Strategies for Accurate Quantitation of Volatiles from Foods and Plant-Origin Materials: A Challenging Task

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Abstract

The volatile fraction of foods and of plant-origin materials provides functional information on sample-related variables and gas-phase extractions are ideal approaches for its accurate chemical characterization. However, for gas-phase sampling, the usual procedures adopted to standardize results from solvent extraction methods are not appropriate: headspace (HS) composition depends on the intrinsic physico-chemical analyte properties (volatility, polarity, partition coefficient(s)) and matrix effect. Method development, design and expression of the results are therefore challenging. This review article focuses on volatile vapour-phase quantitation methods (Internal Standard normalization, Standard Addition, Stable Isotope Dilution Assay, Multiple Headspace Extraction) and their suitability in different applications. Because of the analyte informative role, the different ways of expressing results (Normalized Chromatographic Area, Percent Normalized Chromatographic Areas and Absolute Concentrations) are discussed and critically evaluated with examples on quality markers in chamomile, process contaminants (furan and 2-methyl furan) in roasted coffee and key-aroma compounds from high-quality cocoa.

Key-words:

Food volatiles; gas chromatography-mass spectrometry; accurate quantitation of volatiles; multiple headspace solid phase microextraction

Introduction

The volatile fraction of foods and of plant-origin materials is a mine of functional information concerning sample-related variables: plant genotype and phenotype expression, pedoclimatic and harvest conditions, post-harvest processing and treatments, shelf-life storage conditions, and the effects of transformation/processing technologies. This fraction mainly consists of organic compounds with molecular weight generally below 350 *u*, characterized by medium-to-high Log Ko/w ; they are readily released or vaporized from the condensed phase (solid or liquid) under suitable conditions (temperature, pressure, solubility in the medium, ion strength, etc.[1]

Thanks to their physical-chemical properties, volatiles can easily be extracted or sampled, through gas-phase extraction approaches, also known as headspace (HS) sampling. Sampling from the vapour phase can be carried out under equilibrium or non-equilibrium conditions, and provides information about analytes distribution and concentration in the original sample on the basis of compound-specific partition coefficients K_{hs}.[1] K_{hs} is a constant, temperature dependent, related to the ratio of the analyte concentration in the sample condensed phase (solid or liquid) *vs*. that in the vapour phase – Equation 1.

Eq. 1 $$K_{hs} = \frac{C_0}{C_g}$$

where: C_0 is the analyte concentration in the sample and C_g is the analyte concentration in the vapour phase or headspace.

Headspace sampling can be achieved through different approaches, mainly static (S-HS) or dynamic (D-HS), as well as in fully-automated systems combining analyte extraction, separation by gas chromatography (GC), and detection, either by mass spectrometry (MS) or by other suitable detectors (flame ionization detector FID, electron capture detector ECD or other element-specific detectors).

To achieve suitable sensitivity and, therefore, to access the highest possible level of information, provided by an almost comprehensive mapping of the food or plant volatilome [2–4], high concentration capacity (HCC) techniques are of great help. In this scenario, adequate selectivity and extraction capability, to meet the required method sensitivity and specificity, can be achieved through static or dynamic accumulation of volatiles on polymers, operating in sorption and/or adsorption modes.

Headspace solid phase microextraction (HS-SPME) is undoubtedly the most popular HCC approach [3,5–10], since it is easy to standardize and can be fully integrated within the analysis system, thus making it an ideal solution for high-throughput screenings and comparative studies.

An HS-SPME system (and more in general an HCC-HS system) implies the distribution of analytes across three separate physical phases: the sample, the headspace, and the layer of polymer coating the fiber. Analyte recovery from the HS of a sample depends on two closely related but distinct equilibria: the first is the sample/HS equilibrium, which influences HS composition (measured by its distribution coefficient K_{hs}), and the second is the HS/fiber equilibrium (measured by its distribution coefficient, K_{fh}). The amount of analyte extracted from the fiber (n) at equilibrium is expressed as:

Eq. 2 $$n = \frac{K_{hs}K_{fh}V_fV_sC_0}{K_{hs}K_{fh}V_f + K_{hs}V_h + V_s}$$

where C_0 is the initial concentration of the analyte in the sample, K_{fh} is the fiber/headspace distribution coefficient; K_{hs} is the headspace/sample distribution coefficient, V_s is the sample volume, V_f is the fiber coating volume, V_h is the headspace volume

The equation shows that the amount of an analyte extracted by the SPME device and its initial concentration in the sample are in direct proportion, thus making HS-SPME appropriate for reliable quantitative analysis. The highest sensitivity (i.e. recovery) for an analyte is achieved when

sampling is carried out in equilibrium conditions. However, the dynamics of adsorption during the SPME process, as described in the theoretical model [11], refers to the linear relation between *n* and C_0 ; thus, even in non-equilibrium conditions, reliable quantitation is possible. Theory also suggests which are the influential parameters to be tuned to achieve analyte recovery maximization: nature of SPME polymeric coating; coating volume, extraction temperature, sample agitation, pH and ionic strength, phase ratio , and extraction time.

Headspace sampling approaches: challenges for accurate quantitation

One of the most common practices related to volatile fraction characterization, also referred to as profiling [12], is cross-sample comparison. Analytes and/or informative markers are identified in each sample headspace profile, and compared through quantitative indicators generally based on chromatographic peak areas (Raw Areas, Percentage Area), peak volume percentage for comprehensive two-dimensional GC (GC×GC) (Raw Volume, Percentage Volume), or internal standard (IS) normalization (Normalized Area, Normalized Volume). These approaches are based on analyte relative/normalized responses and, although accepted by the scientific community for several application fields [13], may be inaccurate or misleading if considered as analyte(s) concentration indicators.

These indicators are suitable for the cross-sample analysis of solid or liquid samples, provided that the effect of the condensed phase (matrix effect) on analyte release into the HS is comparable or known. In many cases, the matrix effect may have a dramatic influence on analyte recovery, and lead to erroneous conclusions [7,11–14] .In solid or heterogeneous samples multiphasic equilibria in the condensed phases are established.

Solid samples are characterized by a heterogeneous composition and structure; thus native volatiles may be partitioned (absorbed) or adsorbed into the solid particles network, conditioning

93 their release and equilibration with the HS. This is a very important factor when the focus of the
94 investigation (profiling or fingerprinting) is generalized to all detectable compounds, since analytes
95 may show widely different K_{hs} values and adsorption behaviour.
96 All parameters concurring to produce the phenomenon known as the matrix effect on volatiles
97 release must be clearly characterized, or at least verified, in order to achieve reproducible, accurate
98 and, above all, meaningful results. Suitable procedures capable of compensating/modelling the
99 matrix effect are known as quantitation approaches and are generally based on external or internal
100 calibration with authentic standards or stable isotopologues of target analytes. The most widely
101 used techniques are:
102 ✓ external calibration in matrix-matched blank samples – suitable for liquid samples;
103 ✓ standard addition (SA) by spiking the sample with known incremental amounts of analyte(s);
104 ✓ stable isotope dilution assay (SIDA), which is a specific application of the SA;
105 ✓ multiple headspace extraction (MHE).
106 Whichever approach is applied, HS linearity conditions must be established [1] during sampling
107 for an accurate quantitation and to avoid errors. This condition is verified when the amount of
108 condensed sample (liquid or solid), under the applied sampling conditions is enough to release the
109 minimal analyte amount that matches method sensitivity and, at the same time, does not saturate
110 the HS. Linearity is easily achievable for trace and sub-trace target analytes, or at least in those
111 studies where the focus is on a few compounds, but it becomes challenging in multi-analyte
112 quantitation.
113 The following paragraphs illustrate the most common quantitation approaches adopted in
114 HS-SPME sampling, while discussing their suitability in studies where analyte concentration in the
115 sample must be assessed with appropriate accuracy.

116 The application section presents case-studies selected from the authors' research activity, in
117 which headspace quantitation was implemented through different approaches depending on the
118 aims of the study, and the results will be critically analysed for their information potential.
119
120 **Normalized responses: normalized peak areas and area percent responses**
121 The use of an internal standard (IS), or multiple internal standards, is a recommended
122 practice in GC [13] and, for HS-SPME sampling, it enables chromatographic response data to be
123 normalized and recoveries of the accumulating polymer(s) compared, compensating for differences
124 due to random instrumental errors. The IS must be an exogenous compound, with certified purity
125 and stability, not present in the samples under study. It should mimic analyte(s) physico-chemical
126 properties (e.g. volatility and polarity) and share chemical functionality(ies) with the analytes of
127 interest. Co-elution issues must be taken into consideration, especially in those applications in which
128 MS is not adopted for detection, and analyte response cannot be isolated by interferents through
129 specific *m/z* fragments.
130 The IS must be added to the sample HS before sampling and in a concentration that falls within its
131 linearity range of response.
132 An interesting alternative consists of the standard-in-fibre procedure introduced in 2005 by
133 Wang et al. [14]; this entails pre-loading the IS into the polymer coating before sampling. It informs
134 about the reliability and efficiency of the accumulating polymer over time, and better exploits the
135 isotropy of absorption and desorption of an analyte into and from the SPME device. This approach
136 is useful in particular for heterogeneous and solid samples and is also suitable for on-site or *in vivo*
137 applications.
138 Normalized areas and area percent responses, the latter calculated after IS normalization,
139 are suitable analyte indicators for comparative purposes provided that the matrix effect is

comparable if not identical. These indicators are not, *per se* quantitative, since they simply relate to the relative amount of analytes transferred to the analytical system. Each analyte is therefore characterized by its own K_{hs}, K_{fh} and detector response factor.

Practical examples of the correct use of normalized responses are given concerning chamomile phenotyping and cocoa potent odorants (see below).

Standard Addition: single-point or multiple-point calibrations

Standard addition was one of the first approaches introduced for HS quantitation that was able to compensate the sample matrix effect at least for liquid samples. It consists of a series of experiments in which the original sample, and sample aliquots spiked with increasing and known amounts of reference compounds, are submitted to the analytical process.

With the approach known as "single-point" calibration, the analyte concentration in the sample is estimated with Equation 3:

$$A_{(0+a)} = (A_0/C_0) \times C_a + A_0 \qquad \text{Eq.3}$$

where: C_0 is the amount of analyte in the sample, C_a the amount of analyte added to the sample, A_0 the instrumental response obtained from analysis of the original sample, and $A_{(0+a)}$ the instrumental response of the analyte obtained from analysis of the spiked sample.

Multiple standard additions, up to 6 levels, are recommended to improve accuracy. In linear regression analysis, C_a and $A_{(0+a)}$ are recorded so that the amount of analyte in the original sample (C_0) is given by the ratio between the intercept (b) and the slope (a), Equation 4:

$$b \,/\, a = A_0 \,/\, (A_0/C_0) \qquad \text{Eq. 4}$$

Standard addition can be implemented by: (*a*) directly spiking the target analyte(s), in a gaseous state, into the sample headspace (gas phase addition - GPA); (*b*) spiking the analyte(s) in solution directly onto the sample (sample phase addition - SPA) or (*c*) spiking stable-isotope-labeled analyte(s) dissolved in a suitable solvent (stable isotope dilution analysis - SIDA) onto the sample. Stable isotope dilution assay, introduced by Grosh and Schieberle in 1987 [15] and extended to HS applications by Steinhaus et al. in 2003 [16] for the accurate quantitation of linalool enantiomers in beer after HS-SPME sampling, is also suitable for solid samples, although some limitations may affect the accuracy of the results. The heterogeneous nature of solids requires longer equilibration times for isotopologues to reach the full multiple partition equilibria with the analyte in all sample compartments/phases. When this condition is not achieved, since the labeled standard is generally spiked to the sample as a solution, it may be more recovered than the native analyte, which may be "trapped" in compartments of the sample, from where its recovery is more difficult.

Conversely, SIDA has some unquestionable advantages given by the very close chemical nature of the analyte and its isotopically labelled standard, thereby eliminating some sources of errors due to sample manipulation or sampling dynamics. These advantages are lacking when using IS with a different chemical nature. Quantitation by SIDA is achieved by spiking the sample with a known amount of labeled standard, provided that the relation between isotopologue ratio and intensity ratio is known, either by a multi-point calibration curve or by a relative response factor (RF) as described by Equation 5.

$$RF = \frac{(C_{analyte}/C_{labeled})}{(A_{analyte}/A_{labeled})} \qquad \text{Eq. 5}$$

where $C_{analyte}$ and $C_{labeled}$ are the concentrations and $A_{analyte}$ and $A_{labeled}$ the instrumental responses.

The unavailability of labeled certified standards or, when available, their relatively high cost, are, to date, the most significant limitations affecting the widespread use of SIDA as quantitative approach in routine applications. However, it is fully compliant with EU guidelines and performance requirements [17] for quantitative methods on food contaminants, as here discussed below for furan and 2-methyl furan in roasted coffee.

Multiple headspace extraction

Multiple headspace extraction consists in a dynamic, stepwise gas extraction carried out to quantitate accurately volatiles in solid or heterogeneous samples where the matrix effect is known to play a relevant role in analyte release. The approach was introduced for S-HS applications and later adapted to HS-SPME [1,18–32]. It can be run as an external standard calibration and consists of three experimental steps:

Step 1. Exhaustive extraction of analytes from calibration solutions within a range of concentrations/amounts, matching real-sample concentrations.

Step 2. Exhaustive extraction of analytes from representative samples (solid, liquid or heterogeneous) to define HS linearity boundaries.

Step 3. Application of the MHE procedure to samples of interest.

With the first two steps, a cumulative instrumental response function is built after repeated consecutive extractions from the HS of the same aliquot of calibration solutions or representative samples. Extractions must reach complete (exhaustive) analyte removal from the sample. Four to six consecutive extractions are recommended to validate the exhaustiveness of the extraction process for all analytes of interest. The decrease of the chromatographic peak area, after consecutive extractions, is exponential with HS linearity [1,22]. This condition refers to the linear function between the analyte concentration in the sample (C_0) and its concentration in the gas phase

(C_g), or between C_0 and the instrumental response (A) obtained when analysing an aliquot of the HS. The actual linear range depends on the analyte partition coefficient (K_{hs}) and its activity coefficient. In general, it ranges between 0.1 and 1% in the sample and can be tuned by modifying sampling temperature, equilibration times, and the ratio between HS (V_h) and condensed phase volume (V_s).

The sum of the instrumental response (As) from each step of HS extraction corresponds to the total response (A_T) as it is generated by the analyte originally present in the sample. Equation 6 is applied to obtain the cumulative instrumental response (A_T):

$$A_T = \sum_{i=1}^{=\infty} A_i = A_1 \frac{1}{(1- e^{-q})} = \frac{A_1}{(1- \beta)} \qquad \text{Eq. 6}$$

where A_T is the total estimated response (chromatographic area), A_1 is the analyte response after the first extraction, and q is a constant associated with the response exponential decay (β) through consecutive extractions.

The sequential steps corresponding to the exhaustive extraction of an analyte from a sample in HS linearity conditions are illustrated in Figure 1 for a HS-SPME approach.

The term q can be obtained by plotting the natural logarithm of the chromatographic peak areas as a function of the number of extractions. From this, a linear regression equation (Equation 7) can be calculated as follows:

$$\ln A_i = a\,(i-1) + b \qquad \text{Eq. 7}$$

where i is the number of extraction steps, b is the intercept on the y axis, and a is the slope.

β (e^{-q}) is analyte and matrix dependent, and is a constant for those samples exerting comparable matrix effects [21,24]. β values can be adopted to confirm, or otherwise, HS linearity; its dependence

234 on K_{hs} provides information on matrix retention behaviour and on the actual release of target
235 analyte under specified conditions.

236 When applied to calibration solutions, MHE provides data for external calibration; curves can
237 be adopted to estimate analyte amount in the sample with a simplified procedure, where the
238 analyte response after the first extraction step (A_1) is sufficient for accurate quantitation [33].

239 The potential of MHE in combination with HS-SPME, also known as MHS-SPME, is illustrated
240 here in two case studies and emphasized for accurate quantitation of process contaminants in
241 roasted coffee samples, and for a multi-analyte quantitation, targeted to potent odorants in cocoa
242 nibs and mass. This latter example also discusses the additional information provided by matrix
243 effect characterization through the β parameter, and the advantages deriving from parallel
244 detection by MS/FID.

245

246 **Materials and Methods**

247 See associated contents.

248 **Applications**

249 ***Comparative studies: chamomile chemotyping***

250 Chamomile (*Matricaria recutita* L.) is a medicinal plant widely used in folk medicine for its
251 well-known spasmolitic, sedative, anti-inflammatory and antiseptic effects [34,35]. The drug consists of
252 the flower-heads while its biological activity is due to the presence of several secondary metabolites,
253 including sesquiterpenoids, coumarins and flavonoids (apigenine and related glucosides) [34]. In this
254 perspective, chemical composition of chamomile is fundamental for quality control and safety
255 assessment; it is generally analyzed by qualifying the essential oil (EO) composition. The EO mainly
256 consists of sesquiterpenoids, namely *trans*-β-farnesene, α-bisabolol, bisabolol oxide A, bisabolol
257 oxide B, α-bisabolone oxide A, chamazulene, and spiroether. To facilitate qualification and to

rationalize chamomile classification according to compositional characteristics, Schilcher [36] defined six different chemotypes of chamomile in function of the sesquiterpenoids distribution in the EO (Table 1). Type A is characterized by bisabolol oxide A as main component, Type B by bisabolol oxide B, Type C by α-bisabolol, while Type D contains comparable amounts of bisabolol oxide A and B and α-bisabolol. The two other chemotypes were not included in the study

The conventional approach to distinguish the chamomile chemotypes entails EO profiling by GC-FID (hydrodistillation + GC-FID). Analysis is preceded by analyte identity confirmation through reference spectra comparison and Linear Retention Index (I^T_S) confirmation. The evaluation of target analytes relative distribution is by percent responses (chromatographic area %) from FID detection. FID shows very stable and reliable Response Factors (RF) over a wide linear range of concentrations [37].

The conventional approach for chamomile EO chemotyping, although reliable and accurate, is time-consuming since, by applying the Pharmacopoeia protocol, it takes at least 4 hours for chamomile dried material hydrodistillation and not less than 30 minutes for a GC-FID/MS EO profiling.

An alternative approach to discriminate chamomile chemotypes has been proposed: the chamomile flower-heads' volatile fraction is characterized by HS-SPME combined with GC-FID and principal component analysis (PCA) targeted to EO markers [38–41]. For this specific application, chemotyping can be considered a comparative cross-sample analysis; thus quantitative data are not strictly necessary, the normalized responses (chromatographic area %) from the FID detector being sufficient for consistent comparisons between samples.

Experimental results, based on markers relative distribution (chromatographic areas %) validated the feasibility of applying a more time effective profiling approach by HS-SPME instead of hydrodistillation at the sample preparation level. The cross-validation was based on 127 samples

282 processed in parallel for EO direct profiling by GC-FID *vs.* automated HS-SPME-GC-FID of dried flower
283 heads. The results are in Figure 2**,** which shows the score plots resulting from PCA on marker relative
284 distribution (chromatographic areas %) resulting from EO GC-FID profiling (Figure 2A) or from direct
285 analysis of chamomile dried plant material by HS-SPME-GC-FID (Figure 2B). Elaborations was on a
286 sub-set of samples from the original study [38]. The total variability explained by the two principal
287 components accounted for 71.34% of variability for EO and 75.16% for HS-SPME. In both cases the
288 four chemotypes form coherent sub-clusters according to their different relative distribution of
289 markers and the three well defined chemotypes (A, B and C) are independently clusterized while
290 chemotype D occupies an intermediate position; this reflects Schilcher's classification, in which
291 chemotype D is not definitively characterized by one prevailing component.
292 These results support the adoption of normalized responses (chromatographic area %) for
293 comparative and discriminatory purposes. The use of the FID detector strengthens the reliability of
294 the results, because RF values are almost identical for analytes with the same formulae [37], while also
295 offering linear responses within a wide range, which is useful when analyte amounts in the sample
296 span over different orders of magnitude.
297
298 ***Accurate quantitation of target analytes: process contaminants in roasted coffee***
299 Furan and its homologous are oxygenated heterocycles present in the volatile fraction after
300 thermal treatment of different food crops and drinks, as one of the Maillard reaction products [42].
301 These compounds, in particular furan, are considered food process contaminants, and thus their
302 presence in food is the object of a constant attention by the U.S. Food and Drug Administration
303 (FDA) and the European Food Safety Authority (EFSA) because of carcinogenic and cytotoxic activity
304 in animals and harmful effects on human health [43]. In fact, the International Agency for Research on

305 Cancer has classified furan as a possible human carcinogen (Group 2B), therefore it must be
306 quantified in food to guarantee safe consumption.

307 In 2004, the FDA proposed a method based on static headspace combined with gas
308 chromatography-mass spectrometry analysis (S-HS-GC-MS) to quantitate furan in different food
309 crops, following the SA approach [45]. The method was updated in two steps, in June 2005, and in
310 October 2006 to extend its application range. Based on this protocol, and to overcome the relatively
311 low sensitivity of S-HS, an enrichment step by HS-SPME with a Carboxen/PDMS fiber was proposed
312 as alternative technique [46–48], further using d4-furan as IS with external calibration as quantitation
313 approach.

314 The authors' group [21] studied the accuracy and precision of the available quantitation
315 approaches for determining furan and 2-methylfuran in roasted coffee and compared them in view
316 of their possible application to on-line monitoring of these process contaminants during the roasting
317 process. The quantitation approaches explored for their performance parameters where SA, SIDA
318 and MHS-SPME; the results were compared and validated *versus* the FDA method taken as
319 reference.

320 The sample set consisted of 150 coffee samples of different varieties (Arabica and Robusta)
321 and origins (Costa Rica, Nicaragua, Colombia, Brazil and Kenya) submitted to different technological
322 processing (roasting, cooling, grinding and degassing). Commercial blends were also included to
323 cover sample variability during shelf-life storage.

324 To improve sampling repeatability, to facilitate the addition of the internal standard and to
325 increase method sensitivity for furan and 2-methyl-furan, both hydrophobic analytes, sampling was
326 on ground coffee suspended in ultrapure water. Details of all procedural steps followed for sampling
327 and calibration are described in the associated contents and reported elsewhere [21].

328 The average concentrations (expressed as mg/kg) and related coefficient of variations (CV%)
329 of furan and 2-methyl-furan for all analyzed samples, resulting from the application of the three
330 quantitation approaches (SA, SIDA and MHS-SPME), were in close agreement with the FDA method,
331 most of them giving CV% values well below 15%, chosen as limit of acceptance in agreement with
332 the EU Decision on analytical procedures for food safety assessment [17].
333 The experimental results from MHS-SPME also suggested that accurate determination of
334 analytes could be achieved by adopting an average β value (see Equation 7), instead of the specific
335 value for each sample, thanks to the homogeneity of the matrix effect of the coffee samples under
336 study. In addition, the possibility of quantifying analytes after a single-step extraction makes the
337 MHS-SPME approach rapid and highly competitive with SA and SIDA.
338 To satisfy the ever increasing demand for rapid analyses, for use in routine control when a
339 large number of samples must be screened, MHS-SPME sampling was also combined with direct MS
340 detection (MS-nose) [21,23], skipping the separation step by GC. Furan and 2-methylfuran quantitative
341 results, as obtained by MHS-SPME-MS were comparable to those from conventional separative
342 methods [21] giving satisfactory coefficients of variation (CV%). Figure 3 shows the very good
343 correlation between quantitative data obtained by MHS-SPME and those from the FDA method
344 (taken as reference) with a regression coefficient value of 0.974.
345 The determination of process contaminants on roasted coffee requires accurate and reliable
346 quantitation [17]. Due to the nature of the analytes (highly volatile and hydrophobic) and to the matrix
347 effect exerted by ground coffee, HS sampling is the route of choice, whereas MHS-SPME, among
348 available techniques, combines lower determination errors, low relative uncertainty (due to high
349 repeatability of the sampling approach) and, once optimized, single shot analysis.
350

Extending method capabilities by MHS-SPME and parallel detection by MS/FID for high-quality cocoa odorants

The cocoa volatilome is very complex [49–53] and, among the several hundred volatiles identified in it, the potent odorants are the analytes that contribute to the characteristic aroma signature, or *aroma blueprint* [54]. The *sensomic* approach enabled key-aroma compounds of some cocoa varieties and chocolate products to be defined [55–57] by adopting the well-established workflow, which includes, as a fundamental step, the accurate quantitation of odorants in the reference food sample. Quantitation of potent odorants, revealed by GC-olfactometry (GC-O) after Aroma Extract Dilution Analysis (AEDA), provides a more consistent evaluation of the actual role played by single odorants and affords efficient aroma recombination studies [58].

High-throughput profiling approaches are required when the investigation embraces the entire volatilome as informative fraction of sample functional characteristics (origin/phenotype, harvest and climate conditions, post-harvest practices, processing). Analytical automation on the entire process from sample preparation to separation and detection in combination with appropriate data processing, makes it possible to screen larger sample sets to achieverepresentative and consistent results.

In this study, accurate quantitation by MHS-SPME combined with GC-MS/FID targeted to several potent odorants, including key-odorants [55–57] and process indicators was addressed. Method flexibility was also discussed, since the quantitation of uncalibrated analytes by FID predicted relative response factors (RRFs). Thanks to the key characteristics of MHS-SPME, accurate quantitative results are achievable with just few analyses per sample and additional information on the matrix effect is obtained, describing the odorants released from the condensed phase (cocoa nibs or mass) [22]. The parallel detection by MS/FID is complementary in nature: (*a*) analyte identity is confirmed by MS fragmentation pattern, and the amount is accurately assessed through

diagnostic ions (MS target ions – Ti profiles) and external calibration; (*b*) the FID response on the parallel detection channel provides additional confirmation on the analyte amount, through its specific response factor, which can also be predicted by combustion enthalpies and molecular formulae [37]. In this way, external calibration can be avoided.

The study focused on the key aroma compounds described by Schieberle and co-workers [55–57]; these include alkyl pyrazines (2,3,5-trimethylpyrazine - TMP, 2-ethyl-3,5-dimethylpyrazine, and 3,5-diethyl-2-methylpyrazine), with *earthy, roasted* notes; short-chain and branched fatty acids (acetic acid, butanoic acid, 2-methylpropanoic acid, and 3-methylbutanoic acid), with *rancid*, *sour*, and *sweaty* notes; Strecker aldehydes (2- and 3-methylbutanal) with *malty, cocoa* and *buttery* notes; phenylacetaldehyde, with *flowery honey-like* note. Additional investigated components are some esters (ethyl-2-methylbutanoate – *fruity*; 2-phenylethyl acetate – *flowery*; ethyl phenylacetate – *honey-like*), linear alcohols (2-heptanol – *green, fatty*), phenyl propanoid derivatives (2-phenylethanol – *flowery*), sulfur-derived compounds (dimethyl trisulfide - *sulfury*), and phenols (guaiacol – *phenolic*), benzaldehyde (*bitter almond-like*), 3-hydroxy-2-butanone/acetoin (*buttery*), ethyl octanoate (*green, fruity*) and (*E*)-2-phenyl-2-butenal discriminant for processing stage.

Performance parameters of the MHS-SPME-GC-MS/FID method are reported in the reference paper [59] while the quantitation results, based on MS external calibration, are illustrated as a heatmap in Figure 4. The results, which are rendered in a relative colour scale (white to brown), correspond to the mean values of three replicated measurements from two sample batches. Hierarchical clustering (HC), based on Euclidean distances, informs about samples compositional similarities and on the analytes common trends within samples. On the basis of these results, odor activity values (OAVs) can be estimated, and the odorant ranking compiled.

To extend the method quantitation potential while keeping the results accurate and reliable, FID response factors can be exploited. The rationale behind the applicability of this quantitation

399 approach is related to HS linearity, the mandatory condition for MHE quantitation. By means of the
400 analyte characteristic β value, A_T is predictable (ref. Equation 6) and thus the actual absolute amount
401 of that analyte in the sample can be estimated through FID RRF. In case of liquid injections, the area
402 ratio between the analyte and the IS added to the sample in known amounts, is
403 normalized/corrected to the predicted RRF estimated from the molecular formula [60,61].

404 The reference equation (Equation 9) to calculate analyte RRFs is as follows:

405

406 $$RRF = 10^3 \ (MW_i/MW_{IS}) \begin{pmatrix} -61.3 + 88.8n_C + 18.7n_H - 41.3n_O + 6.4n_N + 64.0n_S \\ -20.2n_F - 23.5n_{Cl} - 10.2n_{Br} - 1.75n_I + 127n_{benz} \end{pmatrix}^{-1}$$ Eq. 9

407

408 where n_C, n_H, n_O, n_N, n_S, n_F, n_{Cl}, n_{Br}, n_I, and n_{benz} are, respectively, the number of carbon, hydrogen,
409 oxygen, nitrogen, sulfur, fluorine, chlorine, bromine, and iodine atoms and the number of benzene
410 rings. MW_i and MW_{IS} are the molecular weights of the analyte *i* and the IS (methyl octanoate),
411 adopted by de Saint Laumer et al. to the develop the model [60].
412 In the study cited, the analyte-specific RRF was corrected to the TMP/methyl octanoate ratio (i.e.
413 $RRF_{i,TMP}=0.7028/RRF_{i,methyl\ octanoate}$) to adapt the model to TMP.

414 Table 4 reports the RRF values calculated for all analytes externally calibrated and for
415 additional compounds of interest: 3-methylbutyl acetate (isoamyl acetate - *banana-like*), γ-
416 butyrolactone (*creamy*), octanoic acid (*sweaty*), 2-ethyl-5(6)-methylpyrazine (*roasty, nutty*),
417 phenylacetic acid (*honey-like*), phenol (*phenolic*), 2-acetyl pyrrole (*musty*), and 2,3-dihydro-3,5-
418 dihydroxy-6- methyl(4H)-pyran-4-one (DDMP – informative of processing stages [49]). Other analytes
419 2-heptanone, 2-nonanone, and heptanal are known secondary products of lipid oxidation. For these
420 additional compounds, the total chromatographic peak area (A_T) is estimated from four consecutive
421 extractions of the same sample, from which the analyte characteristic β value can be calculated.
422 MHS-SPME quantitation by FID RRFs results are validated for accuracy by cross-matching results

423 between external calibration on MS signals and FID RRFs. Regression analysis, reported in Figure 5,
424 shows good correlation between approaches, and confirms the consistency of FID predicted RRFs
425 for accurate quantitation.

426 At this stage, it is interesting to note that other descriptors, based on analyte responses and
427 not on absolute amounts, would give a different picture concerning the samples volatile fingerprint.
428 Graphs of Figures 6A and 6B, referred to Ecuador cocoa nibs (6A) and mass (6B), show analytes
429 distribution based on relative responses (bars - normalized on IS) or by quantitative results (areas –
430 true amount). The different trends followed by indicators clearly shows that the matrix effect
431 influences HS composition, but if normalized indicators alone are used to derive information about
432 analyte presence in the sample, this leads to erroneous conclusions. For cocoa mass, the
433 intermediate exerting the strongest matrix effect on odorant release, relative indicators reliably
434 inform about odorant distribution in the sample headspace. This could be of relevance for
435 orthonasal perception of odorants.

436 Figure 7 shows the average β values for a selection of key-odorants, determined in cocoa
437 samples of five different origins and at different stages of processing (nibs and mass); cocoa powders
438 are also included to illustrate how this intermediate exerts its matrix effect. As the data clearly
439 shows, cocoa mass strongly retains 2-heptanol, 2,3,5-trimethylpyrazine and benzaldehyde, which
440 possess β values twice as high as those of nibs.

441 In this context, MHS-SPME is mandatory for accurate quantitation of key-odorants; of
442 additional interest are data on actual analyte release from the condensed phase (mass, nibs and
443 powder). β values, between gas-phase or calibration solution and real sample multiple extractions,
444 give access to this phenomenon and may be of help in interpreting sensory analysis data.

445

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625

Figures captions

Figure 1. Sequential steps corresponding to the exhaustive extraction of an analyte from a sample in headspace (HS) linearity conditions for a HS solid phase micro extraction (HS-SPME) approach; from Cordero *et al* [59].

Figure 2. Scores plot resulting from the Principal Component Analysis (PCA) on chamomile marker analytes relative distribution (chromatographic areas %) resulting from essential oil (EO) GC-FID profiling (Figure 2A) or from direct analysis of dried plant material by HS solid phase micro extraction (HS-SPME) GC-FID (Figure 2B). Modified from Rubiolo *et al* [38].

Figure 3. Linear regression analysis on a sub-set of coffee samples analyzed by multiple headspace solid phase micro extraction (MHS-SPME) versus FDA method (taken as reference).

Figure 4. Heatmap illustrating the cocoa quantitation results based on MS external calibration; from Cordero *et al.* [59].

Figure 5. Linear regression analysis on quantitation results obtained by multiple headspace solid phase micro extraction (MHS-SPME)-GC-MS/FID: external calibration on MS signal *vs.* FID predicted relative response factors (RRFs) results; from Cordero *et al.* [59].

Figure 6. Analyte distribution based on relative responses - bars (normalized on IS) or on quantitative results – areas for cocoa nibs (6A) and mass (6B).

Figure 7. Average β values estimated in cocoa samples of five different origins and at different stages of processing (nibs and mass) for a selection of key-odorants; from Cordero *et al*. [59].

Table 1. Chamomile chemotypes according di Schilcher [36].

Chemotype	**Compositional characteristics**	Number of samples*
1 - Type A	bisabolol oxide A as main component	17
2 - Type B	bisabolol oxide B as main component	19
3 - Type C	α-bisabolol as main component	69
4 - Type D	comparable amount of α-bisabolol and bisabolol oxide A e B	22
5	α-bisabolone oxide A as main component	-
6	green essential oil, low amount of matricine	-

*Sub-set of those from the original study [39]

Table 2. Cocoa samples, modified from ref [59].

Origin	Commercial description	Supplier - Trader	Harvest year
Mexico	*Chontalpa Cacao fermentado seco calidad Baluarte*	"Mercados alternativos y solidarios para productos del campo S. de RL. de CV" Calle Exterior Manzana 17 Lote 18 Colonia Fracc. Lomas de Ocuiltzapotlan localidad Villa de Ocuiltzapotlan referencia Tabasco Mexico http://www.lacoperacha.org.mx	2016
Colombia	Fino de Aroma Colombia Premium 1	Newchem Srl, Via M.F. Quintiliano 30 20138 Milan, Italy http://www.newchem.it	2016
Sao Tomè	Superior Cacau Fino, good fermented	Satocao LDA -Morro Peixe, Distrito de Lobata São Tomé e Príncipe - CP 762 http://www.satocao.com	2016
Venezuela	Venezuela Superior fermented Carenero	Daarnhouwer & Co. B.V., Korte Hogendijk 18 1506 MA Zaandam, The Netherlands http://www.daarnhouwer.com/	2016
Ecuador	Ecuador ASS (Arriba Superior Selecto)	Domori S.r.l. - Via Pinerolo 72-74 10060 None (Torino), Italy	2016
Powder	Alkalized cocoa powder 22-24%	Gobino srl, Turin, Italy	

Table 3. Cocoa targeted analytes together with their experimental odor quality, odor threshold (ng/g orthonasal from oily matrix), I^T_S, Ti adopted for quantitation, and calibration range covered (absolute amount of analyte, ng); modified from ref [59].

Target analyte	Odor quality	OT (ng/g)	Exp I^T_S	Ti (*m/z*)	Range (ng)
3-Hydroxy-2-butanone	*Buttery*	800	1250	88	20-5000
2-Heptanol	*Fatty green*	263	1295	80	1-100
2,3,5-Trimethylpyrazine	*Nutty roasted*	290	1365	122	1-50
2-Ethyl-3,5(6)-dimethylpyrazine	*Nutty earthy*	57	1406	135	1-50
Ethyl octanoate	*Green, fruity*	16	1411	88	1-50
Benzaldehyde	*Almond*	350	1478	77	1-50
2-Methylpropanoic acid	*Cheesy*	190	1590	88	20-5000
3-Methylbutanoic acid	*Cheesy*	22	1641	87	20-5000
Ethyl phenyl acetate	*Honey-like*	650	1695	91	1-50
2-Phenylethyl acetate	*Flowery*	233	1767	104	1-50
Guaiacol	*Phenol*	16	1808	109	1-50
2-Phenylethanol	*Sweet, floral*	211	1857	91	1-50
					50-500
(E)-2-Phenyl-2-butenal	-	-	1955	115	1-50

OT – odor threshold; Exp I^T_S – experimental Linear Retention Indices; Ti, target ion.

Table 4. extended list of cocoa informative volatiles together with their experiments ITS, information for predicted FID relative response factors estimation and RRF values adopted for their quantitation; modified from ref [59].

Target analyte	Exp I^T_S	**MW**	**Formula**	n_C	n_H	n_O	n_{Arom}	n_N	**RRF**
Isoamyl acetate	1104	130.19	C7H14O2	7	14	2	0	0	0.63
2-Heptanone	1156	114.180	C7H14O2	7	14	1	0	0	0.76
Heptanal	1184	100.160	C6H12O	6	12	1	0	0	0.73
3-Hydroxy-2-butanone	1250	88.105	C4H8O2	4	8	2	0	0	0.46
2-Heptanol	1295	116.201	C7H16O	7	16	1	0	0	0.78
2-Ethyl-5-methylpyrazine	1353	122.171	C7H10N2	7	10	0	0	2	0.69
2-Nonanone	1360	142.242	C9H18O	9	18	1	0	0	0.81
2,3,5-Trimethylpyrazine (REF)	**1365**	**122.170**	**C7H10N2**	**7**	**10**	**0**	**0**	**2**	**0.69**
3-Ethyl-2,5-dimethylpyrazine	1406	136.198	C8H12N2	8	12	0	1	2	0.82
Ethyl octanoate	1411	172.268	C10H20O2	10	20	2	0	0	0.72
2-Ethyl-3,6-dimethylpyrazine	1425	136.198	C8H12N2	8	12	0	1	2	0.82
Benzaldehyde	1478	106.121	C7H6O	7	6	1	1	0	0.79
2-Methylpropanoic acid	1590	88.110	C4H8O2	4	8	2	0	0	0.46
γ-Butyrolactone	1574	86.090	C4H6O2	4	6	2	0	0	0.42
3-Methylbutanoic acid	1641	102.132	C5H10O2	5	10	2	0	0	0.53
Ethyl phenyl acetate	1695	164.204	C10H12O2	10	12	2	1	0	0.74
2-Phenylethyl acetate	1767	164.200	C10H12O2	10	12	2	1	0	0.74
Guaiacol	1808	124.140	C7H8O2	7	8	2	1	0	0.68
2-Phenylethanol	1857	122.160	C8H10O	8	10	1	1	0	0.84
(E)-2-Phenyl-2-butenal	1955	146.189	C10H10O	10	10	1	1	0	0.84
2-Acetyl pyrrole	1913	109.13	C6H7NO	6	7	1	0	1	0.58
Phenol	1955	94.11	C6H6O	6	6	1	1	0	0.79
Octanoic acid	2065	144.21	C8H16O	8	16	1	0	0	0.70
DDMP	2278	144.13	C6H8O4	6	8	4	0	0	0.35
Phenylacetic acid	2580	136.15	C8H8O2	8	8	2	0	0	0.58

Figure 1

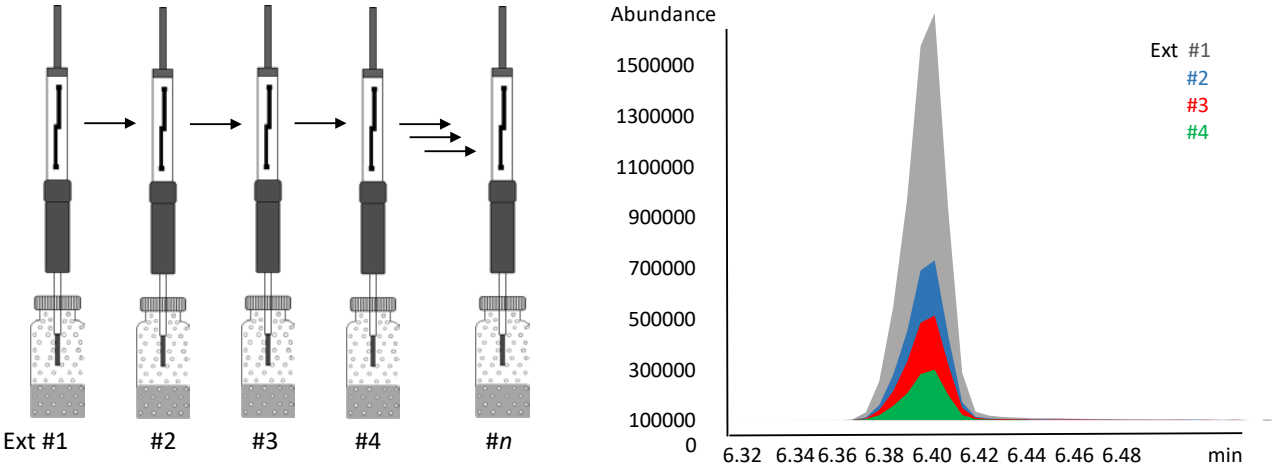

Ext #1
#2
#3
#4
#n
Abundance
1500000
1300000
1100000
900000
700000
500000
300000
100000
0
6.32
6.34
6.36
6.38
6.40
6.42
6.44
6.46
6.48
min
Ext #1
#2
#3
#4

Figure 2

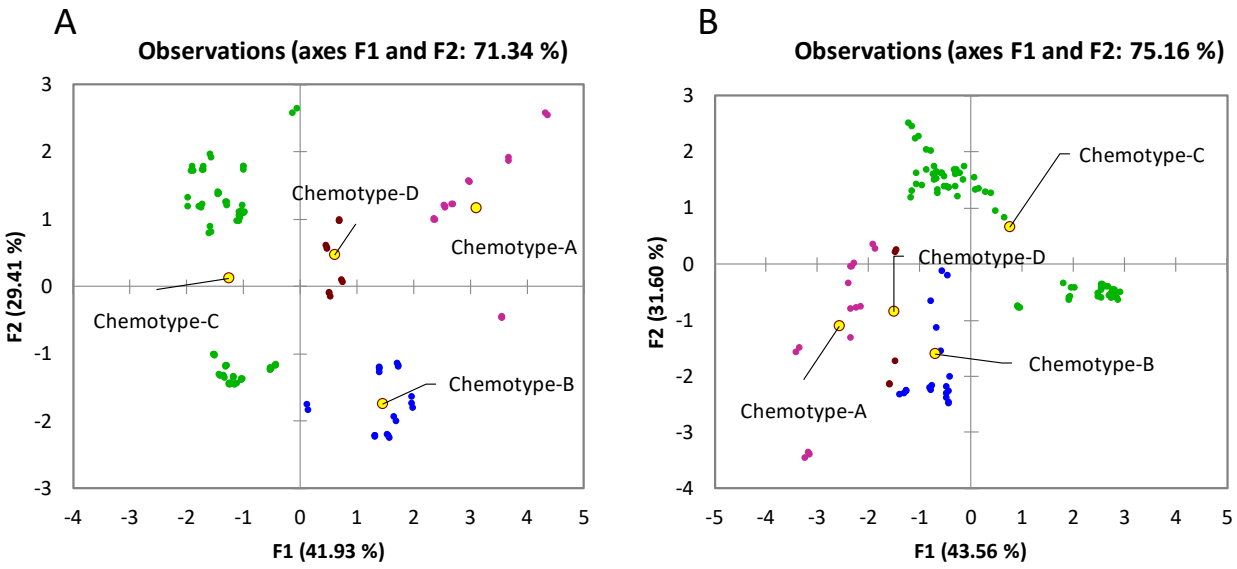

A
Observations (axes F1 and F2: 71.34 %)
Chemotype-D
Chemotype-A
Chemotype-C
Chemotype-B
F2 (29.41 %)
F1 (41.93 %)
B
Observations (axes F1 and F2: 75.16 %)
Chemotype-C
Chemotype-D
Chemotype-B
Chemotype-A
F2 (31.60 %)
F1 (43.56 %)

Figure 3

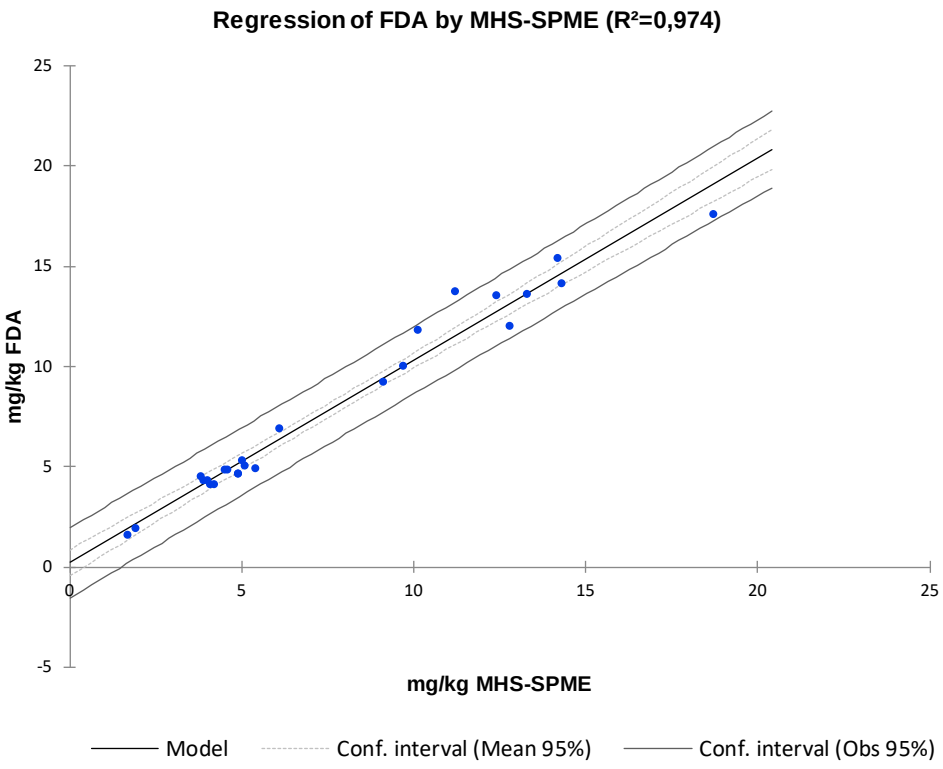

Regression of FDA by MHS-SPME (R²=0,974)
mg/kg FDA
mg/kg MHS-SPME
25
20
15
10
5
0
-5
0
5
10
15
20
25
Model
Conf. interval (Mean 95%)
Conf. interval (Obs 95%)

Figure 4

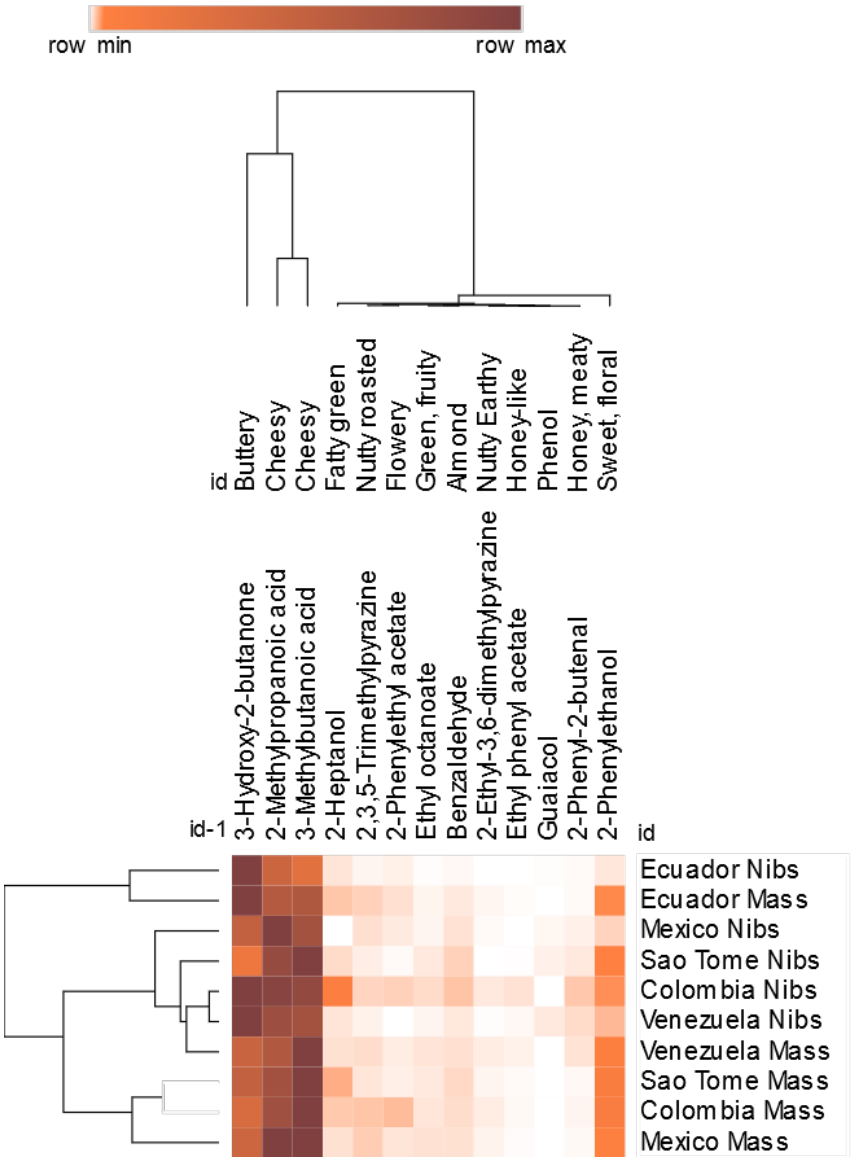

row min
row max
id
Buttery
Cheesy
Cheesy
Fatty green
Nutty roasted
Flowery
Green, fruity
Almond
Nutty Earthy
Honey-like
Phenol
Honey, meaty
Sweet, floral
id-1
3-Hydroxy-2-butanone
2-Methylpropanoic acid
3-Methylbutanoic acid
2-Heptanol
2,3,5-Trimethylpyrazine
2-Phenylethyl acetate
Ethyl octanoate
Benzaldehyde
2-Ethyl-3,6-dimethylpyrazine
Ethyl phenyl acetate
Guaiacol
2-Phenyl-2-butenal
2-Phenylethanol
id
Ecuador Nibs
Ecuador Mass
Mexico Nibs
Sao Tome Nibs
Colombia Nibs
Venezuela Nibs
Venezuela Mass
Sao Tome Mass
Colombia Mass
Mexico Mass

Figure 5

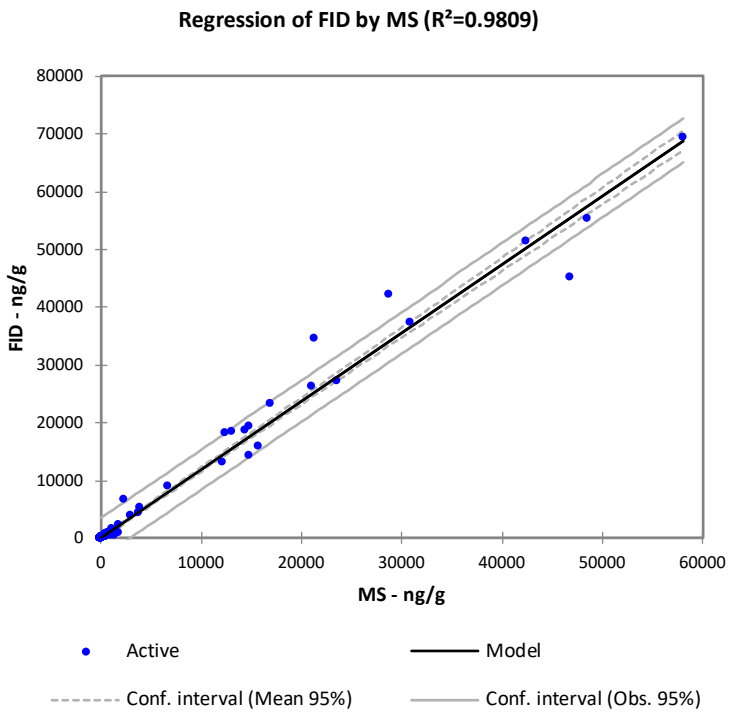

Regression of FID by MS (R^2=0.9809)
80000
70000
60000
50000
40000
30000
20000
10000
0
FID - ng/g
0
10000
20000
30000
40000
50000
60000
MS - ng/g
Active
Model
Conf. interval (Mean 95%)
Conf. interval (Obs. 95%)

Figure 6

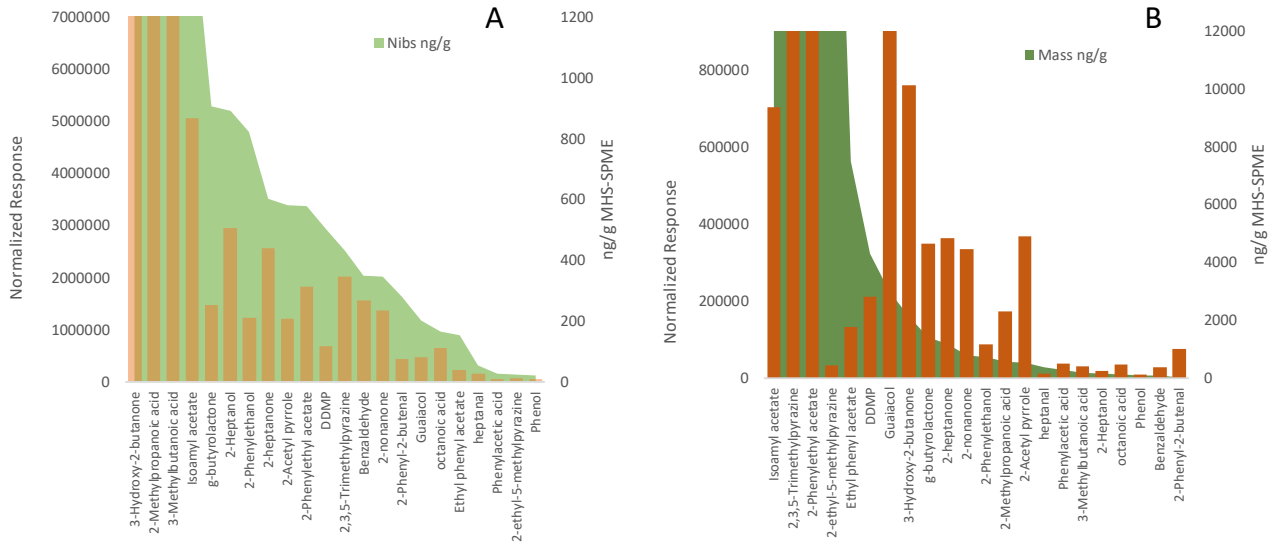
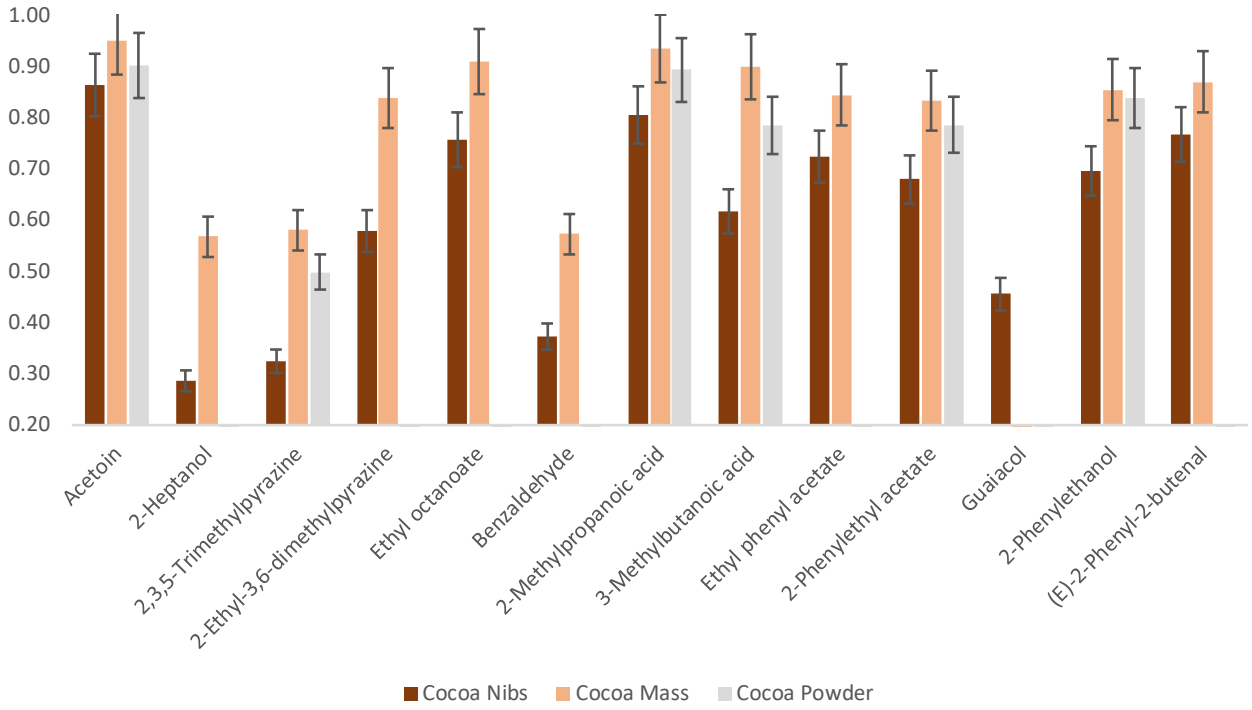
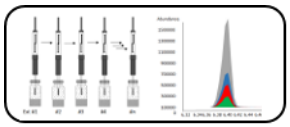

A
Nibs ng/g
Normalized Response
ng/g MHS-SPME
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
1200
1000
800
600
400
200
0
3-Hydroxy-2-butanone
2-Methylpropanoic acid
3-Methylbutanoic acid
Isoamyl acetate
g-butyrolactone
2-Heptanol
2-Phenylethanol
2-heptanone
2-Acetyl pyrrole
2-Phenylethyl acetate
DDMP
2,3,5-Trimethylpyrazine
Benzaldehyde
2-nonanone
2-Phenyl-2-butenal
Guaiacol
octanoic acid
Ethyl phenyl acetate
heptanal
Phenylacetic acid
2-ethyl-5-methylpyrazine
Phenol
B
Mass ng/g
Normalized Response
ng/g MHS-SPME
800000
600000
400000
200000
0
12000
10000
8000
6000
4000
2000
0
Isoamyl acetate
2,3,5-Trimethylpyrazine
2-Phenylethyl acetate
2-ethyl-5-methylpyrazine
Ethyl phenyl acetate
DDMP
Guaiacol
3-Hydroxy-2-butanone
g-butyrolactone
2-heptanone
2-nonanone
2-Phenylethanol
2-Methylpropanoic acid
2-Acetyl pyrrole
heptanal
Phenylacetic acid
3-Methylbutanoic acid
2-Heptanol
octanoic acid
Phenol
Benzaldehyde
2-Phenyl-2-butenal

Figure 7

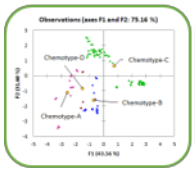

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
Acetoin
2-Heptanol
2,3,5-Trimethylpyrazine
2-Ethyl-3,6-dimethylpyrazine
Ethyl octanoate
Benzaldehyde
2-Methylpropanoic acid
3-Methylbutanoic acid
Ethyl phenyl acetate
2-Phenylethyl acetate
Guaiacol
2-Phenylethanol
(E)-2-Phenyl-2-butenal
Cocoa Nibs
Cocoa Mass
Cocoa Powder

For Table of Contents Only

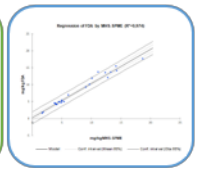


Headspace Quantitative Approaches

Chemotyping



Process contaminants



Cocoa - key-odorants