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# Odorants quantitation in high-quality cocoa by multiple headspace solid phase micro-extraction: adoption of FID-predicted response factors to extend method capabilities and information potential

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**Abbreviations**

DDMP – 2,3-dihydro-3,5-dihydroxy-6- methyl(4H)-pyran-4-one; GC-O – GC-olfactometry; HCC-HS – high concentration capacity headspace; HS – headspace; $I^T_S$ – linear retention indices; MHE – multiple headspace extraction; MHS-SPME – multiple headspace solid phase micro-extraction; OAV – odor activity value; RE – relative error; RRF – relative response factor; Ti – target ion; TMP – 2,3,5-trimethylpyrazine.

## Abstract

This paper focuses on several methodological aspects in the quantitation of volatiles in solid samples by headspace solid phase micro-extraction (HS-SPME) combined with gas chromatography and parallel detection by flame ionization detector and mass spectrometry (GC-FID/MS). Informative volatiles, including key odorants and process markers, from single-origin cocoa samples (Colombia, Ecuador, Mexico, Sao Tomè, and Venezuela) were captured at two processing stages along the chocolate production chain (nibs and cocoa mass). Accurate quantitation was achieved by multiple headspace extraction (MHE) in headspace linearity conditions and by external calibration. Quantitative results on selected analytes (3-hydroxy-2-butanone, 2-heptanol, 2,3,5-trimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine, ethyl octanoate, benzaldehyde, 2-methylpropionic acid, 3-methylbutyric acid, ethyl phenylacetate, 2-phenylethyl acetate, guaiacol, 2-phenylethanol, and (*E*)-2-phenyl-2-butenal) provided reliable information about the key sensory notes of cocoa intermediates (odor activity values) and their origin specificities. Additional information about analytes release by the solid environment (cocoa nibs, mass, and powders) was achieved by modeling decay curves. Parallel detection by MS and FID enabled quantitative cross-validation, and FID-predicted relative response factors (RRFs) extended method quantitation capabilities to additional compounds that were not subjected to an external calibration procedure: 3-methylbutyl acetate (isoamyl acetate), 2-heptanone, heptanal, 2-nonanone, γ-butyrolactone, octanoic acid, 2-ethyl-5(6)-methylpyrazine, phenylacetic acid, phenol, 2-acetyl pyrrole, and 2,3-dihydro-3,5-dihydroxy-6-methyl(4H)-pyran-4-one. This procedure extends method capabilities and information potential with great consistency.



## Keywords

multiple headspace solid phase micro-extraction; predicted FID relative response factors; gas chromatography with parallel detection by MS and FID; high-quality cocoa; key aroma compounds

## 1. Introduction

The number of volatiles that effectively contribute to the aroma of food, the so-called key odorants [1], is relatively small, and complex analytic procedures are required to detect, identify, and quantify odor-active components occurring at trace levels, in some cases below pg $g^{-1}$ [2]. Exhaustive classic approaches based on liquid-liquid extraction, or more effective processes such as solvent-assisted flavor evaporation, closely meet the needs of fundamental studies to isolate, identify, and quantify key odorants [3], but are not practicable in high-throughput studies on large sample sets [4]. Headspace (HS) sampling plays a crucial role in matching in full automation procedures or aroma profiling and fingerprinting. It enables volatiles, including potent odorants, to be recovered from the vapor phase, in equilibrium (or not) with the solid or liquid sample (phase), in a process guided by analyte-specific partition coefficients (*K*) [5]. Moreover, HS sampling is generally done online combined with GC-MS to enable effective quali-quantitative characterization.

Headspace recovery can be implemented by increasing its selectivity and sensitivity with high concentration capacity headspace (HCC-HS) techniques [6]. SPME is the most widely used HCC-HS technique, since it provides effective solutions for high-throughput sampling with full automation and flexibility because of the available commercial devices that combine different extraction sorbents/adsorbents [7]. In addition, HS-SPME is generally adopted for comparative evaluations in studies in which the aroma impact of a food [8] is not established by accurately quantifying potent odorants [9] or process indicators. The most common practice in volatile profiling is the cross-sample comparison in which analytes, and/or informative markers, are analyzed through relative quantitation indicators based on the chromatographic peak area percentage, the peak volume percentage for comprehensive two-dimensional GC (GC×GC), or internal standard (IS) normalization. Although accepted by the scientific community for several application fields, these approaches may result in inaccurate [10] and misleading findings if the aim is to correlate chemical composition with food sensory properties or manufacturing process kinetics.

This consideration is of special significance when a solid matrix is investigated [7,11–14]. Solid samples generally share a common characteristic: a heterogeneous composition and structure. Native analytes can be partitioned (absorbed) or adsorbed in terms of their physicochemical properties, sampling

74 temperature, and related conditions (absolute pressure, presence of additives or modifiers, etc.), making
75 the optimization of multianalyte quantitative methods and reliable quantitative comparisons difficult. This
76 situation is even more complex if the analytes of interest have widely different *K* values, e.g. the ratio of the
77 analyte concentration in the gas phase to that in the condensed phase (solid or liquid).

78 Moreover, HS-GC from solid samples is characterized by low recoveries, most frequently well under
79 1% [5,15]. Reproducible and accurate quantitative results can therefore be achieved only by properly
80 setting the sampling conditions and parameters after the matrix effect on the analytes of interest is known.
81 The matrix effect can be exploited by building calibration solutions in matrix-matched blank samples,
82 spiking the sample with known increments of analyte (i.e., standard addition method), "quenching" the
83 effect of the sample matrix by adding a suitable modifier, or adopting the multiple headspace extraction
84 (MHE) technique [5,15].

85 MHE was adopted to study air-to-water partition coefficients more accurately by overcoming the
86 matrix effect on the release of volatiles exerted by test cells and to overcome the need of calibration
87 solutions for highly-volatiles in studies aimed at defining partition coefficients [16,17]. MHE has therefore
88 demonstrated its advantages in a number of real-life applications. Packaging materials were the focus of a
89 study by Wenzl and Lankmayr [18], who examined the release of straight-chain saturated aldehydes and
90 mononuclear aromatics (benzene, toluene, xylenes, and ethylbenzene) in cellulose-based packaging. Frisell
91 [19] applied MHE-GC to the analysis of hexanal emissions from commercially available packaging board
92 products. Off odors from food contact materials and cork stoppers were investigated by Ezquerro et al.
93 [20–22], who also compared different HCC-HS approaches in view of achieving more accurate quantitative
94 determination from solids. Deng et al. [23] proposed direct quantitation of biogenic volatile organic
95 compounds (terpenoids) from the living leaf of *Pelargonium hortorum in situ*, and several other studies
96 targeted food volatiles, including aroma-active compounds in pasta [24], mushrooms [25], bread crust [26],
97 wines [27–30], coffee [31], roasted hazelnuts [4], and spices [32].

98 Most of the above-mentioned studies combined an HCC-HS technique, such as SPME, with GC-MS
99 separation efficiency and sensitivity; however, for accurate results, HS linearity conditions must be
100 achieved [5] during sampling. This means that the amount of sample under study should be enough to

release, under defined sampling conditions, the minimal amount of analyte to match the method sensitivity and precision while, at the same time, not saturating the HS. This condition is simple to achieve for trace and subtrace target analytes, but becomes challenging in profiling methods where the goal is multianalyte quantitation over a wide range of concentrations. In this context, another attractive possibility is the combination of highly efficient separation by GC with sensitive/specific detection by MS with electron impact ionization and parallel FID, which – thanks to a wider dynamic range of response and the applicability of response factor quantitation principles – extends method quantitation and information potential.

In the extremely challenging context of the present study, i.e. the complex fraction of volatiles from high-quality cocoa of different origins, we aimed to accurately quantify multiple *key aroma* compounds and potent odorants in process intermediates that show different matrix effects by using MHE-HS-SPME-GC with parallel detection by MS and FID. In addition, to extend this accurate quantitation to additional informative compounds not preliminarily calibrated by MHE, we explored the concept of predicted FID relative response factors (RRFs) [10] and cross-validated the quantitative results. We also considered additional information on analytes released from process intermediates as a consequence of the differential matrix effect exerted by solid particles in view of the role this effect plays in potent odorant release in the HS.

## 2. Experimental

### *2.1. Chemicals*

The following chemicals were from Sigma Aldrich (Milan, Italy): IS *n*-heptadecane (*n*-C17) for chromatographic response normalization; dibutyl phthalate and diethyl phthalate (99% of purity) as solvents for MHE calibration solutions and IS, acetone, and cyclohexane as dilution solvents; and *n*-alkanes (*n*-C9 to *n*-C25) for determination of linear retention indices ($I^T_S$).

The following key aroma compounds and potent odorants, selected according to the reference literature [2,33–35] and adopted for external calibration, were from Merck KGaA (Darmstadt, Germany): 3-hydroxy-2-butanone (CAS 513-86-0), 2-heptanol (CAS 543-49-7), 2,3,5-trimethylpyrazine (TMP) (CAS 14667-

55-1), 2-ethyl-3,6-dimethylpyrazine (CAS 27043-05-6), ethyl octanoate (CAS 106-32-1), benzaldehyde (CAS 100-52-7), 2-methylpropionic acid (CAS 79-31-2), 3-methylbutyric acid (CAS 503-74-2), ethyl phenylacetate (CAS 101-97-3), 2-phenylethyl acetate (CAS 103-45-7), guaiacol (CAS 90-05-1), and 2-phenylethanol (CAS 60-12-8). (*E*)-2-phenyl-2-butenal (CAS 54075-09-1) was provided by Firmenich SA (Geneva, Switzerland).

The following reference compounds, for identity confirmation in the predicted FID RRF extended quantitation, were from Merk KGaA (Darmstadt, Germany): 3-methylbutyl acetate (CAS 123-92-2), 2-heptanone (CAS 110-43-0), heptanal (CAS 111-71-7), 2-nonanone (CAS 821-55-6), γ-butyrolactone (CAS 96-48-0), octanoic acid (CAS 124-07-2), 2-ethyl-5(6)-methylpyrazine (CAS 36731-41-6), phenylacetic acid (CAS 103-82-2), 2-acetyl pyrrole (CAS 1072-83-9), and phenol (CAS 108-95-2).

### *2.2. Reference solutions and calibration standards*

Reference stock solutions for analytes subjected to external calibration, IS, and identity confirmation were prepared in acetone as solvent at a 10 g $L^{-1}$ concentration and stored at -18°C for a maximum of 4 weeks.

Solutions for external calibration by MHE-HS-SPME were prepared in diethyl phthalate or dibutyl phthalate by mixing suitable volumes of reference stock solutions. Calibration mixtures were stored in sealed vials, without available HS volume, at -18°C for a maximum of 4 weeks. Calibration solutions were prepared to match the following absolute amounts: 1, 5, 10, 20, 30, 50, 100, 200, 300, 500, 1000, 2000, 5000 ng.

An IS (*n*-heptadecane) working solution for the standard-in-fiber preloading procedure [11] was prepared at 100 mg $L^{-1}$ in diethyl phthalate and stored at -18°C in sealed vials without available HS volume.

### *2.3. Cocoa samples*

Cocoa samples and process intermediates, including some cocoa powders, were provided by Gobino srl (Turin, Italy). Samples were selected on the basis of their specific sensory profile from high-quality productions of different geographic origins. Roasting and refining to obtain cocoa mass were set to

achieve optimal flavor [36]. The list of samples, together with their origin, supplier and harvest year are reported in **Table 1**.

### *2.4. MHE by HS-SPME: sampling conditions*

Divinylbenzene/carboxen/polydimethyl siloxane 1 cm SPME fiber was obtained from Supelco (Bellefonte, PA, USA) and used for MHE-HS-SPME sampling. The standard in-fiber procedure [11] was adopted to preload the IS (*n*-heptadecane) onto the fiber before sampling. A 5.0 µL solution of IS (*n*-heptadecane at 100 mg $L^{-1}$ in diethyl phthalate) was placed into a 20 mL glass vial and subjected to HS-SPME at 50°C for 5 min. After the IS loading step, the SPME device was exposed to the calibration solutions or sample HS for 30 min at 50°C. Extracted analytes were recovered by thermal desorption of the fiber into the S/SL injection port of the GC system at 250°C for 5 min. MHEs from the same sample/calibration vial were conducted by applying the above protocol. The number of successive extractions was set at four to achieve an almost exhaustive extraction for the analytes under study.

### *2.5. GC coupled with parallel detection by MS and FID*

Automated MHE-HS-SPME was performed by using an MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany) installed on a GC-MS system consisting of an Agilent 7890B GC unit coupled to an Agilent 5977B HES (high efficiency source) fast quadrupole MS detector (Agilent Technologies, Little Falls, DE, USA) operating in electron ionization mode at 70 eV. The GC transfer line was set at 270°C. The MS was tuned by using the HES Tune option. The scan range was set to *m/z* 40-300 with a scanning rate of 2,500 amu $s^{-1}$.

We used a SolGel-Wax capillary column (100% polyethylene glycol; 30 m × 0.25 mm $d_c$, 0.25 µm $d_f$) from SGE Analytical Science (Ringwood, Australia). A non-purged “tee” splitter was installed post-column to diverge effluent from the separation column to the FID detector (0.4 m × 0.18 mm $d_c$) and to the MS (0.25 m × 0.1 mm $d_c$), resulting in a 1:1 split ratio.

SPME thermal desorption into the GC injector port was under the following conditions: split/splitless injector in pulsed splitless mode; pressure pulse of 35 kPa. The carrier gas was helium at a

constant flow of 1.5 mL $min^{-1}$. The oven temperature program was as follows: from 40°C (1 min) to 170°C at 3°C $min^{-1}$ and from 170°C to 240°C at 15°C $min^{-1}$ (5 min).

The *n*-alkanes liquid sample solution (50 mg $L^{-1}$ each) for $I^T_S$ calibration was analyzed under the following conditions: split/splitless injector in split mode, split ratio 1:50, injector temperature 250°C, injection volume 1 µL.

Data were acquired by Mass Hunter (Agilent Technologies). Statistical analysis was performed with XLSTAT (Addinsoft, New York, NY, USA).

### *2.6. External standard calibration by MHE-HS-SPME-GC-MS/FID*

Calibration curves were built to cover analyte amounts in the analyzed samples in a range of 1 to 5000 ng for a single odorant.

External standard calibration was done separately on MS total ion current traces by selecting, for each analyte, a specific target ion (Ti) and two qualifier ions for quality match evaluation and on FID by recording the chromatographic peak area for those analytes not affected by coelution issues. Details on the procedure are discussed in section 3.2.

**Table 2** reports the targeted analytes together with their experimental $I^T_S$, odor quality, odor threshold (ng $g^{-1}$ orthonasal from oily matrix) as reported in the literature [2,33,34,37,38], Ti adopted for quantitation, and calibration range covered (absolute amount of analyte, ng).

### *2.7. Basic calculations for accurate quantitation of real samples*

The quantitation of odorants by MHE required preliminary optimization on representative samples to select the amount of sample necessary to obtain HS linearity and good sensitivity for all target analytes. MHE was therefore carried out on 10-15 mg of cocoa nibs, 20-40 mg of cocoa mass, and 50-100 mg of cocoa powders. Optimal amounts were defined on the basis of the achieved exponential decay for all targeted analytes and were as follows: 15 mg nibs; 40 mg mass, and 50 mg powder.

## 3. Results and discussion

### *3.1. Cocoa volatiles and their information potential: aroma and technological markers*

*Theobroma cacao* L. is a tree crop native to tropical forests of the American continent; nowadays, however, most of the world's cocoa is produced in West Africa (Ivory Coast and Ghana), followed by tropical areas of Central and South America and Southern Asia. Several functional variables influence cocoa quality, above all, genotype [39], geographic area of harvest [40], farming practices [42–44], and processing [44–48]. On the other hand, the sensory quality of cocoa (aroma, taste, mouthfeel, and texture) is the key factor in producing premium products that meet consumer preference. Analytic efforts at quality control should therefore be directed to achieving a good understanding of cocoa flavor potential from a market perspective.

Several hundreds of volatiles have been identified in the cocoa volatile fraction [36,40,46,47,49], including potent odorants whose specific distribution provides the characteristic aroma signature, or *aroma blueprint* [50]. The molecular sensory science approach, now called *sensomics*, has characterized the aroma blueprint of different cocoa and chocolate products [2,33,34] by adopting a workflow that includes (a) analyte extraction and isolation, (b) extract concentration, (c) pre-separation and fractionation of extracts to reduce sample dimensionality [51], (d) chromatographic separation and location of odor active compounds by GC-olfactometry (GC-O), (e) identification of odorants by combining retention data with MS fragmentation patterns and odor quality information, (f) accurate quantitation by stable isotope dilution assays, and (g) validation of aroma contributions by recombination and omission experiments (study of possible synergies) [52]. In this procedure, the accurate quantitation of odorants is fundamental, since it enables the objective evaluation of the role played by single odorants. From molecular sensory science principles, those odorants that exceed the odor threshold concentration in the sample, resulting in an odor activity value (OAV) of > 1, are key aromas [1].

When the objective of the investigation is much broader, including the entire volatile metabolome as the informative fraction of the sample's functional characteristics (origin/phenotype, harvest and climate conditions, post-harvest practices, processing), high-throughput profiling is desirable, if not mandatory. Full

234 automation and minimal sample preparation allow large sample sets/batches to be screened while
235 achieving adequate results of representativeness and consistency.
236 In the present study, a quantitative profiling approach based on HS-SPME-GC-MS/FID was adopted
237 to investigate the accurate quantitation of several potent odorants, including some key aroma compounds
238 validated by previous studies [2,33,34] and process indicators, with the flexibility to extend quantitative
239 measurements to uncalibrated analytes based on the concept of FID RRFs. Thanks to the key features of the
240 MHE approach, accurate quantitative results are achievable with few analyses per sample while allowing
241 the retrieval of additional information on the sample matrix effect, which is of considerable value in
242 assessing the release of odorants [4]. The parallel detection by MS/FID provides complementary
243 information, including analyte identity (MS fragmentation signature) and the amount of analytes from
244 specific ion abundances (MS target ions – Ti profiles) or the FID response. The latter has been
245 demonstrated to be correlated with combustion enthalpies and molecular formulae, enabling quantitation
246 without external standards. Principles and details of the adoption of FID RRFs are discussed in section 3.4.
247
248 ***3.2. Quantitation of key aroma compounds and potent odorants from cocoa intermediates***
249 The selection of analytes for quantitative experiments was guided by careful evaluation of the
250 reference literature combined with GC-O experiments performed on cocoa nibs and cocoa mass
251 intermediates [53] for potent odorants.
252 Key aroma compounds described by Schieberle and co-workers [2,33,34] include alkyl pyrazines
253 (TMP, 2-ethyl-3,5-dimethylpyrazine, and 3,5-diethyl-2-methylpyrazine), which impart characteristic *earthy,*
254 *roasted* notes, and short-chain and branched fatty acids (acetic acid, butanoic acid, 2-methylpropanoic
255 acid, and 3-methylbutanoic acid), whose presence, at high concentrations, can impart off flavors from their
256 *rancid*, *sour*, and *sweaty* notes. Strecker aldehydes (2- and 3-methylbutanal), formed during fermentation
257 and roasting, impress *malty, cocoa* and *buttery* notes, and phenylacetaldehyde, derived from L-
258 phenylalanine, is responsible for a pleasant flowery *honey-like* note. Other key analytes are esters (ethyl-2-
259 methylbutanoate – *fruity*; 2-phenylethyl acetate – *flowery*; ethyl phenylacetate – *honey like*), linear alcohols
260 (2-heptanol – *green, fatty*), phenyl propanoid derivatives (2-phenylethanol – *flowery*), sulfur-derived

compounds (dimethyl trisulfide - *sulfury*), and phenols (guaiacol – *phenolic*). This preliminary list was implemented from analytes that contributed to additional sensory notes according to the literature [54–56] or GC-O experiments [55], or because of their informative role in the evolution of volatiles along processing steps [36]. These analytes are benzaldehyde (*almond like*), 3-hydroxy-2-butanone/acetoin (*buttery*), and ethyl octanoate (*green, fruity*); (E)-2-phenyl-2-butenal was discriminant for processing stage.

An external standard calibration strategy was chosen to approach multianalyte quantitation by MHE. It consists of three experimental steps:

*Step 1.* Exhaustive extraction of targeted analytes from reference calibration solutions within a range of absolute analyte amounts, matching real concentrations in real samples.

*Step 2.* Exhaustive extraction of targeted analytes from representative samples (cocoa nibs and mass) to define suitable conditions for HS linearity.

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

The first two steps aimed to define the cumulative instrumental response function through a series of repeated consecutive extractions from the HS of appropriate amounts of the same aliquot of calibration solutions or representative samples, up to complete (exhaustive) targeted analyte extraction from the sample. Preliminary experiments would require up to four to six consecutive extractions to validate the exhaustiveness of the extraction process for all targets.

In practice, the analyte chromatographic peak area decreases exponentially with the number of consecutive extractions, while the partition coefficient ($K$) between the condensed phase (matrix-solid sample) and the HS remains constant, provided that HS linearity is achieved [4,5]. HS linearity is a fundamental condition to achieve accurate quantitative results in any HS application. This condition refers to the linear function between the analyte concentration in the sample ($C_0$) and its concentration in the HS ($C_G$), or between $C_0$ and the chromatographic peak area ($A$) obtained when analyzing an aliquot of the HS. The actual linear range depends on the analyte's solubility (i.e. its partition coefficient) and its activity coefficient. It generally spans concentrations between 0.1 and 1% in the sample; higher sensitivity can be achieved by modifying sampling temperature, equilibration time, and the ratio between the HS ($V_G$) and the condensed phase volume ($V_C$) by exploiting, as already discussed, HCC-HS approaches such as SPME with

single or multipolymer extraction phases [57]. Note that the actual linear range of a given analyte in HS-GC cannot be predicted – it must be determined by experimental measurements [5]. Non-linearity due to adsorption on containers walls have not been taken into account in this study.

The sum of the *A*s from each extraction step corresponds to the total area ($A_T$) of the analyte originally present in the matrix. **Equation 1** is applied to obtain the cumulative instrumental response ($A_T$):

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

where $A_T$ is the total estimated area, $A_1$ is the area detected after the first extraction, and $q$ is a constant associated with the exponential decay (β) of the chromatographic peak area with consecutive extractions. **Figure 1** shows the procedural steps corresponding to the exhaustive extraction of an analyte from a sample by HS-SPME.

**Please insert Figure 1 here**

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 2**) can be calculated as follows:

$$\ln A_i = a\,(i\text{-}1) + b \qquad \textbf{Eq. 2}$$

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 dependent. It is generally constant in samples showing comparable matrix effects [31,58], thereby indicating the extent of the decay across successive extractions while confirming, or not, the HS linearity condition. In addition, its dependence on $K$ offers additional information on matrix behavior and the release of the target analyte in specified conditions. Further details of this aspect are discussed in section 3.3.

312 The multiple extraction procedure, when applied to calibration mixtures at different known
313 concentrations, provides experimental data for external calibration curves. Calibration curves can be used
314 to estimate the analyte amount in the sample with a simplified procedure, where the target analyte
315 chromatographic area ($A_1$) is sufficient for accurate (at given conditions) quantitation in the sample [59].
316 Calibration curves were built to cover the analyte concentration range expected in real samples.
317 **Table 2** reports, for targeted odorants, the calibration range covered (absolute amount of analyte, ng), the
318 calibration function accompanied by its coefficient of determination ($R^2$), and the characteristic RSD%
319 obtained by replicated quantitative measurements of a representative sample and based on MS and FID
320 signals. 2-Phenylethanol required a two-step calibration procedure to match the response linearity of the
321 MS.
322 Note that no reference material was available to validate method trueness [60]; however, based on
323 previous research, the standard addition method on solid samples gave less precise and accurate results
324 than MHE did [4], while the stable isotope dilution assay was not considered because of the commercial
325 unavailability of most of the target analytes. Accuracy was validated by internal cross-matching of MS and
326 FID data (see section 3.4).
327 Calibration curves, based on Ti normalized responses (over *n*-C17 IS), showed good linearity ($R^2$ on
328 average 0.995), and in some cases covered a calibration range of two orders of magnitude, resulting in
329 good method flexibility. Highly volatile analytes, such as 3-hydroxy-2-butanone, have higher imprecision
330 (RSD% 10.1), although this value is still below the limit of acceptability [60].
331 Quantitative results based on MS external calibration are visualized as a heatmap in **Figure 2A** and
332 relate to the set of five cocoa origins for which nibs and cocoa mass were selected for this study. Results,
333 rendered in a relative color scale (white to brown), correspond to the mean value of three replicated
334 measurements from two sample batches. **Supplementary Table 1** reports numerical data, together with
335 uncertainty calculated from intermediate method precision combined with standard calibration error.
336 Hierarchical clustering (HC) based on Euclidean distances facilitates the visualization of results by closely
337 clustering 3-hydroxy-2-butanone, 2-methylpropanoic, and 3-methylbutanoic acids that dominate the others

in absolute amounts. Quantitative data are in line with previous research from Frauendorfer and Schieberle [2,34], although it refers to samples from a different cultivar, i.e. Criollo, not explored in this study.

**Please insert Figure 2 here**

A more realistic picture on the role played by quantitated analytes in terms of sensory contribution to the overall perception is given by the OAV. It is computed as the coefficient of the concentration of a volatile component (e.g. μg $kg^{-1}$) vs. its odor threshold (e.g. μg $kg^{-1}$) in a defined sample. It is a useful parameter for separating an odorant from interfering components. An OAV of 1 is frequently used as a threshold value, although several more parameters need to be considered to judge the odor activity of volatile components [61]. **Figure 2B** illustrates, as a heatmap, the distribution of OAVs for quantitated analytes in the sample set.

From OAV data, the preeminent role of *cheesy* and *buttery* analytes was confirmed. They on average exceed the odor threshold by two to three orders of magnitude, while some pleasant odorants responsible for the *honey-like* (ethyl phenyl acetate), *flowery* (2-phenylethyl acetate), and *sweet-floral* (2-phenylethanol) notes have characteristic trends in nibs and mass samples. HC shows a clear distinction between nibs and mass, suggesting that technological processing plays a role in modulating the quantitative distribution of odorants in the final sample.

This aspect, confirmed by sensory evaluation of samples (data not shown), is further complicated by the release of odorants from the solid matrix. Release can be evaluated by comparing decay curves with the β parameter. In section 3.3, we illustrate the information potential of β, together with practical aspects related to MHE quantitation in real samples.

### *3.3. Matrix effect and release of odorants*

The β averaged value, reported for calibrated analytes in **Table 3**, when determined on a significant number of samples with similar chemical-physical characteristics and texture, enables one-step quantitation by MHE [62]. This is a great advantage in this approach and compensates for the undoubtedly

time-consuming operation of multiple extractions (three to four) from calibration solutions and model samples. On the other hand, the routine application of the standard addition approach, which could be considered an alternative method, requires at least three to four successive analyses of the same sample spiked with known amounts of targeted analytes to build a calibration curve suitable for extrapolating accurate quantitative data [4].

In the present study, analytes β values from all analyzed samples were recorded in Step 2 of the method and their RSD% calculated to evaluate matrix effect homogeneity for cocoa intermediates and to compare, in quantitative terms, the differential release of the odorants from nibs and mass.

Averaged β values ± RSD% associated with nibs and mass are shown in the histogram in **Figure 3**. Results confirm that the matrix effect is independent of cocoa variety and roasting conditions but, as expected, is greatly influenced by the physical properties of the matrix. Cocoa mass, in fact, shows stronger retention of analytes, probably because of the homogeneous dispersion of fat and solid particles obtained during the refining process. The higher β values observed for cocoa mass affect HS composition; although the concentration of odorants in the cocoa mass is generally higher, their relative distribution in the HS may be misleading by suggesting the presence of lower amounts of targeted analytes. **Figure 4** illustrates the differential release of TMP from cocoa intermediates (mass and nibs) compared with its release from cocoa powder: note that a single cocoa powder sample was considered in a comparative example of the release of volatiles.

**Please insert Figure 3 here**

β could therefore add information about the complex phenomenon of aroma perception during food consumption. Independently of their absolute concentration in the sample, odorants are differentially released into the oral cavity, thereby resulting in different perceptions in terms of aroma intensity.

**Please insert Figure 4 here**

### *3.4. Extending the quantitation to noncalibrated analytes by predicted FID response factors*

Complex volatile fractions such as those from roasted matrices [63–66] show a high number of potentially informative components; the possibility of extending the quantitation potential of the analytical method is attractive and of great help for data transferability and long-range studies where different GC platforms could be adopted. On the other hand, most of the validated targeted quantitative methods for aroma compounds are based on MS detection [9]. MS performances satisfy the minimal required sensitivity for aroma compounds that are sometimes present in food at sub-mg $kg^{-1}$ levels [1] and help to overcome coelution issues by selecting specific ion traces for accurate quantitation in the presence of interferents.

In this scenario, the possibility of extending method quantitation to a larger number of analytes without the need for single analyte calibration is practicable only if parallel detection by MS and FID is implemented. FID RRFs based on combustion enthalpies and molecular structure extend quantitation to all reliably identified analytes in a sample, as long as they are not coeluted with interfering compounds [10].

Predicted FID RRFs were validated for GC-FID, GC×GC-FID, and GC×2GC-MS/FID applications by quantifying model mixtures of interest in the fragrance field [67–69]. The alignment of the separation profiles obtained with two parallel detectors allows unified consideration of the results, enables cross-validation of results, and extends quantitative capabilities of the method to uncalibrated compounds.

The principle at the basis of the applicability of FID RRFs to the MHE approach is related to the fact that in HS linearity conditions, the characteristic β value enables one to predict analyte $A_T$, which corresponds to the actual absolute amount of that analyte in the sample. For liquid injections, the area ratio between the targeted compound and the IS added to the sample can be normalized/corrected to the RRF estimated from the molecular formula, and its relative amount can be estimated with great accuracy [10,70].

The reference equation (**Equation 3**) to calculate analyte RRFs is as follows:

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

where $n_C$, $n_H$, $n_O$, $n_N$, $n_S$, $n_F$, $n_{Cl}$, $n_{Br}$, $n_I$, and $n_{benz}$ are the number of carbon, hydrogen, oxygen, nitrogen, sulfur, fluorine, chlorine, bromine, and iodine atoms and the number of benzene rings, respectively. $MW_i$

and $MW_{IS}$ are the molecular weights of the analyte *i* and the IS (methyl octanoate) adopted for the development of the model by de Saint Laumer et al. [10].

The analyte-specific RRF was here corrected to the TMP/methyl octanoate ratio (i.e. $RRF_{i,TMP}=0.7028/RRF_{i,methyl\ octanoate}$) to adapt the model to TMP; note that the IS adopted for MS quantitation, i.e. *n*-heptadecane, was affected by coelution on the FID trace and so was not considered for response normalization.

**Table 3** reports the RRF values calculated for all calibrated analytes and for the additional compounds of interest selected from the volatiles detected by the HS-SPME-GC-MS/FID method: 3-methylbutyl acetate (isoamyl acetate), 2-heptanone, heptanal, 2-nonanone, γ-butyrolactone, octanoic acid, 2-ethyl-5(6)-methylpyrazine, phenylacetic acid, phenol, 2-acetyl pyrrole, and 2,3-dihydro-3,5-dihydroxy-6-methyl(4H)-pyran-4-one (DDMP). Within this extended list are some potent odorants: phenylacetic acid is a key aroma in Criollo cocoa [34] responsible for *honey-like* notes, isoamyl acetate has a *banana-like* odor, octanoic acid has *sweaty* notes, and phenol contributes to the *phenolic* note in some cocoa origins. The 2-ethyl-5-methylpyrazine has a *roasty-nutty* aroma, 2-acetyl pyrrole a *musty* odor, and γ-butyrolactone a *creamy* note. Other analytes (2-heptanone, 2-nonanone, and heptanal) are informative of fat oxidation being a product of fatty acid hydroperoxide degradation, and DDMP was found to be informative of the cocoa processing stage [36].

To validate the consistency and accuracy of RRF quantitation, the quantitative results obtained by applying MHE to the MS traces were compared with those estimated by RRFs. TMP was chosen as the reference compound for peak area normalization on the FID trace. Changing the internal standard will increase the inaccuracy on the RRF but in the study the global accuracy is recorded as relative error % (RE%), with MS as the reference method. RE% was thus calculated as follows (**Equation 5**):

$$RE\% = (M_{m\ RRF} - M_{mMS})/\ M_{mMS} \times 100 \qquad \textbf{Eq. 5}$$

where $M_{m\ RRF}$ is the analyte estimated amount in the sample based on RRF, and $M_{mMS}$ is the analyte estimated amount in the sample based on MS external calibration.

**Table 3** reports the RE% for calibration mixtures at 50 and 20 ng. The accuracy of the results is good, with RE% never exceeding ± 20%, except for acetoin (3-hydroxy-2-butanone), which was

overestimated by 24%. This analyte was also affected by a higher calibration error of MS. By extending accuracy evaluation of the cocoa samples, MS peak purity was considered to verify coelutions; analytes not affected by coelution issues were also quantitated by RRF. Accuracy is shown in the regression graph in **Figure 5A**, where all quantified analytes are computed together. Regression results show good correlation between detectors, while validation (**Figure 5B**), performed randomly on 15 points of quantitation, indicates good concordance (accuracy) of data.

**Please insert Figure 5 here**

To extend the quantitation potential to the extended list of analytes, RRF values were calculated from molecular weight and formula; the total chromatographic peak area ($A_T$) was indeed estimated by recording peak areas from four consecutive extractions of the same sample and calculating the characteristic β value for each analyte; data (±RSD%) are reported in **Table 3**. Quantitative results, combined with those from calibrated analytes, are visualized as a heatmap in **Figure 6A**, while numerical data for additional analytes, together with relative uncertainty, are provided in **Supplementary Table 2**.

**Please insert Figure 6 here**

HC based on the new data matrix, which includes additional odorants and marker compounds, confirms previous observations: the homogeneous composition of cocoa mass (Venezuela, Sao Tomè, Colombia, and Mexico) vs. nibs dominates sample clustering (**Figure 6A**), while key odorants such as 2-methylpropanic acid, 3-methylbutanoic acid, acetoin, and 2-phenylethanol have a homogeneous trend in all samples. Interestingly, other potent odorants such as isoamyl acetate, γ-butyrolactone, and 2-acetyl pyrrole follow a quantitative distribution that is congruent with key aroma compounds. As expected, DDMP is an effective marker of processing: its concentration in cocoa mass is, on average, two orders of magnitude higher than in cocoa nibs.

## 4. Conclusions

MHE combined with HS-SPME enrichment of cocoa solid samples represents a valid complement to classic extraction approaches for the accurate quantitation of a selection of key aroma compounds, potent odorants, and informative volatiles. When GC separation is followed by parallel detection with MS and FID, quantitation can be performed with high selectivity, specificity, and lower detection limits through selected ion traces (Ti and qualifiers) on total ion current data; on the other hand, for analytes that achieve FID detection limits and have good chromatographic resolution (e.g. not affected by coelution issues), RRFs can effectively be applied to extend the quantitation potential of the analytic method without the need for external calibration.

The MHE-HS-SPME approach also enables the evaluation of volatile release kinetics, which represents a valuable parameter for a better understanding of complex samples sensory features where the matrix effect affects HS composition.

Results highlight the relevance, in terms of data representativeness, of HS sampling parameter optimization and of how the matrix effect can affect HS composition, leading to erroneous considerations when normalized indicators, based on chromatographic response, are used. **Figure 6B** shows a heatmap rendering of the relative distribution of analytes considered. As clearly indicated by HC based on Euclidean distances, the normalized response variations (**Figure 6B**) compared with absolute analyte amounts (**Figure 6A**) lead to apparently different conclusions about the compositional similarity-dissimilarity of the samples.

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703

## Figure Captions

**Figure 1:** Procedural steps corresponding to the exhaustive extraction of an analyte from a sample by HS-SPME.

**Figure 2:** (**2A**) Heatmap based on MS external calibration quantitative results. Concentrations (ng/g), rendered in a relative color scale (white to brown), correspond to the mean value of three replicated measurements from two sample batches. (**2B**) Heatmap based on Odor Activity Values calculated on the basis of odor thresholds listed in reference literature. Hierarchical clustering (HC) is based on Euclidean distances after data normalization by Z-score.

**Figure 3:** histograms showing the averaged β values (± RSD%) associated with nibs and mass for selected odorants.

**Figure 4:** differential release of TMP from cocoa intermediates (mass and nibs) compared with its release from cocoa powder. β values (± RSD%) are those calculated on the entire sample set; for cocoa powder a single sample was considered as comparative example.

**Figure 5**: regression graph (**5A**) computing the quantitation results obtained for all analytes by MS (external calibration) and FID (FID-predicted response factors). Validation performed on 15 points of quantitation (5B) refers of good concordance (accuracy) of data (i.e., $R^2$ 0.9809).

**Figure 6**: (**6A**) Heatmap based on FID-predicted response factors quantitative results on the extended list of odorants. Concentrations (ng/g), rendered in a relative color scale (white to brown), correspond to the mean value of three replicated measurements from two sample batches. (**2B**) Heatmap based on normalized responses (normalized chromatographic areas). Hierarchical clustering (HC) is based on Euclidean distances after data normalization by Z-score.

## Table Captions:

**Table 1:** Cocoa samples under study, together with their origin, supplier and harvest year.

**Table 2**: Targeted odorants together with their experimental $I^T_S$, odor quality, odor threshold (ng $g^{-1}$ orthonasal from oily matrix) as reported in the literature [2,33,34,37,38], Ti adopted for quantitation, and calibration range covered (absolute amount of analyte, ng).

**Table 3**: extended list of targeted analytes including potent odorants and technological markers. Analytes are reported together with their experimental $I^T_S$, molecular weight (MW) and formula. Relative Response Factors (RFF) are calculated on the basis of Eq. 3. Accuracy data is reported as Relative Error (RE%) and calculated on calibration solutions at 20 and 50 ng. β values (± RSD%) are calculated on the entire sample set.

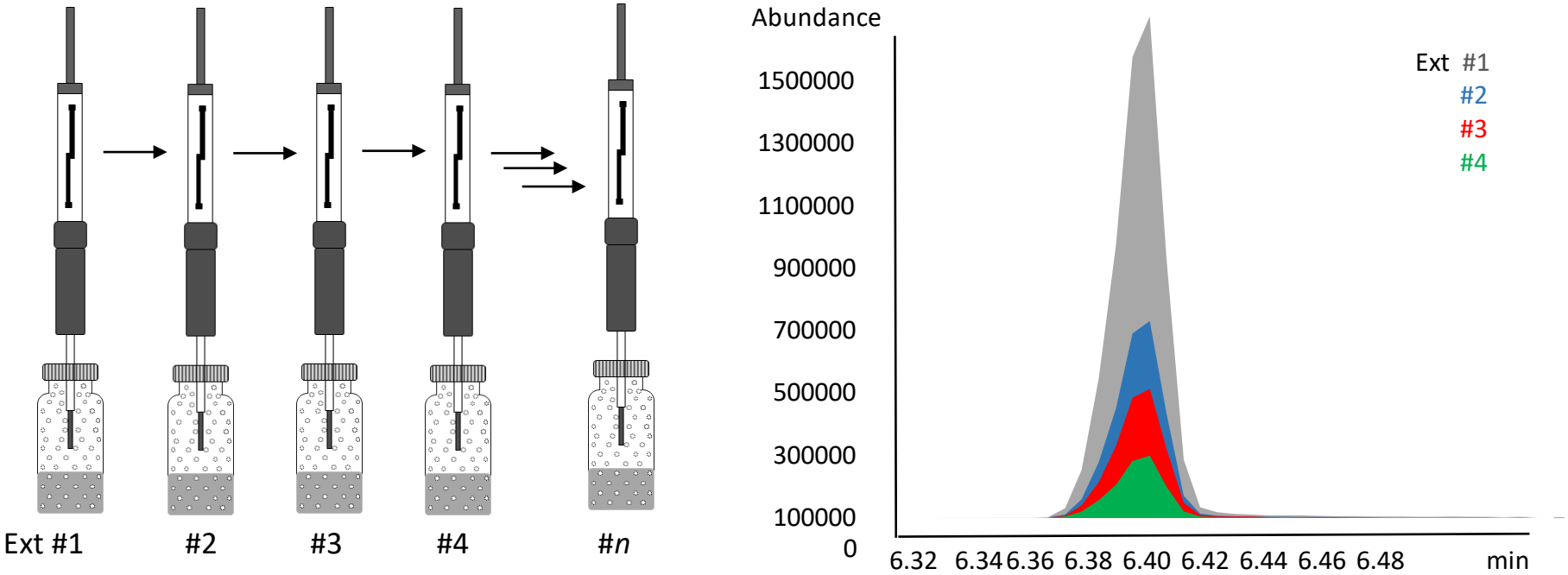
## Captions to Supplementary Tables:

**Supplementary Table 1**: quantitative data based on selected potent odorants and MHE with external calibration on MS signal. The relative uncertainty (Unc.%) is calculated from intermediate method precision combined with standard calibration error.

**Supplementary Table 2:** quantitative data referred to the extended list of analytes obtained by MHE with FID-predicted response factors principle. The relative uncertainty (Unc.%) is calculated from intermediate method precision.

745

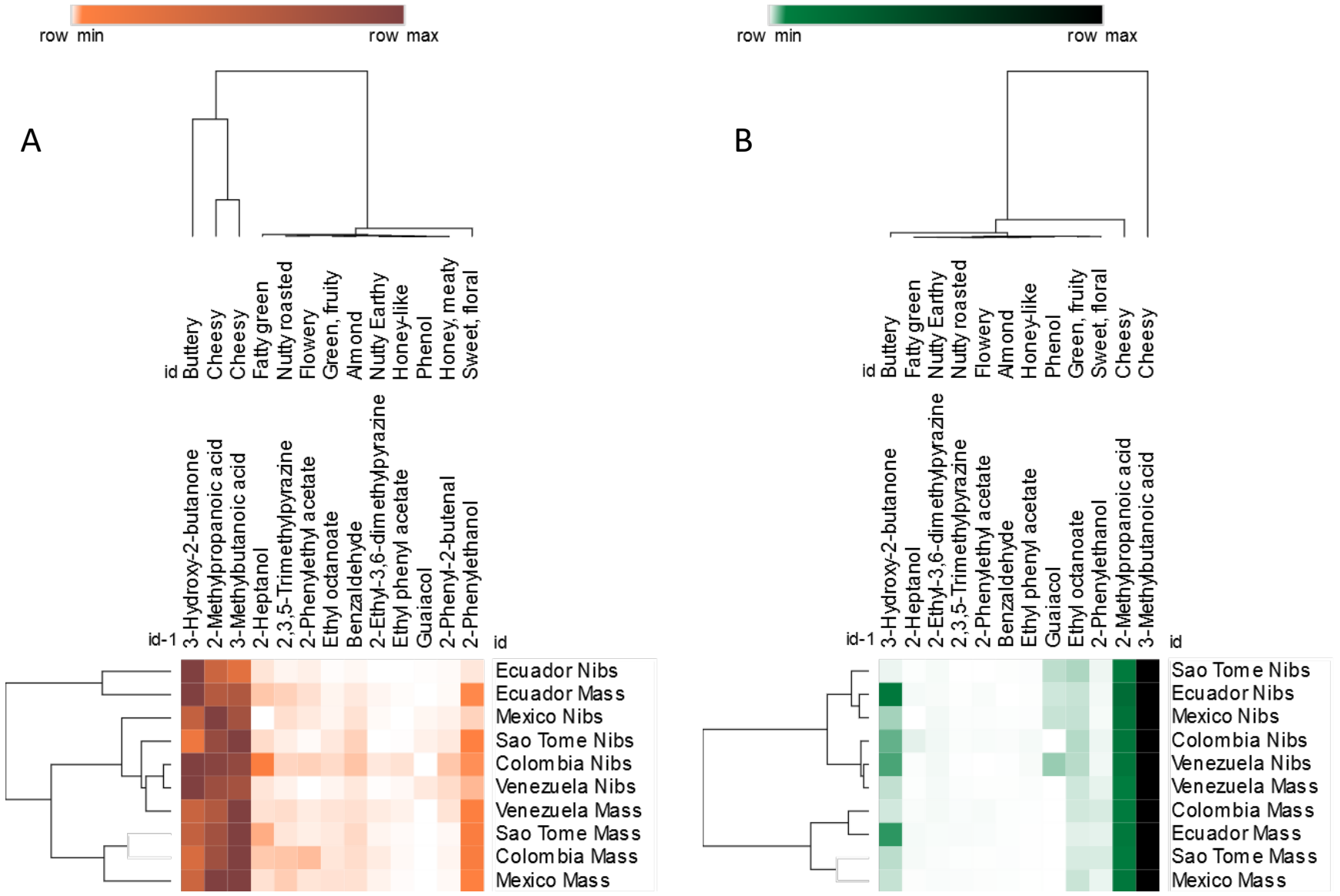
746 **Figure 1**




747

748 **Figure 2**

749




750

751

**Figure 3**

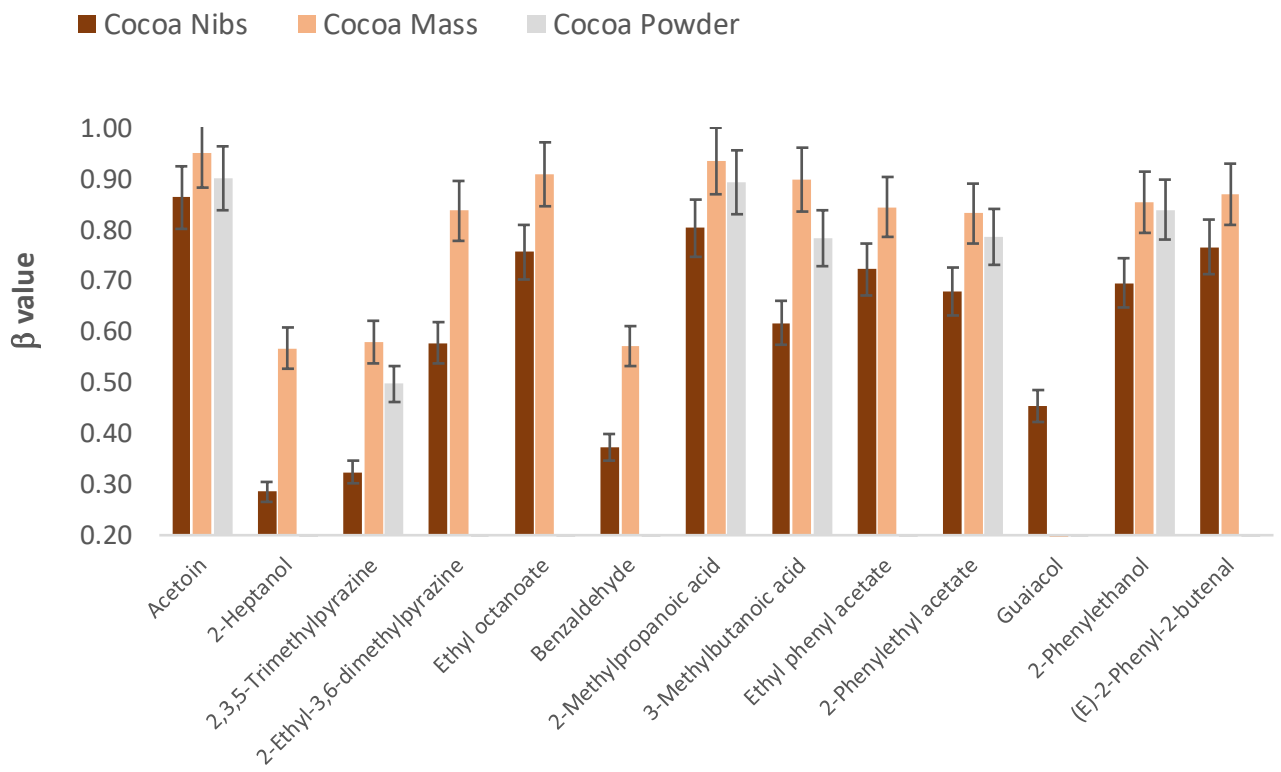

Cocoa Nibs
Cocoa Mass
Cocoa Powder
β value
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
Acetoin
2-Heptanol
2,3,5-Trimethylpyrazine
2-Ethyl-3,6-dimethylpyrazine
Ethyl octanoate
Benzaldehyde
2-Methylpropanoic acid
3-Methylbutanoic acid
Ethyl phenyl acetate
2-Phenylethyl acetate
Guaiacol
2-Phenylethanol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

**Figure 4**

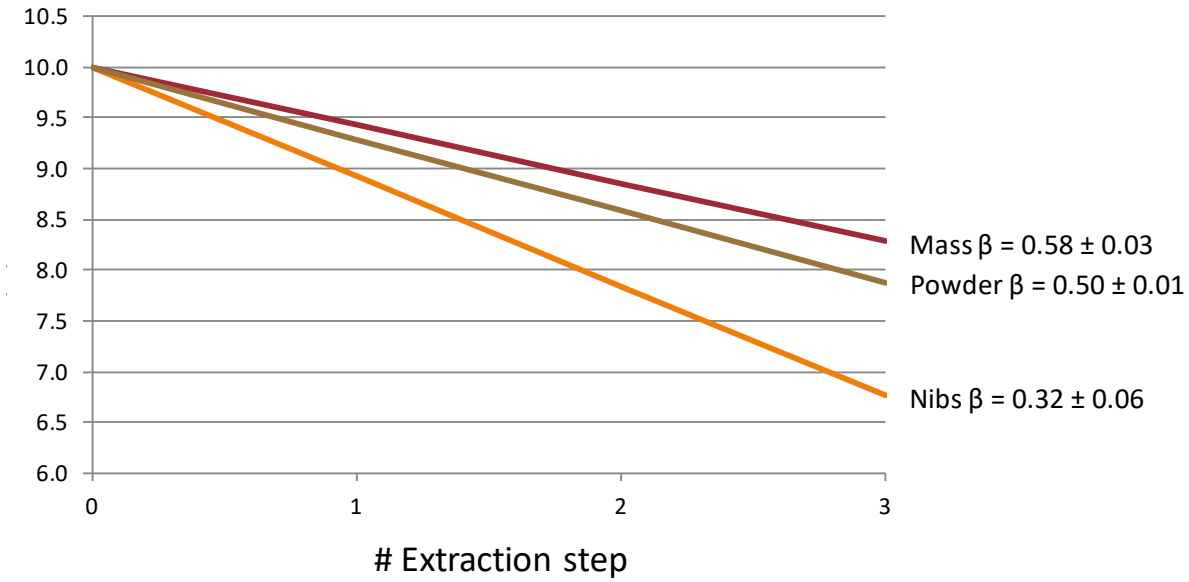

10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
0
1
2
3
# Extraction step
Mass β = 0.58 ± 0.03
Powder β = 0.50 ± 0.01
Nibs β = 0.32 ± 0.06

**Figure 5**

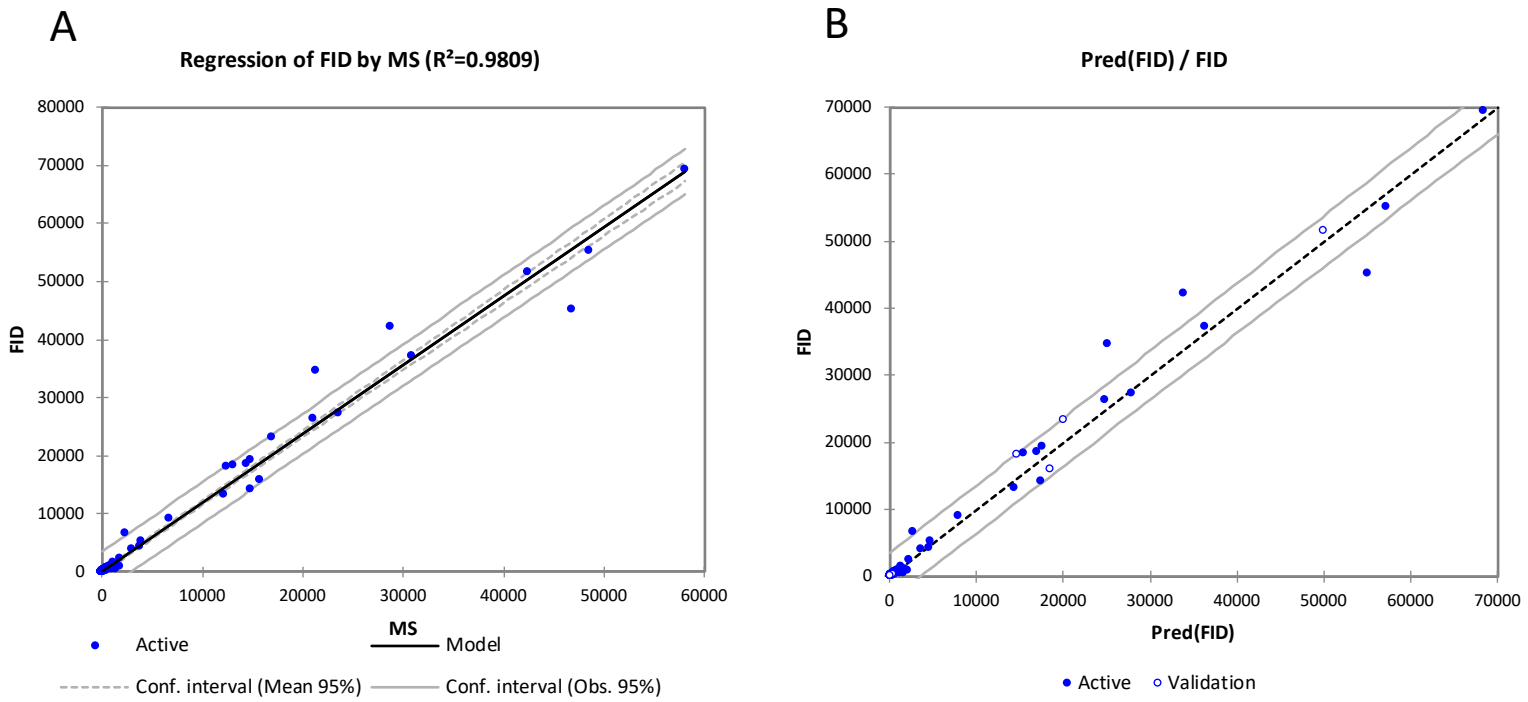
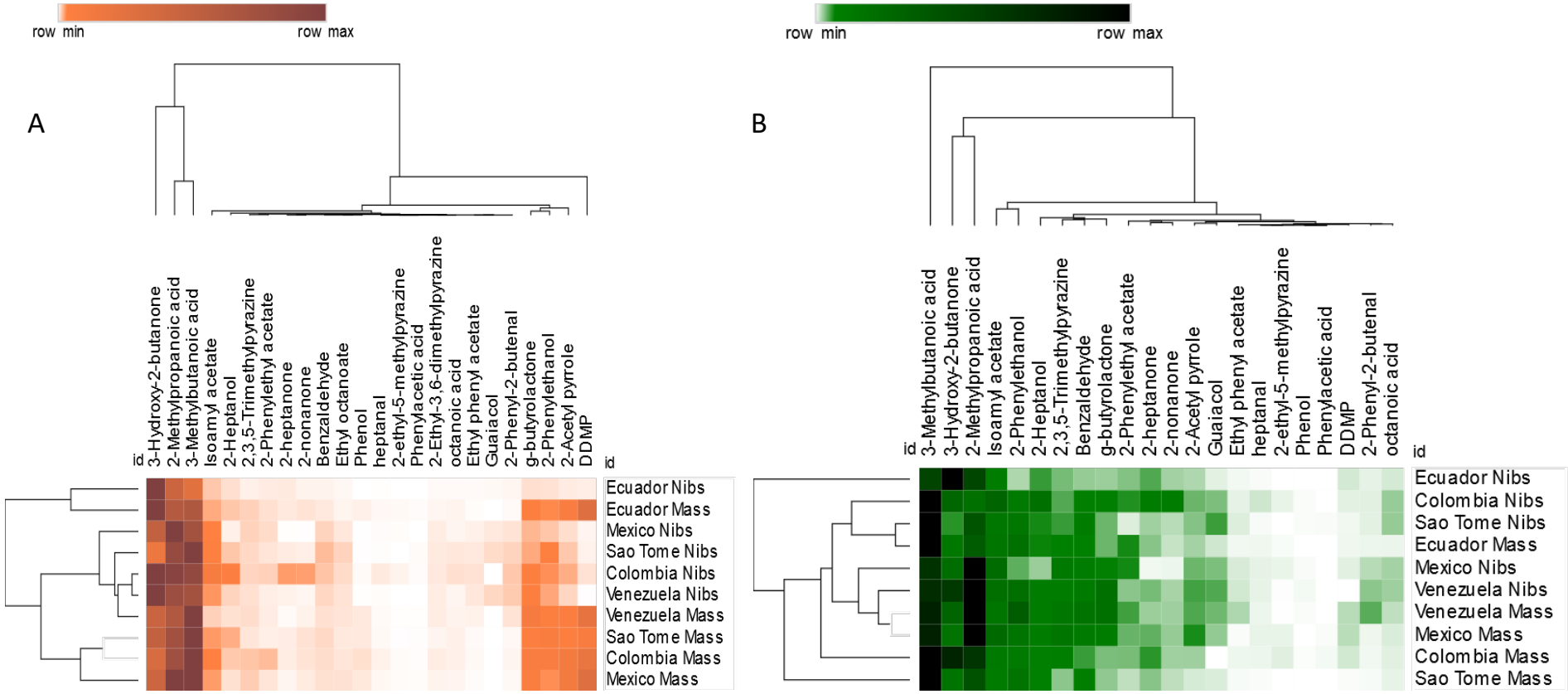

A
Regression of FID by MS (R²=0.9809)
FID
MS
Active
Model
Conf. interval (Mean 95%)
Conf. interval (Obs. 95%)
B
Pred(FID) / FID
FID
Pred(FID)
Active
Validation

**Figure 6**


A
row min
row max
id
3-Hydroxy-2-butanone
2-Methylpropanoic acid
3-Methylbutanoic acid
Isoamyl acetate
2-Heptanol
2,3,5-Trimethylpyrazine
2-Phenylethyl acetate
2-heptanone
2-nonanone
Benzaldehyde
Ethyl octanoate
Phenol
heptanal
2-ethyl-5-methylpyrazine
Phenylacetic acid
2-Ethyl-3,6-dimethylpyrazine
octanoic acid
Ethyl phenyl acetate
Guaiacol
2-Phenyl-2-butenal
g-butyrolactone
2-Phenylethanol
2-Acetyl pyrrole
DDMP
id
Ecuador Nibs
Ecuador Mass
Mexico Nibs
Sao Tome Nibs
Colombia Nibs
Venezuela Nibs
Venezuela Mass
Sao Tome Mass
Colombia Mass
Mexico Mass
B
row min
row max
id
3-Methylbutanoic acid
3-Hydroxy-2-butanone
2-Methylpropanoic acid
Isoamyl acetate
2-Phenylethanol
2-Heptanol
2,3,5-Trimethylpyrazine
Benzaldehyde
g-butyrolactone
2-Phenylethyl acetate
2-heptanone
2-nonanone
2-Acetyl pyrrole
Guaiacol
Ethyl phenyl acetate
heptanal
2-ethyl-5-methylpyrazine
Phenol
Phenylacetic acid
DDMP
2-Phenyl-2-butenal
octanoic acid
id
Ecuador Nibs
Colombia Nibs
Sao Tome Nibs
Ecuador Mass
Mexico Nibs
Venezuela Nibs
Venezuela Mass
Mexico Mass
Colombia Mass
Sao Tome Mass

764 **Table 1**

765

| 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 | |

766

**Table 2**

| Target analyte | Odor quality | OT (ng/g) | Exp $I^T_S$ | Ti (*m/z*) | Range (ng) | Regression equation MS | | | RSD% | Regression equation FID | | | RSD% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *m* | *q* | $R^2$ | | *m* | *q* | $R^2$ | |
| 3-Hydroxy-2-butanone | *Buttery* | 800 | 1250 | 88 | 20-5000 | 0.026 | 2.04 | 0.995 | 10.1 | 0.093 | -0.395 | 1.000 | 11.4 |
| 2-Heptanol | *Green fatty* | 263 | 1295 | 80 | 1-100 | 0.038 | -0.095 | 0.998 | 3.2 | 0.113 | 0.837 | 0.997 | 3.4 |
| 2,3,5-Trimethylpyrazine | *Nutty roasted* | 290 | 1365 | 122 | 1-50 | 0.096 | -0.130 | 0.999 | 3.1 | 0.098 | 1.820 | 0.995 | 4.2 |
| 2-Ethyl-3,5(6)-dimethylpyrazine | *Nutty earthy* | 57 | 1406 | 135 | 1-50 | 0.115 | -0.210 | 0.994 | 3.4 | 0.111 | 2.274 | 0.979 | 3.3 |
| Ethyl octanoate | *Green fruity* | 16 | 1411 | 88 | 1-50 | 0.093 | -0.199 | 0.995 | 4.7 | 0.105 | 1.537 | 0.998 | 3.4 |
| Benzaldehyde | *Almond* | 350 | 1478 | 77 | 1-50 | 0.086 | -0.201 | 0.996 | 2.4 | 0.161 | 2.756 | 0.992 | 5.1 |
| 2-Methylpropanoic acid | *Cheesy* | 190 | 1590 | 88 | 20-5000 | 0.016 | -1.30 | 0.999 | 6.4 | 0.133 | 0.942 | 0.997 | 4.1 |
| 3-Methylbutanoic acid | *Cheesy* | 22 | 1641 | 87 | 20-5000 | 0.016 | -1.20 | 0.996 | 4.9 | 0.048 | 0.201 | 1.000 | 3.1 |
| Ethyl phenyl acetate | *Honey-like* | 650 | 1695 | 91 | 1-50 | 0.112 | -0.226 | 0.991 | 1.3 | 0.060 | 0.607 | 1.000 | 1.6 |
| 2-Phenylethyl acetate | *Flowery* | 233 | 1767 | 104 | 1-50 | 0.115 | -0.255 | 0.986 | 6.2 | 0.150 | 1.373 | 0.996 | 6.4 |
| Guaiacol | *Phenol* | 16 | 1808 | 109 | 1-50 | 0.072 | -0.203 | 0.995 | 1.4 | 0.167 | 0.233 | 0.997 | 3.2 |
| 2-Phenylethanol | *Flowery* | 211 | 1857 | 91 | 1-50 | 0.096 | -0.306 | 0.996 | 7.6 | 0.126 | 1.318 | 0.999 | 2.6 |
| | | | | | 50-500 | 0.034 | 1.99 | 0.992 | | 0.191 | 0.421 | 0.999 | |
| (E)-2-Phenyl-2-butenal | - | - | 1955 | 115 | 1-50 | 0.063 | 0.251 | 0.999 | 1.6 | 0.161 | 0.140 | 1.000 | 3.4 |

OT – odor threshold; Exp $I^T_S$ – experimental linear retention indices; Ti, target ion.

**Table 3**

| Target analyte | Exp $I^T_S$ | MW | Formula | $n_C$ | $n_H$ | $n_O$ | $n_{Arom}$ | $n_N$ | RRF | Accuracy RE% FID vs MS | | β value (±RSD%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | ESTD 20 ng | ESTD 50 ng | Nibs | Mass | Powder |
| Isoamyl acetate | 1104 | 130.19 | C7H14O2 | 7 | 14 | 2 | 0 | 0 | 0.63 | - | - | 0.26 (±0.05) | 0.74 (±0.03) | - |
| 2-Heptanone | 1156 | 114.180 | C7H14O2 | 7 | 14 | 1 | 0 | 0 | 0.76 | - | - | 0.19 (±0.03) | 0.63 (±0.04) | - |
| Heptanal | 1184 | 100.160 | C6H12O | 6 | 12 | 1 | 0 | 0 | 0.73 | - | - | 0.41 (±0.04) | 0.90 (±0.05) | - |
| 3-Hydroxy-2-butanone | 1250 | 88.105 | C4H8O2 | 4 | 8 | 2 | 0 | 0 | 0.46 | 24 | 1 | 0.86 (±0.08) | 0.95 (±0.02) | 0.90 (±0.01) |
| 2-Heptanol | 1295 | 116.201 | C7H16O | 7 | 16 | 1 | 0 | 0 | 0.78 | -9 | 3 | 0.29 (±0.05) | 0.57 (±0.04) | |
| 2-Ethyl-5-methylpyrazine | 1353 | 122.171 | C7H10N2 | 7 | 10 | 0 | 0 | 2 | 0.69 | - | - | 0.52 (±0.03) | 0.82 (±0.01) | - |
| 2-Nonanone | 1360 | 142.242 | C9H18O | 9 | 18 | 1 | 0 | 0 | 0.81 | - | - | 0.30 (±0.04) | 0.88 (±0.03) | |
| **2,3,5-Trimethylpyrazine (REF)** | **1365** | **122.170** | **C7H10N2** | **7** | **10** | **0** | **0** | **2** | **0.69** | **-1** | **-4** | **0.32 (±0.07)** | **0.58 (±0.03)** | **0.50 (±0.01)** |
| 3-Ethyl-2,5-dimethylpyrazine | 1406 | 136.198 | C8H12N2 | 8 | 12 | 0 | 1 | 2 | 0.82 | -3 | 3 | 0.58 (±0.07) | 0.84 (±0.01) | - |
| Ethyl octanoate | 1411 | 172.268 | C10H20O2 | 10 | 20 | 2 | 0 | 0 | 0.72 | 11 | 11 | 0.76 (±0.02) | 0.91 (±0.03) | - |
| 2-Ethyl-3,6-dimethylpyrazine | 1425 | 136.198 | C8H12N2 | 8 | 12 | 0 | 1 | 2 | 0.82 | 15 | 3 | 0.57 (±0.06) | 0.84 (±0.02) | |
| Benzaldehyde | 1478 | 106.121 | C7H6O | 7 | 6 | 1 | 1 | 0 | 0.79 | 14 | 17 | 0.37 (±0.05) | 0.57 (±0.02) | 0.55 (±0.03) |
| 2-Methylpropanoic acid | 1590 | 88.110 | C4H8O2 | 4 | 8 | 2 | 0 | 0 | 0.46 | -5 | -10 | 0.80 (±0.09) | 0.93 (±0.01) | 0.89 (±0.04) |
| γ-Butyrolactone | 1574 | 86.090 | C4H6O2 | 4 | 6 | 2 | 0 | 0 | 0.42 | - | - | 0.43 (±0.02) | 0.95 (±0.03) | - |
| 3-Methylbutanoic acid | 1641 | 102.132 | C5H10O2 | 5 | 10 | 2 | 0 | 0 | 0.53 | -3 | -2 | 0.62 (±0.08) | 0.90 (±0.01) | 0.78 (±0.09) |
| Ethyl phenyl acetate | 1695 | 164.204 | C10H12O2 | 10 | 12 | 2 | 1 | 0 | 0.74 | 19 | 13 | 0.72 (±0.02) | 0.84 (±0.01) | - |
| 2-Phenylethyl acetate | 1767 | 164.200 | C10H12O2 | 10 | 12 | 2 | 1 | 0 | 0.74 | 2 | -2 | 0.68 (±0.01) | 0.83 (±0.02) | 0.79 (±0.01) |
| Guaiacol | 1808 | 124.140 | C7H8O2 | 7 | 8 | 2 | 1 | 0 | 0.68 | 7 | 12 | 0.45 (±0.07) | - | - |
| 2-Phenylethanol | 1857 | 122.160 | C8H10O | 8 | 10 | 1 | 1 | 0 | 0.84 | 13 | -6 | 0.70 (±0.14) | 0.85 (±0.01) | 0.84 (±0.03) |
| (E)-2-Phenyl-2-butenal | 1955 | 146.189 | C10H10O | 10 | 10 | 1 | 1 | 0 | 0.84 | 5 | 14 | 0.77 (±0.03) | 0.87 (±0.02) | - |
| 2-Acetyl pyrrole | 1913 | 109.13 | C6H7NO | 6 | 7 | 1 | 0 | 1 | 0.58 | - | - | 0.48 (±0.06) | 0.98 (±0.09) | - |
| Phenol | 1955 | 94.11 | C6H6O | 6 | 6 | 1 | 1 | 0 | 0.79 | - | - | 0.46 (±0.03) | 0.89 (±0.10) | - |
| Octanoic acid | 2065 | 144.21 | C8H16O | 8 | 16 | 1 | 0 | 0 | 0.70 | - | - | 0.19 (±0.01) | 0.88 (±0.05) | - |
| DDMP | 2278 | 144.13 | C6H8O4 | 6 | 8 | 4 | 0 | 0 | 0.35 | - | - | 0.44 (±0.05) | 0.84 (±0.06) | - |
| Phenylacetic acid | 2580 | 136.15 | C8H8O2 | 8 | 8 | 2 | 0 | 0 | 0.58 | - | - | 0.47 (±0.03) | 0.81 (±0.03) | - |

$I^T_S$ – experimental linear retention indices; MW – molecular weight; $n_C$, $n_H$, $n_O$, $n_N$, $n_{Arom}$, – number of carbon, hydrogen, oxygen, and nitrogen atoms and number of aromatic rings, respectively; RRF – relative response factor; RE% – relative error %; ESTD – external standard; DDMP – 2,3-dihydro-3,5-dihydroxy-6-methyl(4H)-pyran-4-one.