehyde area values that fit well in the predicted interval. In relation to (Z)-3-hexenol acetate, its value in this confirmatory assay was slightly under the lowest predicted value,

but considering that this compound has the narrowest confidence interval (together with benzaldehyde) the model optimization predictions could be accepted.

In conclusion, the use of 30 g of weight sample, 49 minutes of extraction time with EnviCarb phase, elution with 5 mL hexane - diethyl ether (1:1) mixture followed by 5 mL diethyl ether and 1.6 L min^{-1} of nitrogen flow were selected as the best extraction conditions for the purge and trap methodology developed in this study.

3.3. Validation

Once optimized, the method was validated in terms of linearity using solvent standard solutions, and in terms of specificity, precision and detection limit using real samples. Although "Piel de sapo" muskmelon type was used for method optimisation, preliminary experiments showed that "Charentais" muskmelon presented a higher number of volatile compounds compared to the rest of the cultivars, so it was selected for validation studies. Linearity was tested by injecting (in triplicate) 7 concentration level standards ranging from 10 ppb to 5 ppm (nominal concentration). The calibration curves generated by plotting peak areas (Q ion signal) versus concentration (ng mL^{-1}) of each standard showed acceptable correlation coefficients (r^2) > 0.995 for all the compounds studied.

Precision was calculated by obtaining the instrumental, intra-day and inter-day precision (expressed as %RSD) of peak. Instrumental precision (n=6) ranged from 2% to 12%. Intra-day (n = 6) precision showed RSD (%) below 20% except for octyl acetate and beta-ciclocytral. As could be expected, the inter-day RSD were higher than intra-day precision, especially for benzyl acetate, hexyl acetate, phenethyl acetate, octyl acetate, propyl butyrate, amyl acetate, (Z)-3-Hexen-1-ol acetate, and ethyl hexanoate that showed a RSD greater than 20%. During method development and validation we considered the feasibility of using an internal standard to improve precision and account for recovery, but when using D_3-Methyl salicylate as a surrogate only a few compounds improved, while the rest of them showed a big deterioration in precision. These RSD results were better than those reported for a liquid-liquid extraction method coupled to GC-MS (Aubert & Bourger, 2004), and

similar to those obtained by our group using the SPME technique for some coincident compounds in tomatoes samples (Beltran et al., 2006). All the values for precision and linearity are shown in detail in **Table 1**. Detection limits (LODs) were calculated as the concentration of a peak having a signal/noise ratio of 3 (**Table 3**).

3.4. Determination of real samples

The applicability of the developed methodology was tested by extracting a number of muskmelon and watermelon samples, which were expected to have different volatile profiles. For this purpose 5 muskmelon types ("Charentais", "Galia", "Piel de Sapo", "Tendral" and "Amarillo") and one watermelon cultivar were evaluated.

Figure 3 and **Table 3** show results obtained by applying the dynamic headspace purge and trap developed methodology making use of EnviCarb cartridges with the optimized extraction conditions. As it was expected, a strong genotypic profile effect was observed, with changes both in single and relative analyte contents in the muskmelon types or watermelon analysed. "Charentais" muskmelon showed a more complex volatile profile when compared with other muskmelon types, with 47 compounds detected, while the rest had around or slightly below 30 compounds detected. (**Table 3**, column 4 to 9). All these samples contain ethyl 2-methyl butyrate, (Z)-3-nonen-1-ol, (Z)-6-nonen-1-ol and (E,Z)-2,6-nonadienal, which are reported to have a muskmelon-like flavour (Saftner et al., 2006; Verzera et al., 2011).

Several studies reported that 2-methyl propyl acetate, ethyl-2-methyl butyrate, ethyl butyrate, ethyl hexanoate, butyl acetate, hexyl acetate, 1-hexanol, (Z)-3-hexen-1-ol, ethyl-3-(methylthio)propanoate, and benzyl acetate were the main organic volatiles observed in some cultivars of "Charentais" muskmelons (*Cucumis melo* var *cantalupensis*) in concentration ranges similar to the ones found in this study (Aubert & Bourger, 2004; Kourkoutas et al., 2006; Obando-Ulloa & Moreno, 2008). The "Charentais" melon profile was differentiated from the others by the presence of butyl acetate, ethyl-3-(methylthio)propanoate, ethyl butyrate, amyl acetate, heptyl

acetate, methyl butyrate, 2-phenylethanol, ethyl hexanoate, phenethyl acetate, methyl hexanoate, isobutyl butyrate, butyl isobutyrate and octyl acetate compounds. Geranylacetone was only present in watermelon samples and compounds like (Z)-3-nonen-1-ol, β-ionone, (E)-2-heptenal and (E,Z)-2,6-nonadien-1-ol were present in higher concentration in watermelon compared to other samples. "Tendral" muskmelon type contained the highest concentration of (Z)-6-nonen-1-ol. "Piel de sapo" muskmelon type presented with the highest concentration of (E,Z)-2,6-nonadienal and (Z)-6-nonenal.

Regarding the analytical methodology used to evaluate aromatic profiles, in the literature several works can be found, dealing with SPME, in which only semiquantitive data, expressed as peak area or percentages (Beaulieu & Grimm, 2001; Kourkoutas et al., 2006; Lamikanra, Richard, & Parker, 2002; Obando-Ulloa & Moreno, 2008; Saftner et al., 2006) are reported, making use of a single external standard (Beaulieu, 2005) to calculate relative responses or by using standard additions (Verzera et al., 2011). In our opinion, the method developed in the present study has improved compound quantitation allowing the use of external calibration with a mixture of standards in pure solvents, avoiding the need for using standard additions method or matrix matched calibration. Recently SBSE technique (Amaro et al., 2012) has been used to carry out studies in order to differentiate samples through similar quantitation, using external standard calibration. However, the application of this technique requires the use of dedicated equipment (Twister and thermal desorption unit over an Agilent GC), and cannot be applied when lacking of this equipment. Additionally, as the validation of the method has not been thoroughly discussed by Amaro et al. (2012) it is not easy to compare both similar methods. Advantages of the proposed purge and trap technique compared to SPME and SBSE are: the possibility of extracting several samples at the same time, saving analysis time and reducing overall costs and the fact that a final volume of extract compatible with every GC system is obtained allowing it to be stored and injected several times and in different systems, as High Resolution Mass Spectrometry (HRMS), for further elucidation of unknowns in retrospective analysis. Our laboratory configuration permitted the extraction of up to 6

samples at a time; but this is completely scalable and could be upgraded to more than 15 at a time in routine laboratories.

The method developed is useful, reproducible and advantageous compared with previous methods, by being easier, less expensive and with higher sample throughput. The data obtained agreed with that in the literature, and thus suggested that the method is a relevant tool to differentiate the volatile profiles in muskmelon and watermelon breeding programs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version

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Figure captions

Fig. 1: a) Total ion chromatogram (TIC) of a standard mixture of 61 volatile compounds and b) TIC of a Charentais muskmelon sample extract. See **Table 1** for peak identification

Fig. 2: Response surface plots showing the effect of A: sample weight (g), B: extraction time (min) and C: gas flow (% max of the scale, 1.6 L min^{-1}) on the peak area of prominent volatile compounds found in muskmelon and watermelon. The selected conditions with the weighted desirability function to maximize global extraction are represented with a vertical black solid line.

Fig. 3: Profile (mean content, ng g^{-1}) of prominent volatile compounds detected in different cultivated types of muskmelon and watermelon.