Direct analysis of volatile compounds during coffee and tea brewing with Proton Transfer Reaction Time of Flight Mass Spectrometry

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CONTRIBUTION TO THE MANUSCRIPTS THAT FORM THIS CUMULATIVE THESIS

José A. Sánchez-López has been author of the following manuscripts. His contribution to each one is described below.

Insight into the Time-Resolved Extraction of Aroma Compounds during Espresso Coffee Preparation: Online Monitoring by PTR-TOF-MS
Analytical Chemistry, Volume 86, Issue 23, **2014**, Pages 11696–11704.
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José A. Sánchez-López designed and performed all the experiments. He also performed the data analysis and prepared the manuscript. His work to this publication accounts for approximately 90%.

Extraction kinetics of coffee aroma compounds using a semi-automatic machine: On-line analysis by PTR-ToF-MS
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José A. Sánchez-López was involved in the design and execution of the experiments. He performed the data analysis and prepared the manuscript. His work to this publication accounts for approximately 80%.

Rapid and direct volatile compound profiling of black and green teas (camellia sinensis) from different countries with PTR-ToF-MS
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José A. Sánchez-López was involved in the design and execution of the experiments. He collaborated on the data analysis and the preparation of the manuscript. His work to this publication accounts for approximately 40%.

Extraction Dynamics of Tea Volatile Compounds as a Function of Brewing Temperature, Leaf Size and Water Hardness: On-Line Analysis by PTR-ToF-MS.
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José A. Sánchez-López was involved in the design and execution of the experiments. He performed data analysis and prepared the manuscript. His work to this publication accounts for approximately 70%.

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José Antonio Sánchez López

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Abstract

Coffee and tea are the two most consumed beverages in the world. Reasons for consuming them are varied and include the energy boost from their caffeine content, multiple health benefits and, above all, a pleasant sensory experience while drinking a cup of tea or coffee. Aroma is one of the main contributors to the sensory perception, and therefore of utmost importance for both coffee and tea. This thesis work focuses on the development of simple, fast, sensitive and reliable methods based on PTR-TOF-MS for the analysis of volatile organic compounds and how they are extracted into the liquid phase during the brewing of both coffee and tea.

In a first study, an on-line method to analyze extracted volatiles directly from the coffee flow was developed and applied to the study of single dose coffee capsules. Volatile concentration could be followed at 1 Hz resolution during the 42 seconds that the coffee extraction last. Differences in extraction between compounds were revealed, implying an aroma profile that changed with extraction time. Coffee capsules could be differentiated according to their extraction profiles by unsupervised statistical methods (Principal component analysis, PCA; and Hierarchical Cluster Analysis, HCA).

A follow up study was performed using a semi-automatic coffee machine and varying the brewing parameters (temperature and pressure). The different brewing conditions resulted in different time-intensity profiles that could be differentiated by PCA. Furthermore, all the compounds extracted were grouped according to their extraction profiles into 5 families by HCA and Self Organizing Three Algorithm (SOTA), with compounds in the same family sharing their physicochemical characteristics (mainly water solubility and volatility). An increase in either brewing pressure or temperature resulted in an increase in compound extraction. However, differences were only significant in the second part of the extraction (after 10-15 seconds) and more pronounced in the less polar compounds.

In the case of tea, a preliminary study was performed in order to obtain the volatile profiles of a large number of commercial teas. In that study, an automated headspace sampling method was used in combination with PTR-TOF-MS to allow the screening of a large amount of samples in a short time. The aroma profiles of 63 black teas and 38 green teas were analyzed for both full leaves and the infusion obtained after brewing the leaves. Differences between leaves and infusions were found, indicating incomplete extraction of some compounds (e.g. terpenes) and formation of others (e.g. alcohols) during hot water extraction. Using multivariate analysis, black and green teas were successfully discriminated. Also the origin of the samples could be partially discriminated although with some miss-assignments, mainly between neighboring countries.

The extraction of volatiles from tea leaves and how it is affected by several parameters (leaf size, temperature, brewing time and water composition) was further studied. Tea aliquots were taken every 30 seconds during five minutes and the headspace was analyzed with PTR-TOF-MS coupled to an autosampler. An increase in brewing temperature resulted in increased volatile extraction, with differences more pronounced at longer brewing times. Reduced leaf size resulted in faster extraction, a difference that was more significant during the first minute of brewing. On the other hand, water mineralization had low impact on the extraction kinetics and the volatile aroma content in the cup. Using PCA and HCA, not only the impact of brewing parameters was assessed but also different sets of brewing conditions resulting in analogous volatile profiles (i.e. same aroma profile) were identified.

The positive outcomes of this thesis support the use of PTR-TOF-MS to follow the extraction of volatile aroma compounds during preparation of hot beverages, both on-line and off-line. The use of multivariate methods on the dynamic data increased the applicability of the methods allowing differentiation of samples according to its composition (e.g. coffee capsules), country of origin (e.g. tea origins) or parameters used for preparation (e.g. temperature-pressure combinations).

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1. Coffee

Coffee is not only one of the most consumed beverages around the world, it is also one of the most important commodities[1]. According to the International Coffee Organization (ICO), more than 140 million of green coffee bags (60kg/bag) were produced in 56 different countries during 2013[2]. Brazil was the biggest producer with more than 30% of the worldwide coffee production. Although some countries consume more coffee than they produce (e.g. Philippines) this is not a general feature. Most of the coffee produced is exported. Taking a look at the two main coffee producers in 2013, Brazil and Vietnam, they exported 60% and 90% of their total production respectively. Although coffee consumption is higher in non-producer developed countries, not all imported coffee is meant to be consumed in the country. Those countries roast the imported green coffee, adding value to the product, and re-export it as roasted coffee. Therefore, coffee has a huge economic importance not only for the coffee producing countries but worldwide.

1.1. Green Coffee.

The coffee plant, or coffee tree, is indigenous of Ethiopia and belongs to the *Rubiaceae* family, genus *Coffea*. Despite the existence of more than 100 species of the *Coffea* genus, only two of them are of commercial importance: *Coffea Arabica*, commonly known as Arabica coffee and accounting for 62% of the world coffee production; and *Coffea canephora* var. *robusta*, known as Robusta coffee [3,4]. The coffee plant is easily damaged by frost, does not tolerate temperatures higher than 30 °C and needs a minimum of 1500 L/m^2 of rainfall per year[4]. These requirements limit the regions where coffee can be cultivated. Hence coffee is mainly produced in the area between the Tropics of Cancer and Capricorn, a region commonly known in the coffee industry as *"The bean belt"*.

The coffee plant starts to produce white and fragrant flowers after 3-4 years, which develop into the fruits (or berries). Coffee berries need 7-11 months of maturation from flowering, changing their color from green to dark read with ripening. The coffee berry is formed by an exocarp (skin), mesocarp (mucilage), endocarp (husk) and the seeds (coffee beans). Each coffee berry usually contains two coffee beans, each one wrapped with a perispem that is commonly called *silver skin*.

Harvesting starts when most of the cherries are ripe and it is performed by selective handpicking of the ripe cherries or non-selective stripping of all the cherries at the same time (either manually or mechanically). Selective hand picking results in high quality but it needs extensive work force therefore not being feasible in big plantations. There is a general believe that non-selective stripping results in lower quality coffee but, with post-harvesting classification of the berries, top-quality coffee can be obtained independently of the harvesting method[3].

Post-harvesting process to separate the beans from the other parts of the coffee berry is carried out at origin by the dry or the wet processes[3,4]. The dry process is more economical as the cherries are sun dried (or dried using mechanical driers) during several weeks. Then the dried husks are removed leaving the bean with the silver skin attached. Wet process is more complex and requires high amounts of water, but it generally provides better quality coffee. Pulp is removed in a pulper prior to fermentation in a water tank, which enzymatically breaks down the mucilage surrounding the beans allowing its separation. After washing away the mucilage, coffee beans are dried – either at sun or at mechanical driers.

1.2. Composition of green coffee beans

The chemical composition of green beans presents high variability as it is generally the case with biological materials. Variations in green coffee composition have been attributed to both agricultural factors linked to geographical origin (soil[5,6], altitude[7,8] or other environmental conditions[8,9]) and to the post-harvesting process[9–11]. Table 1 provides a general overview on the non-volatile constituents of green coffee beans. Comprehensive data about the composition of green beans can be found in the reviews of Illy (1995)[3], Flament (2002)[12] and Clarke (2012)[4].

Table 1. Main non-volatile compounds reported in raw green beans.

NITROGENOUS COMPONENTS			
Alkaloids			
caffeine	*liberine*	*methylliberine*	*paraxanthine*
theacrine	*theobromine*	*theophylline*	*trigonelline*
Proteins and free amino acids			
*alanine**	*b-alanine*	*g-aminobutyric acid*	*arginine*
asparagine	*aspartic acid*	*cystathionine*	*cysteine*
cystine	*glutamic acid*	*glutamine*	*glycine**
histidine	*1-(or 3-)methylhistidine*	*hydroxyproline*	*isoleucine**
*leucine**	*Lysine*	*methionine*	*ornithine*
*phenylalanine**	*pipecolic acid*	*proline*	*pyroglutamic acid 5-oxoproline*
serine	*taurine*	*threonine*	*tryptophan*
*tyrosine**	*valine**		

CARBOHYDRATES			
Monosaccharides			Oligosaccharides
xylose (a-D-)	*glucose (a-D-)*	*rhamnose*	*sucrose*
arabinose (a-D-)	*galactose (a-D-)*	*fructose (a-D-)*	*raffinose*
ribose (a-D-)	*mannose (a-D-)*	*psicose*	*stachyose*

LIPIDS					
Free Fatty Acids		Sterols		Diterpenes	
linoleic acid	*stearic acid*	*sitosterol*		*cafestol*	*16-O-methylcafestol*
palmitic acid	*arachidic acid*	*stigmasterol*		*kahweol*	*dehydrocafestol*
oleic acid	*linolenic acid*	*campesterol*		*cafestal*	*deydrokahweol*
				kahweal	

CHLOROGENIC ACIDS			
5-caffeoylquinic acid	*5-feruloylquini acid*	*5-p-coumaroylquinic acid*	*3,5(4,5 and 2,4)-dicaffeoylquinic acid*

Data compiled from Clifford and Willson (1985)[13] *and Flament (2002)[12]. * indicates Strecker-active amino acids*

In general terms, green beans have weights between 100 and 200 mg with water content from 9 to 13% in weight. Different composition has been reported for Arabica and Robusta varieties. Arabica contains more lipids (~15%), sucrose (6-9%) and trigonelline (0.6-1.3%) than Robusta (10, 3-7 and 0.3-0.9% respectively). On the other side, Robusta contains more caffeine (2.2-2.4%) and chlorogenic acids (~10%) than Arabica (1.2-1.3% and ~6% respectively). For both coffee varieties, the main constituents are carbohydrates which account for 50% of the green bean's dry weight.

Green coffee beans have a weak aroma that resembles that of peas or green bell peppers. Although the volatile composition of green beans is less complex than that of roasted beans, around 300 volatile compounds have been identified to date, of which 100 are unique to green beans. As green beans are not used for consumption, most of the work done on volatile constituents had the objective of identifying defective beans[14–16] or contaminants[17,18].

1.3. Coffee roasting.

Roasting is a crucial process that highly impacts the quality of the final coffee product. It basically consists on heating the green coffee beans to obtain a desired taste and aroma, with a texture that allows grinding and further extraction of the roasted coffee beans. The main aspects of bean roasting are highlighted in Figure 1.

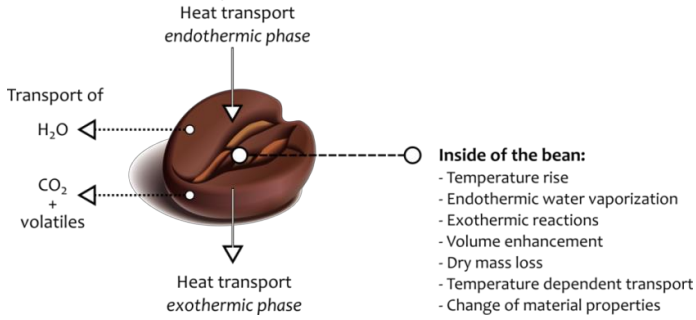

Figure 1. Roasting of coffee beans – main aspects[19,20]

The coffee roasting process can be basically divided in three different steps: (i) drying of the beans, (ii) roasting phase and (iii) cooling of the beans. The drying step is an endothermic process and happens when the coffee bean temperature is at around 100°C; free water evaporates and the bean reduces its moisture from around 12% to a few percent. At this time the first changes in the beans are observed, with a color change from green to pale yellow. The chemical reactions taking place at this stage are endothermic but when the bean moisture is lower than 6%, the temperature keeps rising up to ~150-190 °C and the chemical reactions in the bean become exothermic (pyrolytic reactions). These reactions lead to the production of large amounts of gas that increases the pressure inside the bean cell walls, resulting in swelling of the beans and formation of coffee aroma. The high pressure inside the beans produces the break of some cells generating a characteristic sound known as *"first crack"*. If beans are roasted for longer time, the beans become dark roasted and start to release oils to the surface after a second small popping (*"second crack"*). The final step of roasting is the cooling down of the beans in order to stop all the reactions, which can be done either with air or water quenching[21].

The changes that coffee beans experiment during roasting can be divided into physical or chemical transformations. The main physical changes are the increase in volume (up to 100%) and reduction of the

density from 550-700 g·L^{-1} in green beans to 300-450 g·L^{-1} in roasted beans[19]. Furthermore, the cellular structure of the bean is damaged with formation of microporous and a fractured structure in the cell walls, which allows the mass transfer of compounds from the inner cavities to the surface (e.g. oil) and it accelerates degassing (loss of CO_2 and volatile compounds)[22].

The chemical changes occurred during roasting are crucial, as they produce a large number of volatile compounds that contribute to the final aroma of roasted coffee. More than 900 compounds have been identified in roasted coffee to date[23]. The main reactions that lead to aroma formation are summarized in Figure 2 and include: Maillard reaction, between reducing sugars and amino acids that leads to the formation of volatile compounds and melanoidins (colored compounds); Strecker degradation, reaction between and amino acid and a α-dicarbonyl to form aminoketones; trigonelline thermal degradation, forming compounds with pyridine or pyrrole rings; phenolic acid degradation, to form phenol derivatives; oxidative degradation of lipids, resulting in aldehydes, ketones and terpenes; breakdown of sulfur amino acids, resulting in sulfur containing volatiles; and degradation of hydroxy amino acids that mainly produce alkylpyrazines[4,21].

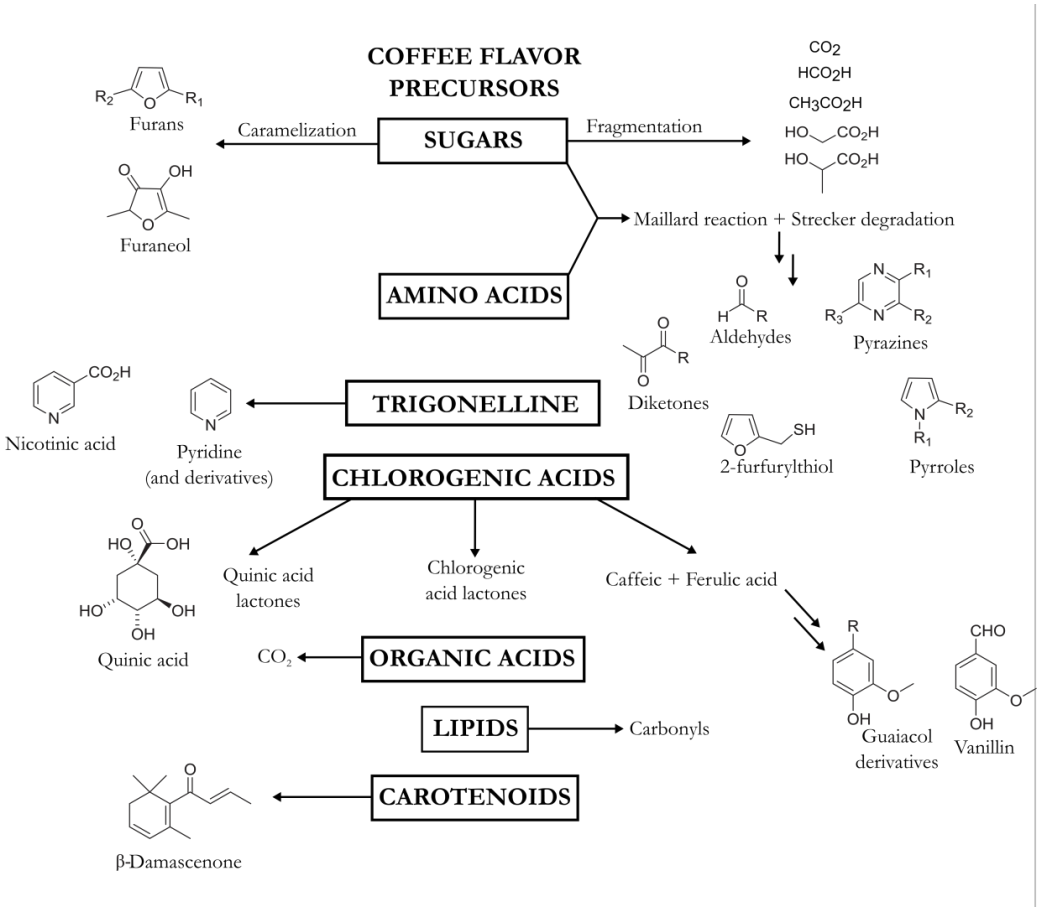

Figure 2. Main volatiles produced during roasting from non-volatile precursors. Adapted from Yeretzian et al 2002[21]

Despite the large number of volatile compounds found in coffee, only a small fraction is responsible for the characteristic aroma of roasted coffee. The aroma composition of coffee depends on several factors like origin, post-harvesting processes and roasting; therefore, the compounds reported as important odorants in coffee are highly determined by the specific coffee analyzed and the methods used to

determine the contribution of each compound to the overall coffee aroma. Sunarharum and co-workers recently reviewed the chemical components that are responsible of Arabica coffee flavor and ended up with a short list of the main volatiles contributing to the aroma of Arabica coffee (Table2)[24].

Table 2. Compounds reported as important odorants of roasted Arabica coffee. Adapted from Sunarharum et al. (2014)[24].

Aldehydes	**Thiols**	**Phenolic compounds**
2-methylbutanal	*3-mercapto-3-methylbutylformate*	*Guaiacol*
2-methylpropanal	*2-furfurylthiol*	*4-ethylguaiacol*
3-methylbutanal	*2-methyl-3-furanthiol*	*4-vinylguaiacol*
E-2-nonenal	*3-mercapto-3-methylbutylacetate*	*vanillin*
Acetaldehyde	*3-methyl-2-butene-1-thiol*	
Propanal	*methanethiol*	**Pyrazines**
p-anisaldehyde		*2,3-dimethylpyrazine*
phenylacetaldehyde	**Thiophenes**	*2,5-dimethylpyrazine*
	3-methylthiophene	*2,3-diethyl-5-methylpyrazine*
Acids		*2-ethenyl-3,5-dimethylpyrazine*
2-methylbutyric acid	**Thiazoles**	*2-ethenyl-3-ethyl-5-methylpyrazine*
3-methylbutyric acid	*2,4-dimethyl-5-ethylthiazole*	*2-ethyl-3,5-dimethylpyrazine*
		2-ethyl-3,6-dimethyl-pyrazine
Esters	**Furanones**	*2-methoxy-3,5-dimethylpyrazine*
ethyl-2-methylbutyrate	*dihydro-2-methyl-3(2H)-furanone*	*2-methoxy-3,2-methylpropylpyrazine*
ethyl-3-methylbutyrate	*2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone*	*2-methoxy-3-isopropylpyrazine*
	3-Hydroxy-4,5-dimethyl-2(5H)-furanone	*3-ethenyl-2-ethyl-5-methylpyrazine*
Furans	*4-Hydroxy-2,5-dimethyl-3(2H)-furanone*	*3-isobutyl-2-methoxypyrazine*
Furfural	*5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone*	*6,7-dihydro-5-methyl-5H-cyclopentapyrazine*
2-((methylthio)methyl)furan	*5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone*	*ethylpyrazine*
2-furanemethanol acetate		
2-methylfuran	**Ketones**	**Pyridines**
5-methyl-2-furancarboxaldehyde	*1-octen-3-one*	*pyridine*
furfurylmethyl ether	*2,3-hexadione*	*pyrrole*
Fufurylformate	*2,3-butanedione*	*1-methyl pyrrole*
Furfuryldisulfide	*2,3-pentanedione*	
	3,4-dimethylcyclopentenol-1-one	**Terpenes**
Sulfur-containing compounds	*4-(4'-hydroxyphenyl)-2-butanone*	*linalool*
dimethyl trisulfide	*1-(2-furanyl)-2-butanone*	*limonene*
bis(2-methyl-3-furfuryl)disulphide		*geraniol*
Methional	**Norisoprenoids**	
	E-β-damascenone	

1.4. Coffee brewing.

Brewing is the last step of coffee preparation and it is crucial to obtain the desired quality in the final beverage. It basically consists the percolation of hot water through a bed of ground roasted coffee particles. Water not only dissolves the soluble components of coffee but also sweeps along solids and oils present in the surface of the ground coffee particles, which will impact the final organoleptic properties of the coffee brew.

Brewing methods can be mainly classified in: (i) decoction methods, in which the hot water is kept in contact with the ground coffee for a considerable amount of time (e.g. boiled coffee, Turkish coffee, Vacuum coffee, French press); (ii) infusion methods, where water flows through the ground coffee bed

with shorter contact times (e.g. filter coffee, Napoletana coffee); and (iii) pressure methods, where a relative high pressure is used to create a flow through the compacted coffee bed (e.g., Moka, espresso)[25]. The method used for coffee brewing will determine the final characteristics of the cup including caffeine content[26–29], antioxidants[27–29], lipids[30] or volatile composition[28,29,31]. The only coffee brewing method in the scope of this thesis is espresso brewing; thus, it will be described in detail.

Espresso coffee can be defined as a beverage prepared on request by percolation of hot water (90 ± 5 °C) under pressure (9 ± 2 bar) through a compacted cake of roast and ground coffee beans (6 ± 1.5 g) for a short defined time (30 ± 5 s)[25]. The extraction conditions used for brewing espresso are responsible for the characteristics of the final beverage that make espresso unique and different from other coffee beverages. As defined by Petracco, *"espresso is a polyphasic beverage, prepared from roast and ground coffee and water alone. It is constituted by a foam layer of small bubbles with a tiger-tail pattern, on top of an emulsion of microscopic oil droplets in an aqueous solution of sugars, acids, protein-like material and caffeine, with dispersed gas bubbles and colloidal solids."*[3]

The methods to brew an espresso can be grouped in three main categories: use of a bar machine, coffee pods and single dose coffee capsules. The last two are responsible of the increase in espresso coffee domestic consumption. Both methods allow preparing an espresso with high reproducibility just by pressing a button, in a clean and easy way. As a drawback, the selection of coffees is limited and the coffee machines designed for both pods and capsules do not usually allow the user to adjust the water temperature or pressure for extraction.

The first step in coffee brewing is grinding of the roasted coffee bean. Grinding is generally considered an independent step from brewing but, the requirement of freshly ground beans in order to get a top quality espresso together with the high impact that grind size has in regulating the flow of water through the coffee cake, sustain the need of considering both processes –grinding and brewing- together. Grinding of the roasted coffee beans produces not only physical but also chemical changes that impact the final beverage. The most obvious effect is the fragmentation of the bean in small particles (0.2-650 μm) increasing the effective surface area for extraction and destroying the cellular structure, therefore liberating most of the trapped CO_2[32]. The finer the grind, the highest the loss of CO_2[33] and consequently of volatile aroma compounds. Particles bigger than 50 μm can still contain intact cell cavities filled with the gases generated during roasting which are responsible for the formation of "crema" (coffee foam)[22,32]. Another physical effect of grinding is the release of coffee oil and its deposition on the coffee particles surface. Coffee oil is extracted and found as emulsified oil droplets in the final beverage, contributing to the mouthfeel and aroma lingering typical from espresso beverages[34]. The increased surface exposed to oxygen upon grinding, also results in an increased oxidation rate of coffee lipids and aroma compounds, negatively affecting the final flavor of the beverage. Therefore, it is crucial to brew the espresso immediately after grinding the roasted coffee beans. In the case of pods and capsules systems, the manufacturer has to provide a packaging that ensures the freshness of the product until its consumption.

In the case of semi-automatic bar machines, the roast and ground coffee is dosed into a porta-filter holder and tamped. Tamping consists of the application of pressure on the ground coffee in order to eliminate the void spaces between coffee particles. The dose, particle size of the ground coffee and the compacting of the bed determine the hydraulic resistance of the system, therefore controlling the flow of

water through the coffee cake[19]. Different flows imply different contact times between the coffee particles and water, thus affecting the extraction yield of the different coffee compounds. A non-uniform coffee bed favors the creation of water channels of high flow, reducing the extraction efficiency.

Two main mechanisms can be distinguished in the extraction of soluble substances from the coffee bed into water: washing out of the solutes from the surface of the coffee particles and diffusion from inside the particles to the solution. In the case of espresso extraction, where the contact time between the hot water and the coffee particles is short, washing out can be considered the main effect[19,35] but diffusion still plays an important role[36]. As different compounds have different water solubility and diffusion coefficients, their extraction rates differ, varying the composition of the cup of coffee over the extraction time[37]. Parameters that affect the water/coffee contact time, the solubility of compounds in water or their diffusion will also affect the extraction efficiency.

Several authors have studied the effect of different extraction parameters on the composition and quality of the final espresso beverage. The particle size of ground coffee showed an inverse correlation with the extraction of solids, soluble compounds, caffeine and lipids[38,39]. Finer grounds resulted in higher compaction of the coffee bed and therefore higher water/coffee contact times, consequently increasing the extraction yields. Regarding the organoleptic characteristics of the espresso coffee, higher levels of volatile compounds could be determined in espresso coffees prepared from fine grounds, with roasty, fermented and woody notes; while coarse particles produced coffees that were described as burnt or rubbery by the sensory panelists[38]. Closely related to the coffee dose is the coffee/water ratio, in other words, the amount of water used to extract the ground coffee. Coffees prepared with high coffee/water ratios had higher density, viscosity, surface tension and total solids content[40]. Total lipids and diterpene content also showed a positive correlation with coffee/water ratio[39]. Additionally, higher coffee/water ratio resulted in higher caffeine and chlorogenic acids extraction which gave bitterness and astringency to the beverage. Although the volatile composition was not significantly affected by the coffee/water ratio, samples with higher ratio were described as less fresh and presented a burnt, acrid and fermented flavor.

Water is the second main component of coffee. Three parameters attributed to water will affect the extraction of espresso coffee: water composition, pressure and temperature. The water used to brew coffee needs to be free of any impurity that can impact the organoleptic properties of the final cup[41]. Water is generally softened in order to reduce calcium and magnesium content as these cations produce insoluble salts that precipitate, affecting the heat transfer efficiency and damaging the coffee machines[19]. The most common way of water softening is the exchange of Ca^{2+} and Mg^{2+} ions with sodium ions, although this replacement by Na^{+} was found to dramatically affect the extraction time, increasing it up to 80%[42]. Furthermore, the binding energy of Na^{+} with some compounds (e.g. caffeine or eugenol) is higher than that of water according to quantum-chemical calculations, therefore sodium ions negatively affect the extraction of those compounds[43]. Another water component that influences both extraction and final characteristics of coffee is the hydrogen carbonate ion. Both carbonate CO_3^{2-} and hydrogen carbonate HCO_3^{-} ions can be neutralized by coffee acids and produce CO_2. The dissolved carbon dioxide produced from hydrogen carbonate affects the compaction of the coffee bed, increasing the extraction time and affecting the coffee crema. Higher CO_2 content produces higher foam volume in the cup but the foam has an undesired texture when too much CO_2 is released, with big bubbles that collapse quickly, negatively affecting foam persistence[32,44].

Water temperature and pressure can be easily adjusted by the barista in semi-automatic bar machines in order to optimize coffee extraction. Increase of water temperature results in an increased extraction yield and higher foam volume and persistence. Coffees extracted at high temperatures also show larger amounts of caffeine, lipids and volatile compounds that impact the final flavor of the beverage[39,45,46]. Significantly higher values for sensorial descriptors have been found between coffees extracted at 90 or 100°C, including color intensity, body, texture, olfactive intensity, bitterness or astringency amongst other attributes[46]. Extraction efficiency also shows positive correlation with pressure. Increase in total solids, lipids or chlorogenic acids has been reported between 7 and 9 bar[47], and higher diterpenes were extracted at 11 bar[39]; however, other compounds as caffeine were not significantly affected by pressure. Decrease of diterpenes, total lipids and chlorogenic acids with pressures higher than 11 bar have also been reported and attributed to a differences in compaction of the coffee bed that resulted in a reduced flow[47]. The higher extractability of compounds with high water temperature and pressure has been related with two different processes. The first one is the change in polarity of water with temperature and pressure that allows medium and low polarity compounds to dissolve in water and it improves mass transfer from the beans to water. The second effect is the higher penetrability of water into the coffee particles due to pressure that can assist the extraction of compounds that remain inside the ground coffee pores[48].

The composition of espresso coffee changes with time. Despite this widely known fact, most research has been focused on the final cup and only few studies have investigated the extraction kinetics for espresso coffee. Kinetics of extraction have been determined for compounds with health related properties as caffeine, chlorogenic acids or acrylamide. Despite the high water solubility of these compounds, only 70-80% of the total content in the beans is extracted during the short time in which espresso is prepared (~25s)[19,25,49]. Figure 3 summarizes the results from Ludwig and co-workers studying the extraction of caffeine and chlorogenic acids during espresso brewing (45 mL in 24 seconds). The first fraction corresponds to the first 8 seconds (~17mL) and it contained around 70% of the total amount extracted for monocaffeoylquinic acids and caffeine, and half of the dicaffeoylquinic acids[27]. After those 8 seconds, extraction was much slower for all the compounds. The same behavior has been observed in other studies with caffeine[50], chlorogenic acids and melanoidins[51]; with the accumulate concentration during extraction following a 2-parameter hyperbolic curve of formula $y = a \cdot x/(b+x)$. Kinetics of 20 volatile aroma compounds have also been studied for a single dose coffee capsule system[52]. By extracting capsules to different volumes (10 to 150 mL) and the subsequent analysis of the coffee headspace, Mestdagh and co-workers found the same hyperbolic curves and an inverse correlation between the slope at the beginning of the curve and the polarity of the compounds. The more polar the compound the faster it was extracted from ground coffee[52]. Figure 3 shows the concentration of three volatile compounds at different extraction volumes (from 0 to 160 mL). The different slopes at the beginning of the curve for each compound reflect different extraction rates. Therefore, the volatile profile in the final beverage is highly dependent on the volume (and therefore time) in which the coffee is extracted, highlighting the importance of time resolved data in order to study coffee the brewing process.

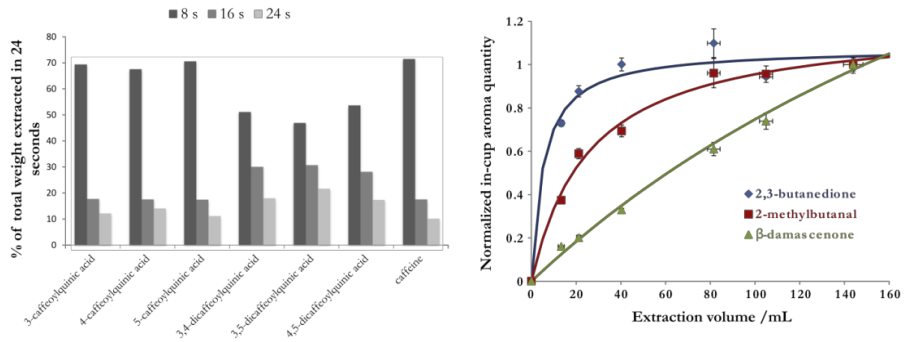

Figure 3. Dynamics of espresso brewing. Left: Extraction of non-volatile compounds. Fractions were taken every 8 seconds and had volumes between 14-17mL and data is presented as % of the total amount extracted [27]. Right: Extraction of volatile compounds. Coffees were brewed to different final volumes and the headspace concentration determined by GC-MS[52].

In order to better describe the percolation process and the extraction of soluble compounds, Bandini and co-workers created a computational model to simulate the motion of water across a compacted coffee bed and the extraction of soluble compounds from the coffee particles. The results of the simulation are shown in Figure 4. In the case of water, the number of elements leaving the system follows an initial growth that stabilizes with time. For soluble compounds, extraction is more efficient at the beginning and it slowly decreases with time. These results are in agreement with experimental results[53].

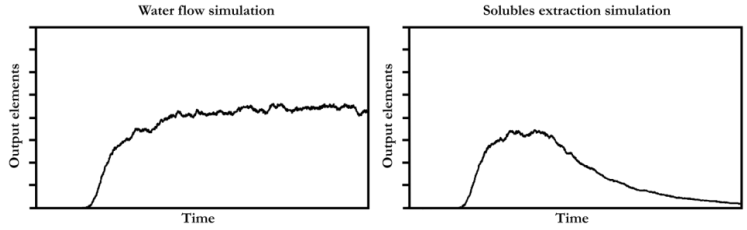

Figure 4. Water (left) and soluble compounds (right) exiting the ground coffee bed system in a computational model for espresso brewing.[53]

2. Tea

2.1. Economic importance

The Food and Agriculture Organization (FAO) of the United Nations (UN) recognizes 50 tea producing countries in the world, although the first ten (China, India, Kenya, Sri Lanka, Vietnam, Turkey, Iran, Indonesia, Argentina and Japan) accounted for more than 90% of the total production in 2013. Tea production has been almost doubled during the last 20 years. That increase has been attributed to an increased consumption in the producing countries and to an increment in the exports to other countries, mainly the United States of America, Russian Federation and United Kingdom. According to FAO data, 2 million tons of tea were exported on 2013, accounting for 5.7 billion USD (United States Dollars)[54].

2.2. Tea classification

The tea plant presents high heterogeneity on its vegetative (somatic) characteristics which has made difficult its taxonomical classification. Two major varieties with economic importance are cultivated: *Camellia sinensis* var. *sinensis*, which represents the majority of green teas from China and Japan, and

Camellia sinensis var. *assamica*, from which black tea is produced[55]. Owing to that heterogeneity, together with the large differences between producing countries in terms of climate (temperature, day length, humidity), soil, and growing, harvesting and processing practices, tea is normally commercialized with the name of the production region (often including also the name of the plantation and the harvesting season).

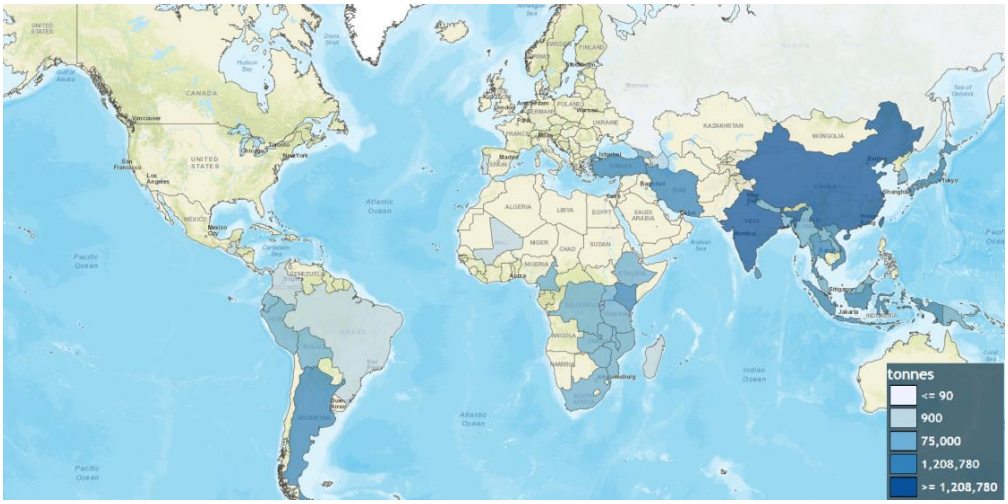

Figure 5. Tea producing countries color-coded according to 2013 FAOSTAT data.

Tea is mainly classified according to its fermentation degree: green (non-fermented), white (lightly fermented), oolong (semi-fermented) and black tea (fermented). The tea manufacturing process is highly variable, with differences not only between regions but within producers. Most times, producers have specific steps in the manufacturing processes that are kept secret, as it represents the main differentiator from competition. The main steps in tea manufacturing are: harvesting (plucking), fixing (enzyme inactivation), withering, rolling and drying.

It all begins with the plucking of the two youngest leaves and a bud from the tea plant. For high quality tea, plucking is done manually to avoid removal of stalks. The so-called fermentation is in fact a combination of enzymatic reactions induced by endogenous oxidative enzymes (e.g. polyphenol oxidase or peroxidases). Therefore, in the case of the non-fermented green teas, enzymes need to be deactivated after harvesting and the tea leaves are then subjected to a thermal treatment, being pan firing and steaming the most commonly used. The thermal treatment used for fixing will impact the chemical composition and organoleptic properties of the final tea[56]. After fixing, and while still hot, the leaves are rolled in order to break the leaf cells and liberate the inner content, developing the tea aroma. To keep the quality of the tea until brewing, leaves are finally dried. For those teas in which fermentation is required, leaves are withered after plucking. This process can last for few hours to several days, either at ambient conditions in the sun or indoors in heated rooms. In the case of black tea, withering is performed until the water content of the leaf is around 60%. Then, leaves are rolled until 80-90% of the leaf cells are broken and they are kept at controlled conditions of temperature and humidity so the enzymatic reactions can take place. The leaves turn darker and the aroma evolves from green to fruity as the chemical reactions evolve. Once the fermentation is finished, the tea leaves are dried[56,57].

2.3. Tea Aroma

Tea quality is assessed by the color and aroma of the dry leaves together with the aroma and taste of the infusion obtained after brewing the tea leaves with hot water. Therefore, the volatile content of teas is of utmost importance. More than 600 volatiles have been reported in tea, and their concentrations in tea leaves depend on the tea cultivar, environmental conditions and leaf processing. Extensive research has been performed on the volatile content of tea since the early 90's and the current knowledge of tea flavor has already been reviewed[57–59].

The main pathways leading to volatile compounds in tea are depicted in figure 6. Saturated and unsaturated C6 and C9 aldehydes and alcohols are formed from enzymatic oxidation of fatty acids, mainly by lipoxygenase. Therefore, any change in the enzymatic activity (e.g. seasonal variations) also alters the concentration of these compounds which are accountable for the green notes of tea aroma. Methyl jasmonate also derives from fatty acids, although its biosynthetic pathway has not been yet elucidated in tea plants[58]. The main volatile terpenes found in tea are geraniol, linalool and linalool oxides. They are key compounds in the aroma of black tea, contributing to the floral aroma[60]. Other compounds contributing to the floral and fruity aroma of tea are those derived from L-phenylalanine: benzaldehyde, benzylalcohol, phenylethanol, phenylacethaldeyde and coumarin, the last one with a pleasant sweet odour.

Carotenoids are also important precursors of aroma volatiles in tea. The total carotenoid content in the tea leaves is variable but it can be up to 10% in dry weight[61]. Enzymatic and thermal degradation of carotenoids lead to formation of important aroma compounds as ionones and damascenones[62]. Finally, there are a large number of volatile compounds present in glycosidically-bound forms, which increases their solubility but reduces their volatility. Enzymatic hydrolysis releases some of those volatiles during tea processing[63], while others can be hydrolyzed during the hot water extraction of the tea leaves[60].

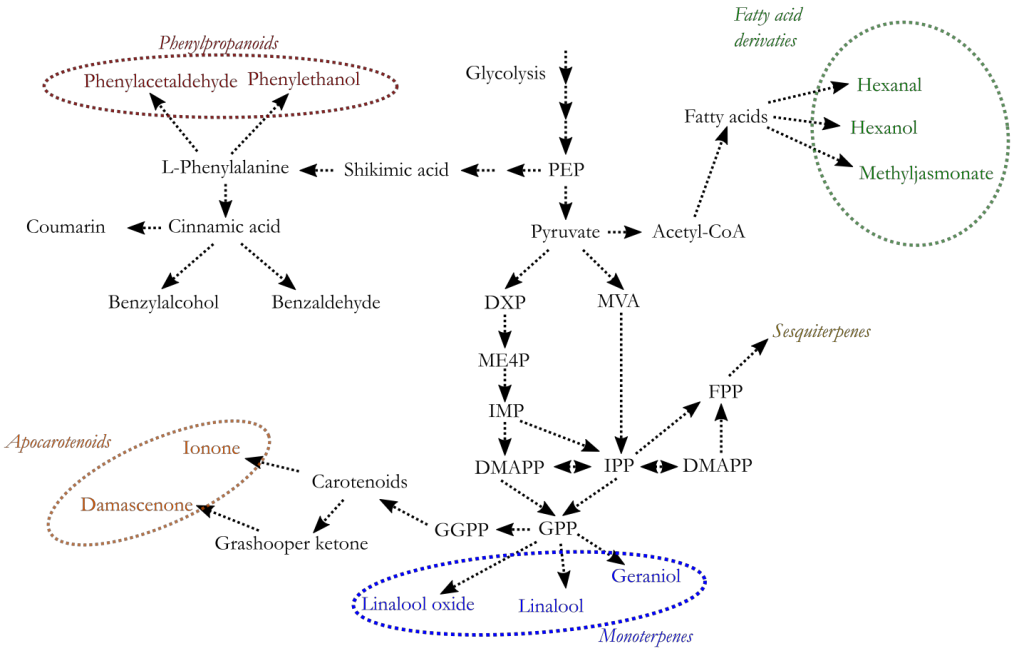

Figure 6. Simplified proposed biosynthetic pathways of tea volatiles. From Yang et al. 2013[58].

Most of the research on tea volatiles has been focused on the identification of new compounds or the description of the volatile composition of a specific tea. Wang and co-workers determined the volatile composition of 87 different teas (8 white, 27 green, 27 oolong, 15 black and 10 Pu-erh)[64]. Although the volatiles analyzed were similar for all teas, their concentration varied largely. All tea types had linalool and linalool oxides among the five compounds in highest concentration. Geraniol was also among the most abundant compounds for all the teas with exception of pu-erh teas. As not all volatiles are relevant to the aroma of tea, some authors have identified the key aroma compounds in tea by using flavor dilution technique in combination with GC-Olfactometry[65,66] (Table 3).

Differences in the compounds contributing to aroma have been found not only between different tea types but also between the tea leaves and their infusions. Most of the differences found were attributed to the lower concentration of compounds in the beverage compared to the leaves. Interestingly, some alcoholic volatiles like geraniol were found in higher concentration in the hot water infusions than in the leaves, probably due to thermal hydrolysis of their glycosides[60].

Table 3. Potent odorants of green and black teas identified by flavor dilution.

Compound	Aroma descriptor	Compound	Aroma descriptor
(E)-2-nonenal[1,2]	green	*3-methylnonane-2,4-dione*[1,2]	green
(E)-methyl jasmonate[2]	floral	*4,5-dihydro-3(2H)-thiophene*[2]	roasty
(E,E)-2,4-decadienal[1,2]	fatty	*4,5-epoxy-(E)-2-decenal*[1]	metallic
(E,E)-2,4-heptadienal[1,2]	fatty	*4-hydroxy-2,5-dimethyl-3(2H)-furanone*[1,2]	caramel like
(E,E)-2,4-nonadienal[1,2]	fatty	*4-mercapto-4-methyl-2-pentanone*[2]	meaty
(E,E)-2,4-octadienal[1]	fatty	*4-nonanolide*[2]	sweet
(E,E,Z)-2,4,6-nonatrienal[1]	oat flake like	*4-octanolide*[2]	sweet
(E,Z)-2,6-nonadienal[1,2]	cucumber like	*5-octanolide*[2]	sweet
(E,Z)-2,6-nonadienol[1]	cucumber like	*bis(2-methy-3-furyl) disulfide*[1]	meaty
(Z)-1,5-ocatadien-3-one[1,2]	metallic	*butanoic acid*[1]	sweaty
(Z)-3-hexenol[1,2]	green	*coumarin*[2]	sweet
(Z)-3-hexenal[1]	green	*ethyl-2-methylbutanoate*[1]	fruity
(Z)-4-heptenal[1,2]	hay like	*eugenol*[2]	spicy
(Z)-jasmone[2]	green	*geraniol*[1,2]	floral
(Z)-methyl jasmonate[2]	floral	*geranylacetone*[2]	floral
1-octen-3-one[1,2]	mushroom like	*guaiacol*[2]	burnt
2,3,5-trimethylpyrazine[2]	nutty	*hexanal*[1]	grassy, green
2,3-butanedione[1,2]	buttery	*hexanoic acid*[1]	sweaty
2,3-diethyl-5-methylpyrazine[2]	nutty	*indole*[2]	animal like
2,3-pentanedione[2]	buttery	*jasmine lactone*[2]	sweet
2-acetyl-1-pyrroline[2]	popcorn-like	*linalool*[1,2]	floral
2-acetyl-2-thiazoline[2]	popcorn-like	*methional*[1,2]	potato like
2-acetylpyrazine[2]	roasty	*methyl anthranilate*[1]	grape like
2-aminoacetophenone[2]	grape like	*nonanal*[2]	orange like
2-ethyl-3,5-dimethylpyrazine[2]	nutty	*octanal*[1,2]	orange like
2-ethyl-3,6-dimethylpyrazine[2]	nutty	*p-cresol*[2]	phenolic
2-isobutyl-3-methoxypyrazine[2]	earthy, musty	*pentanoic acid*[1]	sweaty
2-methoxyphenol[1]	smoky	*phenyl acetic acid*[1]	honey like
2-methoxy-4-vinylphenol[2]	spicy	*phenylacetaldehyde*[1,2]	honey like
2-phenylethanol[1]	honey like	*trans-4,5-epoxy-(E)-2-decenal*[1]	metallic
3-ethylphenol[1]	phenolic	*vanilin*[1,2]	vanilla like
3-hydroxy-4,5-dimethyl-2(5H)-furanone[1,2]	seasoning	*β-ionone*[1]	violet like
3-methylbutanal[1,2]	malty	*β-damascenone*[1,2]	honey like
3-methylbutanoic acid[1]	sweaty	*γ-nonalactone*[1]	coconut like

[1] *Volatiles identified in Darjeeling Black tea by Schuh et al.(2006) [60]*

[2] *Volatiles identified in Sen-cha, Kamari-cha and Longjing green teas by Kumazawa et al. (2002)*[72]

Volatile compounds have also been used to discriminate among various teas. Using only five volatiles (trans-2-hexenal, benzaldehyde, methyl-5-hepten-2-one, methyl salicylate and indole), it was possible to separate non-fermented teas green teas (34 samples) from fermented ones (22 samples)[67]. In another study, seventy-five oolong teas from five different varieties were also discriminated by their volatile content[68]. Similar approaches based in either GC-MS or eNoses have been successfully applied for the discrimination of teas according to their quality[69–71].

Although with some discrepancy in the discrimination efficiency, attempts of discriminating tea origin by their volatile content have also been reported. Togari and co-workers applied pattern recognition techniques to volatiles extracted from 44 different samples and analyzed by GC-MS. They could classify black teas according to 3 origins, but classification was not possible for green or oolong teas[73]. Other study reported successful classification of green teas from two different regions in China also based on GC-MS data[74]. Baldermann and coworkers analyzed 38 samples belonging to 5 different origins. They could discriminate teas according to fermentation (green, oolong and black teas) by cluster analysis. Although most samples from same origin were clustered together, some samples were misclassified and was not possible to relate origin with volatile profile[75]. Lee et al. neither found any relationship between the country of origin and the volatiles measured in the headspace of 24 different green teas by SPME/GC-MS[76]. All these difficulties in discriminating tea samples points to the complexity and variability of their volatile composition.

2.4. Tea brewing.

Tea brewing consists of the immersion of tea leaves in hot water during a defined period of time. Although this process might seem simple a priori, the optimal brewing conditions depend on the kind of tea, culture, social environment and individual preferences. Therefore tea brewing can go from a ceremonial preparation like the Japanese tea ceremony to pouring boiling water in a mug containing a tea bag in UK, to exemplify two extreme situations. The main factors affecting the quality of the final tea infusion are the water/tea ratio, leaf shape and size, water composition, water temperature and brewing time.

As it happens in the case of coffee, work studying the kinetics of extraction of tea compounds has been focused on caffeine and other health related compounds, mainly polyphenols. Detailed studies on the kinetics of caffeine and theoflavins extraction have been performed by several authors. Spiro and Jago stated that, according to the two phase and steady-state theories, the extraction kinetics for soluble compounds in tea leaves should obey first-order kinetics (Equation 1)[77].

$$\ln\left(\frac{C_\infty}{C_\infty - C}\right) = K_{obs}t \qquad \textit{Eq. 1}$$

Where C is the concentration of the compound at time t, C_∞ is the concentration at equilibrium and K_{obs} is the overall rate constant.

In a series of papers, Spiro, Price and other authors have used equation 1 to fit experimental data and describe how the kinetics and equilibria of tea infusions are affected by the leaf size[78], water composition[79–81] or extraction temperature[82–84] amongst other factors[85–88]. Their results suggest that the rate determining step for extraction of tea components into water is the diffusion of the component through the leaf matrix to the surface. Therefore, the extraction rate will depend on

compound structure, mass, solubility, water temperature and the concentration gradient between the tea leaf and the solution.

Leaf size had a minimum effect on the equilibrium concentrations of caffeine and theaflavin[89] but it affected their extraction kinetics[78]. Smaller particle size resulted in larger rate constants. For full leaves, extraction takes part mainly through the two large surfaces of the leaf, but the effect of the edges becomes important when the particle size is reduced, increasing the surface area of the leaf, and enhancing the extraction rate. In the case of fine ground tea leaves, caffeine was found to be extracted fast from leaves into the infusion, with no significant differences between 0.5 to 30 minutes infusion time [90]. The enhanced extraction of soluble compounds from fine ground leaves resulted in increased antioxidant activity and higher concentrations of polyphenols and flavonoids in the final beverage. However, as it often happens with flavor, those infusions were found extremely bitter and astringent, therefore being less pleasant than infusions prepared from full leaves[91].

The dissolved mineral content of water also impacted the extraction kinetics of caffeine[80] and theflavins[81] but had no influence on the equilibrium concentration of those compounds[79]. In the case of caffeine, minerals can modify its solubility affecting extraction kinetics. A decrease on caffeine extraction rate was found with NaCl, NaOAc, KCl or $CaCl_2$ that correlates with a decrease in caffeine solubility in the presence of those minerals. On the other hand, higher extraction rate is obtained with Bu_4N^+ ions which increase caffeine solubility[80,92]. In the case of theaflavins, addition of salts slightly increased extraction rate with no significant differences between the different salts, with the exception of $CaCl_2$ which highly decreased the extraction rate. This pronounced decrease in extraction with $CaCl_2$ have also been observed for caffeine, polyphenols and the total organic carbon content of tea infusions, and it has been related to the formation of Ca-pectin complexes. These complexes modify the cell wall properties, affecting the diffusion of compounds, hence hampering extraction[93].

In the case of volatile compounds, research has focused on the identification of the volatiles present in the final infusion (after brewing) and not on the extraction process itself. To the best of our knowledge, no studies have been reported about the kinetics of volatile compounds extraction from tea leaves into the infusion and how this extraction is affected by different brewing parameters (e.g. temperature, leaf size or water composition).

3. Aroma.

While the biological purpose of eating food is nutrition, it also has a very important hedonic element. The pleasure associated with food consumption is a result of the stimulation of different receptors and it involves the five human senses. The multimodal nature of food sensation –commonly referred as flavor– implies not only the combination of different senses but the interaction between them (crossmodality) to form the final flavor perception[94]. Among all the attributes that result in flavor perception, aroma plays a key role. Aroma substances are those volatile compounds that are detected at the sensory receptors located in the olfactory epithelium, inside the nasal cavity. Odorants can reach the olfactory receptors during inhalation through the nose (orthonasal) or via the throat (retronasal) after they are released due to oral food processing and also after swallowing.

To date, more than 8000 volatile compounds have been identified in food products, exhibiting a broad range of physicochemical properties. In a single foodstuff, the volatile fraction varies from few hundred compounds in the case of simple natural products (e.g. strawberries) to more than 900 in complex

products (e.g. roasted coffee)[95]. Despite the large number of volatiles present in foods, only a small fraction is responsible for the final aroma as their concentration has to be higher than their odor threshold (OT) in order to be relevant for sensation. The OT is defined as the lowest amount of an aroma compound needed in order for humans to recognize its odor. OT concentrations are compound dependent and vary several orders of magnitude, from few pptv to ppmv. The OT also depends on the medium, temperature, the route of perception (orthonasal or retronasal) or the interactions with other aroma compounds present in the food product. Therefore, in order to allow comparison between compounds, OT values are generally calculated in water or air by orthonasal evaluation. A common practice to determine which compounds contribute to the final aroma of a certain food is to calculate their odor activity values (OAV), which is the ratio between the concentration of the compound and the OT value in that specific food[96]. Compounds with OAV>1 are present in a higher concentration than their thresholds and will most probably contribute to the aroma.

3.1. Aroma analysis.

The large number of aroma compounds, their chemical diversity, the low concentrations at which they can be sensorially significant and the varied physicochemical characteristics of the food matrices complicate aroma analysis. Unfortunately, there is not a universal method to study aroma and a careful choice of the analytical procedure has to be done depending on the objective of the study, food material and volatiles of interest. The main purposes of volatile analysis in food are: (i) identification of the key compounds contributing to the aroma of the product, (ii) obtaining the volatile profile of the sample, (iii) detection of any off-odors that affect the final quality of the product, (iv) monitoring changes in time (aroma production, release or degradation), (v) linkage of volatile content to sensory attributes and (vi) checking the authenticity of the food product or existence of fraud related to its composition[97]. Independently of their objective, analytical methods for aroma analysis can be divided into two different processes: sampling or isolation of the volatile fraction and analysis of the volatile fraction.

3.2. Isolation of the volatile fraction.

The first step in aroma analysis is the separation of the volatile fraction from other compounds present in the sample. The complexity of this step depends on the number of volatiles that are object of study, their concentration, the complexity of the matrix and the technique that will subsequently be used for the analysis; it can also be a rather simple process such as the withdrawal of a fraction of the headspace above the food product, or a very elaborate process involving the combination of extraction, distillation and concentration steps. The main methods for aroma isolation will be described, pointing out their advantages and disadvantages.

3.2.1. Distillation and Solvent extraction.

The two simplest methods to separate aroma compounds from the food matrix are direct extraction of the volatiles from the food matrix with an organic solvent, and distillation. Distillation can be performed by heating the food matrix, applying vacuum or a combination of both. The high volatile compounds are then condensed on a cooled trap. When high vacuum is used for distillation, water is also drawn into the distillate and a further extraction with an organic solvent is needed. Nickerson and Likens developed a method for the simultaneous distillation and extraction of volatiles with organic solvents[98]. Simultaneous distillation-extraction (SDE) is one of the most flexible aroma isolation techniques but the use of high temperatures for distillation might result in the generation of artifacts. More recently, Engel and co-workers developed a new distillation system called Solvent Assisted Flavor Evaporation (SAFE)

that reduces artifact formation[99]. The main problem of both SDE and SAFE resides in the bias introduced by the different solubility of the compounds in the different solvents. Figure 7 shows how the volatile recovery varies depending on the solvent used for extraction, thus resulting in completely different volatile profiles.

3.2.2. Static Headspace (SHS).

In SHS, the sample is placed in a closed vessel, leaving some air between the sample and the lid of the vessel. Then the sample is heated at a fixed temperature during a certain time period, until equilibrium is reached between the sample and the air in the headspace above it. At that point, a few mL of air are drawn for analysis. Static headspace is, a priori, the best method for aroma analysis as it directly reflects the genuine volatile composition that arrives to the olfactive receptors (orthonasally). The main drawback of SHS is its lack of sensitivity for aroma applications as it is difficult to detect compounds at low concentrations in a small volume of the headspace air. Considering that several important aroma compounds have very low threshold values and occur at very low concentrations in the headspace, the lack of sensitivity of the SHS technique represents a real limitation in aroma applications. As it will be discussed later, one of the most common techniques for aroma analysis is gas chromatography coupled to mass spectrometry (GC-MS). When SHS is coupled to GC-MS, the detection limit is in the ppb range while the concentration of most aromas in the headspace is in the ppt range, therefore allowing detection of only the major volatile compounds[100]. In order to overcome these problems, SHS can be used with high sensitivity instruments (e.g. GC-Chemoluminesence, PTR-MS, eNose) or the headspace can be concentrated into a sorbent (e.g. purge and trap, SPME) in order to increase sensitivity.

3.2.3. Dynamic Headspace (DHS)

A continued flow of an inert gas is bubbled through the sample or blown above its surface in order deplete the maximum amount of volatiles into the headspace. The gas leaving the vessel is then passed through a trap where volatiles get retained either by condensation (cryogenic trap) or adsorption (adsorptive polymer trap). This is the reason why DHS is commonly referred as purge-and-trap method. Volatiles trapped are then released by fast heating and drawn by a carrier gas into the analytical instrument (e.g. GC-MS). This method is fast, simple, and easily automated, but still has some disadvantages. The main problem of cryogenic traps is that they will also condensate water which might need to be separated again from the volatile fraction. The use of adsorptive polymer traps overcome the problem of trapping water but, as the different compounds can have different adsorption affinities for the polymeric phase, these traps alter the volatile profile of the headspace analyzed. For example, the widely used TENAX trap has high affinity for nonpolar compounds but low for polar ones, therefore resulting in selective enrichment of the first ones and loss of important aroma compounds.

3.2.4. Solid-phase micro-extraction (SPME)

Solid phase micro extraction was developed in the early 90s at Pawliszyn group as a solvent free sample preparation technique[101], and it has become one of the most popular techniques to extract volatiles from samples' headspace and thermally desorb them into a GC. It consists of a fused-silica fiber (inert) coated with a polymeric stationary phase incorporated into a syringe-like device. In the case of headspace sampling, the syringe is placed inside the vessel containing the sample and the stationary phase is then exposed to the air in the headspace. When the stationary phase is exposed, partition of the volatiles between the headspace and the polymeric coating occurs. Once equilibrium between the fiber coating and the headspace is reached, the fiber is removed and directly placed in a GC injector for thermal

desorption. This procedure can be done manually or easily automated using an autosampler. As the principle of SPME is the partition of the analyte between fiber and headspace of the sample, fiber coatings can be selected to have high affinity for specific compounds and a wide range of stationary phases with different polarities is commercially available. The high surface area of the coating, together with its high affinity for organic compounds, results in concentration of the volatiles in the fiber, thus substantially increasing the sensitivity of headspace sampling[102]. Despite SPME being one of the most used techniques for aroma analysis, it also has some associated problems. As it is an equilibrium technique, the volatile profile obtained is dependent of the sampling parameters (temperature, time), the absorption/adsorption properties of the stationary phase used and the competition of different volatiles for the adsorption sites of the fiber[103]. Another important drawback is the stability of the fiber over time and the differences between fibers of different batches that complicate the comparison of results from different fibers. These disadvantages can be overcome with the use of isotopically label standards that allow the quantification of the compounds and correct for differences in fibers performance, making SPME a valuable tool for target analysis.

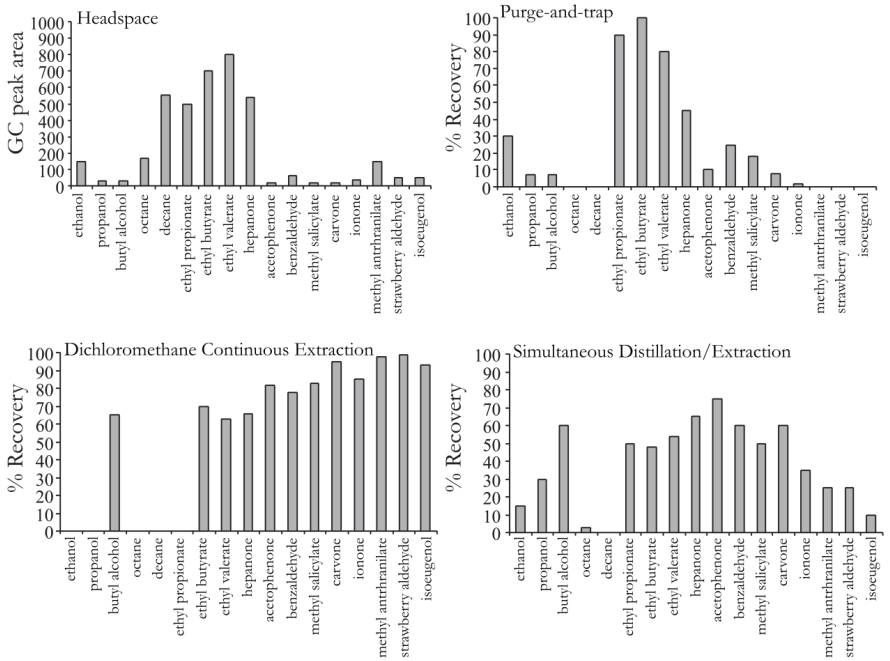

Figure 7. The recovery of volatiles depends on the isolation method and has to be chosen wisely depending on the objective of the analysis. Figure adapted from Reineccius 2005 [104]

3.3. Analysis of the volatile fraction.

3.3.1. Gas chromatography (GC)

Modern gas chromatography is commonly attributed to the invention of Martin and James presented in 1952[105]. The contribution of GC to volatile analysis is beyond doubt. GC can be considered a reference method for trace volatile analysis and therefore for aroma analysis. This technique basically consists in the separation of volatiles from a mixture by partitioning between a gas phase (mobile phase) and a solid or liquid one (stationary phase). Nowadays the stationary phase is generally a thin layer of a viscous liquid coating the inside of a capillary column. The analyte is injected upstream of the column

and drawn by a carrier gas through the column. The different interactions between the volatile compounds and the stationary phase result in separation of the compounds which elute the column at different times (retention time). Those interactions will depend on the column coating and temperature, thus the column is kept inside an oven to control temperature over time. At the end of the column, a detector produces a signal each time a compound is eluting.

3.3.2. Gas Chromatography Mass Spectrometry (GC-MS)

Several detectors can be coupled to GC, each one with its own strengths and drawbacks. The selection of the GC detector will depend on its selectivity for the analyte of interest, its sensitivity, linear range or limit of detection. GC detectors have been reviewed by Colón and Baird[106] and only the mass spectrometer (MS) detector will be discussed here as it is the most commonly used for aroma analysis. The basic configuration of an MS detector consists of an electron impact (EI) ionization source, a quadrupole mass filter, an electron multiplier and an ion counter. Standard electron ionization uses 70 eV which is higher than the energy needed to ionize most organic compounds. Therefore, the excess of energy results in high fragmentation of the organic molecules. Fragmentation could be considered a negative aspect of EI but the mass spectra obtained gives structural information about the compound and it can be used as its fingerprint. The GC retention time together with the mass spectra obtained by EI-MS can be compared with libraries (e.g. FFNSC GC/MS library) for compound identification.

GC-MS is a reliable and well stablished technique in aroma analysis. Its main advantages are the separation of the compounds, that can be improved by the use of multi-dimensional GC (i.e. GCxGC); and the possibility to identify compounds by their mass spectra. But it also has some limitations. The first one is the lack of sensitivity, which makes almost imperative the isolation and concentration of the aroma compounds prior to analysis. A second limitation is the analysis time. The time needed for chromatographic separation, together with the time needed for pre-concentration of the compounds, limits the amount of samples that can be analyzed in a daily basis. This time constraint has a big relevance when fast changes in volatile composition need to be monitored as it happens in the case of flavor generation or release.

3.3.3. Gas chromatography – olfactometry (GC-O)

GC can also be used to detect which compounds contribute to the aroma of food. When it comes to aroma analysis, the reference detector is indeed the nose. Considering that the human nose is more sensitive for some odor active compounds than any other detector coupled to GC, techniques were developed where the detector was replaced by a real human nose: GC-Olfaction (GC-O). The GC-O approach consists in sniffing the effluent of the GC column in order to identify the aroma quality at the respective retention times for odor active molecules[66]. Generally, the effluent is split in two, one part going to the sniffing port and the other to a detector (e.g. MS), so the chromatographic peaks can be associated to an odor descriptor[107]. GC-O is a qualitative technique per se, but quantitative data can be obtained in combination with dilution analysis methods (e.g. AEDA, CHARM). Those methods consist in the sequential dilution of the aroma extract until the odor cannot be detected. The dilution for the lowest concentration at which a substance can still be smelled is known as Flavor Dilution (FD) factor. It is assumed that the higher the FD of a compound in the mixture, the higher its contribution to the aroma of the foodstuff will be[65].

3.3.4. Direct injection mass spectrometry (DIMS)

Analytical techniques based on direct injection mass spectrometry (DIMS) have been successfully used to monitor volatile organic compounds in different fields, including food analysis. They overcome some of the limitations of GC-MS methods as they allow on-line analysis of volatiles in real time, without any pre-treatment and with high sensitivity. As no chromatographic separation takes place, it is crucial that the ionization is soft (i.e. does not result in compound fragmentation) to ease the interpretation of the recorded mass spectra. It is also important to use mass spectrometers with fast response (≤1 Hz) to follow fast dynamic processes and with high mass resolution to discriminate between isobaric compounds (i.e. same nominal mass). Some of the main soft ionization DIMS methods will be discussed in this section.

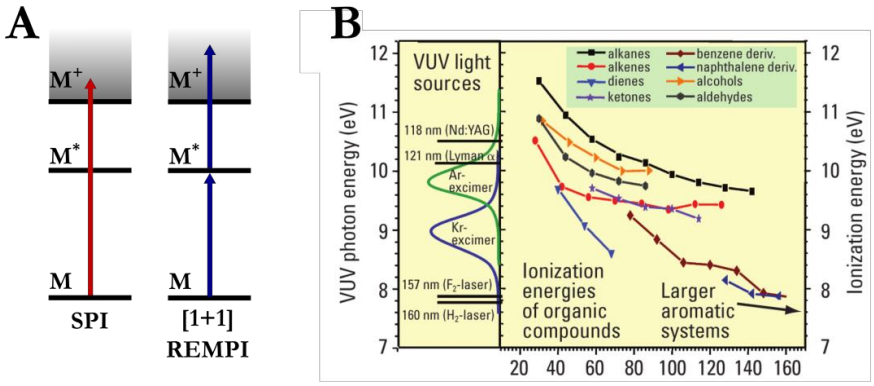

Figure 10. A) Schematic mechanism of SPI and REMPI ionization (M: electronic ground state; M^*: excited intermediate state; and M^+: ionized product). B) Ionization energies for some compound groups and energy of VUV light sources. Adapted from Hanley and Zimmermann[108]

3.3.4.1. Photoionization

Pulsed lasers can be used for gas-phase soft ionization of organic molecules with high sensitivity. The two main photoionization methods are resonance-enhanced multi-photon ionization (REMPI) and vacuum ultraviolet-single photon ionization (VUV-SPI). In REMPI, the energy of a single photon is lower than the ionization potential (IP) of the molecule, therefore the absorption of at least a second photon is needed to ionize the molecule. Although ionization is possible without excitation via an intermediate state, the ionization is strongly enhanced when the energy of the first photon is in resonance with an excited state of the neutral molecule (Figure 10A)[109]. Therefore, REMPI has a great selectivity because the wavelength can be selected in order to ionize only some of the molecules of a mixture (e.g. aromatic compounds), which can be used to discriminate between isomers[110,111]. This high selectivity makes REMPI a valuable ionization scheme for targeted analysis but restricts its use as a universal ionization method. By contrast, SPI, while retaining some selectivity, results in soft ionization of all organic compounds and can be considered a universal soft ionization method. It is based in the absorption of a single VUV photon. This photon has energy between 8 and 11 eV and most compounds have ionization energies lower than 10 eV (Figure 10B). This will result in ionization of the molecule without fragmentation due to the small excess of energy. At the same time, common inorganic constituents of air have higher ionization energies and are not ionized by SPI (O_2 12.06 eV, N_2 15.58 eV, CO_2 13.77 eV and H_2O 12.62 eV), making VUV-SPI a versatile tool to directly and selectively analyze

volatile organic compounds in gas samples[108]. Photoionization mass spectrometry has been mainly applied to direct online monitoring of gas samples and some applications in food sciences will be presented in section 5.

3.3.4.2. Atmospheric Pressure Chemical Ionization (APCI)

Ionization of volatile organic compounds by proton transfer reaction (PTR) via the hydronium ion (H_3O^+) is a universal mechanism. For compounds with higher proton affinity than that of H_3O^+ (Table 4) the following reaction occurs:

$$H_3O^+ + R \underset{k}{\rightarrow} H_2O + RH^+ \qquad \textit{Eq. 2}$$

The simplest method to produce hydronium ions is APCI. Ions are generated from air due to a corona discharge (e.g. N_2^+) and react with the water vapor present in air to generate H_3O^+ and water clusters $(H_20)_nH^+$[112]. Ion-molecule reactions in the gas phase result in the ionization of the volatile compounds by proton transfer, adduct formation or charge transfer.

Table 4. Proton affinities (PA) of some compounds.

Molecule	PA (kJ mol^{-1})	Molecule	PA (kJ mol^{-1})
O_2	421	ethanol	776
N_2	494	phenol	817
CO_2	541	acetaldehyde	769
O_3	626	propanal	786
H_2O	691	acetone	812
NH_3	854	2-pentanone	833
methane	544	acetic acid	784
ethene	681	propanoic acid	797
benzene	750	acetonitrile	779
methanol	754	dimethyl sulfide	831

Source: Hunter and Lias 1998[113]

One of the limitations of APCI is the complexity of the mass spectra due to the presence of different ionization agents that can produce other species than the protonated molecule RH^+; this complicates the identification of the compounds based on their m/z values. With a proper design and controlling the instrumental conditions, it is possible to obtain a mass spectra containing mainly the protonated molecular ions[114]. APCI has been successfully used for headspace analysis[115,116], in-vivo aroma release during consumption[117,118] and food authenticity[119].

3.3.4.3. Selected Ion Flow Tube (SIFT)

Selected ion flow tube mass spectrometry (SIFT-MS) has been the technique of choice to study the kinetics of ion-molecule reactions. In SIFT, reactant ions (e.g. H_3O^+, NO^+) are selected from all the ions formed in the ion source via a quadrupole mass filter and then injected with a carrier gas (He) into the flow tube. Analyte volatiles are also injected via a separate inlet into the flow tube where the ionization reaction takes place:

$$A^+ + R \underset{k}{\rightarrow} B^+ + C + D \ldots \qquad \textit{Eq. 3}$$

Finally, the formed ions are detected by a mass spectrometer. By introducing a known concentration of analyte gas (R) via a flow meter, the decay of the primary ion (A^+) as a function of the analyte flow rate can be measured and used to calculate the reaction rate coefficient (k). Similarly, for reactions with

known k, it is possible to calculate the concentration of the volatile compounds in a mixture from the intensities of the product ions and the primary ion.[120]. A limitation of SIFT-MS is its sensitivity. Due to the primary ion selection process, a low ion number density is found in the flow tube. However, sub-ppb concentrations can be measured at expenses of increasing the ion counting time of the mass detector. SIFT-MS applications for real-time quantification of VOCs have been reported in different fields including food sciences, and they have been reviewed by Smith and Španěl[120].

3.3.4.4. Proton transfer reaction (PTR).

Proton transfer reaction mass spectrometry (PTR-MS) was developed in the 1990s by Werner Lindinger's group [121–123]. PTR-MS can be considered as an evolution of SIFT-MS with the aim of increasing its sensitivity for quantification of organic volatile compounds in complex gas mixtures. There are a few and specific modifications in the design which drastically modify the performance of PTR-MS in comparison to SIFT-MS. The first one is the ion source which has been replaced to ensure an almost pure source of H_3O^+, eliminating the need for a mass filter. Another difference is the replacement of the flow tube by a much shorter drift tube in which the analyte gas is delivered without the need of using helium as a carrier gas. The kinetic energy is provided to the ions by an electric field inside the drift tube. This allows increasing the amount of analyte gas and therefore the sensitivity of the instrument. The negative effect of using an electric field is that the additional collision energy can result in some fragmentation. PTR-MS has been the technique used in this thesis for the analysis of volatile compounds and therefore it will be described in more detail.

3.3.4.4.1. Components of PTR-MS instrument.

PTR-MS instruments consist on three basic parts: the ion source, where the hydronium ions are formed; the drift tube, where the proton transfer reaction takes place; and the mass spectrometer, to separate ions according to their m/z ratio. (Figure 11).

3.3.4.4.1.1. Ion source.

The ion source is the region of the instrument where the hydronium ions, needed for the proton transfer reaction, are produced. Although some alternative ion sources have been suggested (direct current discharge[124], circular glow discharge[125] or radioactive[126] ion sources) most of the PTR-MS instruments are equipped with a hollow cathode discharge ion source. Two main parts can be differentiated in the ion source: the hollow cathode region (HC) and the source drift region (SD) (Figure 11). Water vapor is introduced in the HC region where it is ionized by electron impact and converted in the following ions[127]:

$$e^- + H_2O \rightarrow H_2O^+ + 2e^- \quad \textit{Eq. 4}$$

$$e^- + H_2O \rightarrow OH^+ + H + 2e^- \quad \textit{Eq. 5}$$

$$e^- + H_2O \rightarrow O^+ + 2H + 2e^- \quad \textit{Eq. 6}$$

$$e^- + H_2O \rightarrow H^+ + OH + 2e^- \quad \textit{Eq. 7}$$

$$e^- + H_2O \rightarrow {H_2}^+ + O + 2e^- \quad \textit{Eq. 8}$$

To ensure a high purity (>99.5%) of hydronium ions leaving the ion source, a SD region is included. In the SD region, the ions previously formed by electron impact undergo ion-molecule reactions that lead to the production of H_3O^+ (or an ion that can be further converted into H_3O^+)[122]:

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH \quad \textit{Eq. 9}$$

$$OH^+ + H_2O \rightarrow H_3O^+ + O \quad \textit{Eq. 10}$$

$$OH^+ + H_2O \rightarrow H_2O^+ + OH \quad \textit{Eq. 11}$$

$$O^+ + H_2O \rightarrow H_2O^+ + O \quad \textit{Eq. 12}$$

$$H^+ + H_2O \rightarrow H_2O^+ + H \quad \textit{Eq. 13}$$

$$H_2^+ + H_2O \rightarrow H_3O^+ + H \quad \textit{Eq. 14}$$

$$H_2^+ + H_2O \rightarrow H_2O^+ + H_2 \quad \textit{Eq. 15}$$

Impurity ions can be formed in the SD region due to some air entering from the drift tube region. These ions are mainly O_2^+ and NO^+ and, although in relative low quantities (<1%), can undergo reactions with the analyte in the drift tube complicating the interpretation of the mass spectra.

3.3.4.4.1.2. Drift tube.

The drift tube can be considered the main part of PTR-MS instruments as it is where the ion-molecule reactions take place (mainly proton transfer), via collision between hydronium ions and analyte molecules. Reactions happening in the drift tube will be briefly discussed in section 4.2.4.6.

The drift tube consists on a series of electrodes to which a voltage is applied, generating a uniform electric field along the drift tube. The analyte gases are introduced upstream of the drift tube where they mix with the positive ions coming from the ion source. These positive ions reach a constant velocity as a result of two opposite forces: acceleration due to the applied electric field and collisions with the neutral gas molecules. A portion of those ions leave the drift tube through an orifice, and are guided by a transfer lens system into the mass spectrometer.

The drift tube can be heated up to 120 °C in order to avoid condensation of volatiles. It is generally operated at pressure values around 2 mbar with an electric field strength (E) near 60 $V \cdot cm^{-1}$. These parameters are commonly combined and referred to as the reduced electric field value of the drift tube (E/N), where N is the gas number density (cm^{-3}). E/N values are expressed in Townsends (Td, 10^{-17} $V \cdot cm^{-2}$) and can be easily adjusted in PTR-MS instruments in order to control the proton transfer reaction[128]. High E/N values result in higher collision energies between H_3O^+ and the analyte molecules resulting in undesired fragmentation that can complicate the interpretation of the mass spectra. PTR-MS instruments are commonly operated at E/N values between 100 and 140 Td.

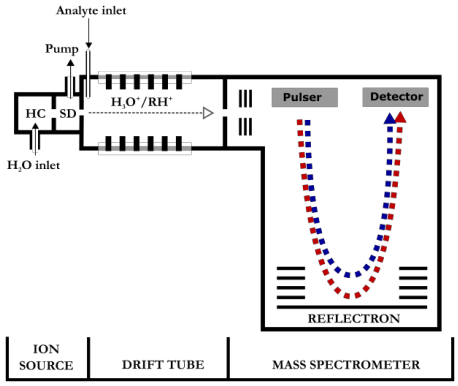

Figure 11. Scheme of a PTR-TOF-MS instrument

3.3.4.4.1.3. Mass spectrometer.

In PTR-MS, volatile compounds are directly injected into the drift tube without previous separation. The function of the mass spectrometer is to separate the ions generated in the drift tube according to their m/z ratio. Both mass accuracy and mass resolution of the mass spectrometer are crucial in PTR-MS for identification of compounds according to their exact mass and discrimination between near-isobaric compounds (compounds with the same nominal mass).

Mass accuracy is defined as the difference between the mass measured in the mass spectrometer and the theoretical mass of the compound. It is generally expressed in ppm and calculated according to:

$$mass\ accuracy = \frac{(m/z_{measured} - m/z_{theoretical})}{m/z_{theoretical}} x\ 10^6\ ppm \qquad Eq.\ 16$$

High mass accuracy is needed to assign the elemental composition (i.e. molecular formula) to each individual ion. As the mass of the ion increases, the number of possible molecular formulas increases as well; therefore, a higher mass accuracy is needed for a reliable compound identification. For example, the theoretical mass for protonated 2,3-butanedione is 87.0446. If the mass accuracy is 15 ppm, this means that the measured mass is in the range 87.0446 ± 0.0013. In that range, only one molecular formula can be assigned ($C_4H_7O_2$), therefore the mass accuracy is good enough to determine the elemental composition. If protonated caffeine (m/z 195.0882) is measured with the same mass accuracy, the difference between the measured mass and the theoretical one could be up to 0.003 amu. In that range, 16 different molecular formulas can be assigned to the measured mass, implying that the accuracy is not enough for compound identification. In some cases, previous knowledge about the compounds that might be present in the sample can be used to tentatively identify the compound when the mass accuracy is not high enough to get a unique molecular formula from the experimentally measured mass.

Another important parameter of the mass spectrometer is the mass resolution, which is the ability to distinguish between two different peaks. Mass resolution is calculated as the quotient between the measured m/z and the width of the peak (Δm) at a specific fraction of total height (usually 50%). As it can be seen in Figure 13 (left), with high mass resolution it is possible to distinguish two different peaks: m_1 and m_2. As the resolution decreases, the peak width increases to the point in which only one peak can be distinguished. In the case of low mass resolution instruments (e.g. quadrupole), nearly-isobaric compounds mix up in the same peak, preventing compound identification. As an example, a commercial PTR-ToF-MS instrument was capable to distinguish between 2,3-butanedione and 3-methylbutanal in a coffee sample (Figure 13 – center).

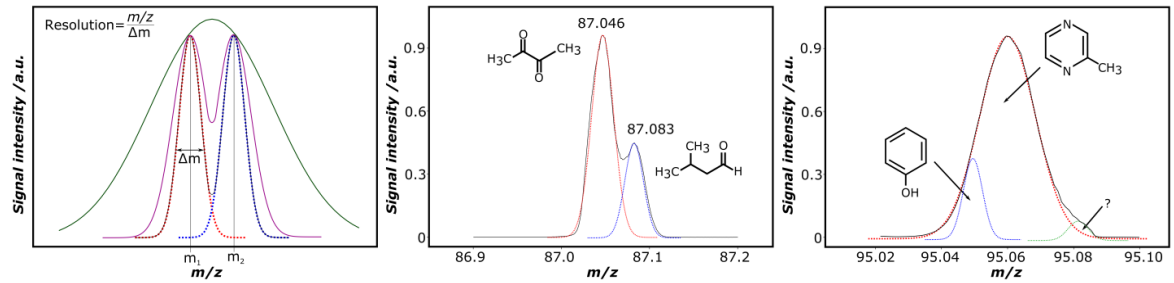

Figure 13. Left: Scheme showing the effect of different mass resolution in peak separation. Center: two nearly isobaric compounds (2,3-butanedione and 3-methylbutanal) in a coffee sample analyzed by PTR-ToF-MS. Right: Mass peak tentatively identified as methylpyrazine in a coffee sample analyzed by PTR-ToF-MS which might hide other compounds as phenol. Although native molecules are drawn, the mass peaks measured in PTR-ToF-MS correspond to the protonated molecules MH^+.

Hence, in PTR-MS, high mass resolution is needed in order to separate and identify the compounds in the sample. Three main types of mass analyzers have been used in PTR-MS instruments: quadrupole, ion trap and time of flight (TOF) mass spectrometers.

The first PTR-MS instruments were equipped with a quadrupole filter due to its robustness, compact size, low cost and high sensitivity and dynamic range[121,128]. Ion separation via quadrupole mass filters is based on the stability of the trajectory of the ions in oscillating electric fields as a function of their m/z ratio. This working principle has some advantages and disadvantages. When a quadrupole is operated in single ion monitoring (SIM) mode (i.e. only one m/z is selected), it has a duty cycle of 100%. That means that all ions of that specific m/z introduced in the mass spectrometer will be detected, therefore resulting in high sensitivity. The problem arises when more than one m/z needs to be monitored or when all the m/z in a defined range have to be measured (full scan). As only one m/z travels through the quadrupole at a time, with all the others being excluded, the duty cycle is drastically reduced. For example, for a full scan between 0 and 500 amu, the duty cycle will be 1/500 = 0.2%, decreasing both the sensitivity and the scan speed. Another drawback of quadrupole filters is the low mass resolution. Although quadrupoles can reach resolutions up to 0.1 amu, most quadrupoles have low resolution and are able to discriminate only nominal masses[129]. The low mass resolution prevents compound identification, as near-isobaric compounds (i.e. compounds with same nominal mass) will be detected together. The slow scan speed and low mass resolution make quadrupoles not suitable for the online analysis of complex gas mixtures experimenting fast changes.

Ion trap mass spectrometers have been also coupled to PTR[130–132]. Ion traps also use oscillating electric fields but in this case they are used to store ions. Ions over the full mas range can be stored and scanned in milliseconds, resulting in a duty cycle >90%, which can go up to >99% by increasing the trapping time[131]. This fast response (<1s for a full spectrum) represents a huge advantage over quadrupole filters for monitoring fast dynamic processes. Another advantage is the possibility to perform collision induced dissociation, allowing identification of isobaric compounds[131]. The last advantage of ion traps over quadrupoles is the possibility to use lower electric fields in order to increase sensitivity. Low E/N values result in cluster formation, but those clusters are fragmented after isolation in the ion trap[132]. Despite the advantages of ion traps, the number of PTR-MS equipment using this mass spectrometer is rather low.

The actual tendency is to equip PTR-MS with a TOF mass spectrometer. In PTR-TOF-MS, m/z ratio is calculated using the time that ions take to move in a field-free region from the source to the detector. Ions coming from the drift tube enter the pulse extraction region were they are focused and accelerated into the field-free section. As the same kinetic energy is applied to each ion, their velocities will be inversely proportional to the square root of the mass, therefore taking different times to reach the detector[129]. In order to increase the mass resolution, a reflectron TOF-MS is preferred to a linear one (Figure 11). The reflectron is an electrostatic reflector that corrects for differences in the kinetic energy of the ions leaving the extraction region. Those ions with higher kinetic energy (red path in Figure 11), will penetrate deeper and will spend more time in the reflector. Consequently, slow and fast ions will reach the detector at similar times[129].

The possibility of getting a full spectrum in milliseconds, the high ion transmission, high sensitivity (pptv to ppqv range) and the high mass resolution (6000 – 10000 m/Δm) of commercial PTR-TOF-MS instruments[133,134] justify its choice for real-time online monitoring of fast processes in food industry (e.g aroma formation and aroma release). Figure 14 shows the mass spectra recorded for the headspace of ground roasted coffee. The full mass spectrum was acquired with a commercial PTR-TOF-MS instrument in one second. More than 200 mass peaks were found in the range 19-200 m/z. Thanks to the mass accuracy of the instrument, 100 compounds were tentatively assigned to mass peaks and the mass resolution allowed discrimination of compounds like furan (m/z 69.034) and isoprene (m/z 69.070) or furfural (m/z 97.028) and dimethylfuran (m/z 97.065). Unfortunately, PTR-TOF-MS cannot discriminate isobaric compounds as ethylmethylpyrazine and trimethylpyrazine (m/z 123.092). It also has problems to detect near isobaric compounds when the intensity of one of them is high. In figure 13 (right), a mass peak at m/z 95,060 was tentatively identified as methylpyrazine. In that coffee sample it was expected to find also phenol at m/z 95.049, but the high intensity of the methylpyrazine mass peak made separation impossible. In that case, higher mass resolution or previous separation of the compounds via fast-GC approaches is needed in order to discriminate between the compounds.[135]

3.3.4.4.2. Reactions in PTR-MS

The main reaction happening in the drift-tube of a PTR-MS instrument is non-dissociative proton transfer and it is defined by the reaction:

$$H_3O^+ + R \underset{k}{\rightarrow} H_2O + RH^+ \qquad \textit{Eq. 2}$$

This reaction is fast, usually taking place at collisional rate, and it is thermodynamically spontaneous when the proton affinity (PA) of the compound R is higher than the PA of hydronium ions. It enables ionization of most organic compounds (with exemption of alkanes) while not reacting with the common constituents of air. Although PTR is considered a soft ionization technique, some fragmentation is observed in the final mass spectrum. Some molecules can undergo dissociation after proton transfer, resulting in fragmentation into a charged fragment (F^+) and a neutral one (N):

$$H_3O^+ + R \rightarrow H_2O + (RH^+)^* \qquad \textit{Eq. 17}$$

$$(RH^+)^* \rightarrow F^+ + N \qquad \textit{Eq. 18}$$

An example of the dissociative ionization is the dehydration of alcohols, aldehydes or carboxylic acids[128]:

$$H_3O^+ + C_nH_{2n+1}OH \rightarrow 2H_2O + {C_nH_{2n+1}}^+ \qquad \textit{Eq. 19}$$

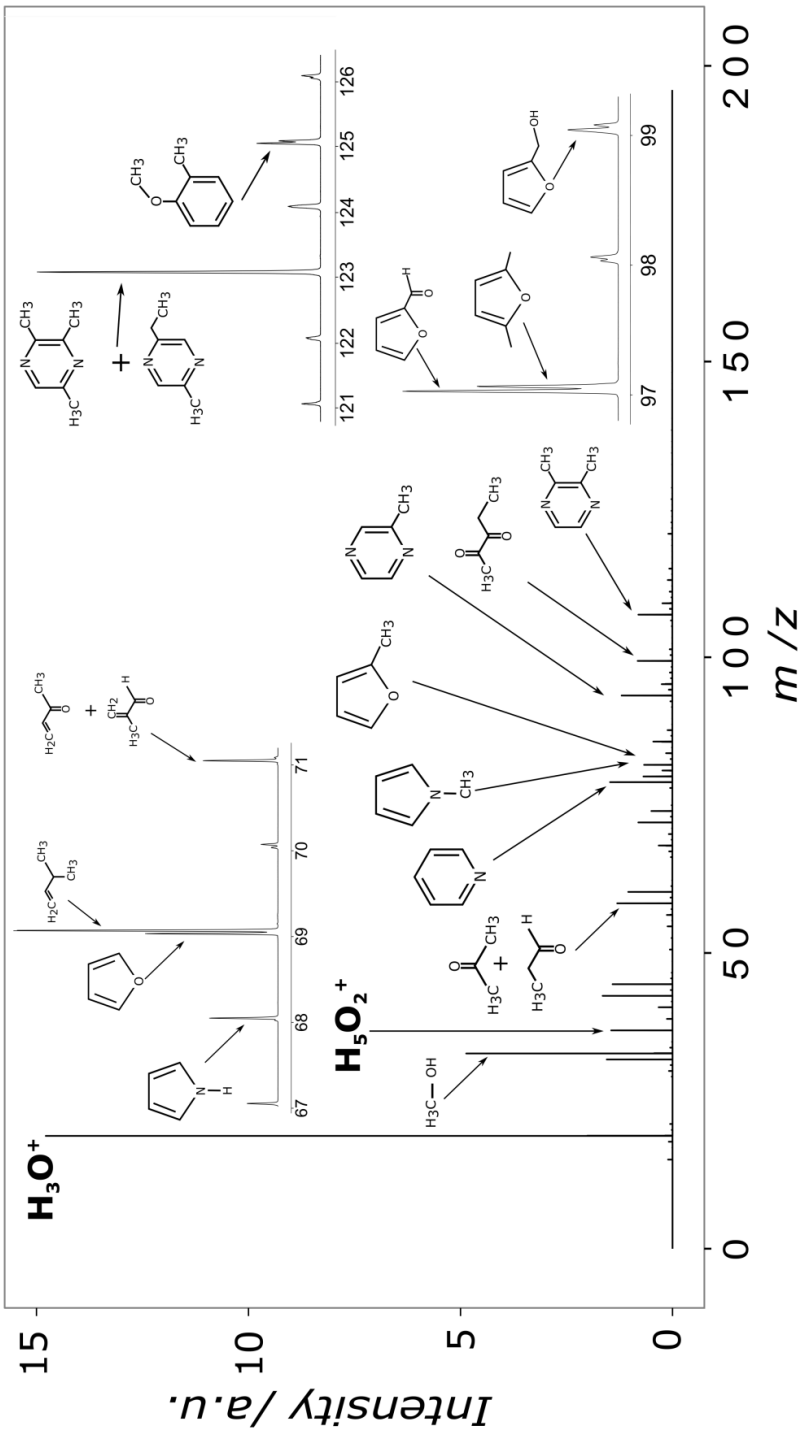

Figure 14. PTR-TOF-MS spectrum of roasted ground coffee headspace highlighting some of the main aroma compounds. Tentatively identified compounds are drawn as neutral molecule *M,* although the mass peaks correspond to the protonated compounds $[MH]^+$.

Other possible reaction with the hydronium ion is association, as it happens in the case of heptane and higher alkanes[128]:

$$H_3O^+ + C_nH_{2n+2} + M \rightarrow H_3O^+(C_nH_{2n+2}) + M \qquad \textit{Eq. 20}$$

Where M is a third body.

The main reactions between H_3O^+ and volatile organic compounds have been summarized in the book by Ellis and Mayhew[135] and are presented in Table5.

Table 5. Main product channels for reaction of H_3O^+ with volatile organic compounds at 300K

Functional group	*Reaction products*	*References*
Alkanes	No reaction for C5 and lighter alkanes. C_6 and higher species give only association products ($H_3O^+ \cdot M$) but reaction is much slower than the collisional limit. Cycloalkanes react at the collisional limit	[136,137]
Alkenes	Fast reaction for all alkenes except ethane. 100% MH^+ for small alkenes but fragmentation important for C_7 and higher alkenes	[136–138]
Alkynes	Slow (endothermic) reaction for acetylene but fast reaction for larger alkynes dominated by MH^+ production	[136–138]
Aromatic hydrocarbons	Yield almost exclusively MH^+	[137]
Alcohols	C_3 and higher alcohols show an increasing tendency to undergo dehydration on protonation. Dehydration is the only product channel for tertiary alcohols	[139,140]
Ethers	Main product MH^+, although dissociative channels grow in importance as the complexity of the ether increases	[141]
Aldehydes	100% production of MH^+ of C_3 and lower aldehydes, but increasing tendency to eject H_2O for C_4 and higher species	[142]
Ketones	Almost 100% production of MH^+ regardless of chain length	[142]
Carboxylic acids	Dominated by MH^+ formation, but accompanied by small amount of dehydration product	[143]
Esters	MH^+ formation is main channel for small esters but increasing propensity for major ion fragmentation, particularly once the alcohol conjugate is a propyl or higher unit.	[143,144]
Nitriles	100% production of MH^+	[145]
Amines	MH^+ main product, with minor channels involving alkene or H_2 loss also found for some amines	[145]
Organosulfur compounds	100% production of MH^+	[146,147]
Organohalides	Most halomethanes undergo slow reaction. Heavier halomethanes undergo a variety of reactions, some of which include termolecular association with H_3O^+. On the other hand, aromatic halides show 100% MH^+ production in fast reactions.	[148,149]

(Source: Ellis A.M and Mayhew C.A. Proton Transfer Reaction Mass Spectrometry: Principles and Applications. 2013)

The ion source of PTR-MS instruments is optimized to produce high purity H_3O^+, but unfortunately, the presence of unreacted water vapor from the source and from the sample leads to formation of water clusters $H_3O^+(H_2O)_n$. Despite the efforts to minimize the presence of water clusters, they are frequently present, especially when analyzing humid samples or operating at low E/N[128]. Proton affinity of water clusters is higher than that of H_2O, therefore some compounds will be ionized via proton transfer from H_3O^+ but not from the clusters $H_3O^+(H_2O)_n$. However, ligand switching reactions can occur:

$$H_3O^+(H_2O) + R \rightarrow H_3O^+(R) + H_2O \qquad \textit{Eq. 21}$$

These reactions lead to ions different from the expected RH^+, complicating the obtained mass spectrum in the case of complex gas mixtures and affecting quantitative analysis.

Recently, a new system has been developed that allows fast switching between H_3O^+, NO^+, O_2^+, Xe^+, and Kr^+ as primary ions. The system is commercially available under the name of Switchable Reagent Ion (SRI). Some advantages are the possibility to analyze compounds that are not ionized by H_3O^+ and to discriminate isobaric compounds due to different fragmentation[150,151]. The main drawback of using other reagent ions is that the ion chemistry is not as well-known as in the case of H_3O^+, thus complicating the mass spectra. Therefore these other reagents have been only used for target analysis or fingerprinting of samples for further classification using multivariate methods[151,152].

4. Applications of direct injection mass spectrometry in food science.

4.1. Food authenticity.

The aim of food authentication is to protect the consumer from fraudulent products. The three main authenticity issues are: (i) economic motivated adulteration of a food product, that comprise substitution or addition of a substance in the product to increase its apparent value while decreasing the cost; (ii) false declaration of geographical origin; and (iii) false declaration of farming regime (e.g. conventional products labelled as organic).

Laboratories dealing with food safety and authenticity require fast and high-throughput analytical techniques that allow fast analysis of a large number of samples. With DIMS techniques it is possible to get a fingerprint of the volatile composition of a sample within a few minutes, and without need of sample preparation. The classification of samples based on those fingerprints is possible even without compound identification, which further simplifies the process. Successful application of DIMS methods for food authentication have been reported in the literature for edible fats[153–156], cheese [157],dry-cured ham[158,159], honey[160], apple juice[119], strawberries[161], chocolate[162] or coffee[152,163–165] amongst others. Only the case of coffee will be discussed here as an example.

Coffee is a natural product that is generally marketed as roasted (or roasted ground) coffee beans. Commercially available coffees can belong to only one coffee variety (Arabica or Robusta), or can be sold as a blend of both varieties. Robusta has a lower market value than that of Arabica coffee; thus the incorporation of higher levels of Robusta than those specified in the label can be considered economic motivated adulteration. Using SPI-TOF-MS, it was possible to discriminate between Arabica and Robusta varieties by the ratio between two different terpenes: cafestol and kahweol[165]. Although the

study needed further statistical analysis, the potential of SPI-TOF-MS for discriminating the content of Robusta in different blends was clear.

Coffee origin is also an issue that affects the consumer. The origin of coffee has an impact in its sensory attributes and also in the price. Using PTR-TOF-MS coupled to an autosampler (Figure 15D) for the high throughput analysis of coffee headspace, roasted ground coffee samples from three different origins (Brazil, Ethiopia and Guatemala) were successfully separated by Principal Component Analysis (PCA - unsupervised method) and the separation was further confirmed by Partial Least Square Regression – Discriminant Analysis (PLS-DA – supervised method)[164]. In a follow up study, the same authors analyzed both the roasted ground beans and the coffee brews prepared with six coffees of different origins (Brazil, Ethiopia, Guatemala, Costa Rica, Colombia and India). In this case, different ions were used for ionization (H_3O^+, NO^+ and O_2^+). All the ions resulted in good classification of the sample, but combination of the information obtained for all the ions via data infusion techniques increased the efficiency of the classification[152]. Different coffee origins have also been distinguished by on-line monitoring of the roasting process with PTR-TOF-MS [163]. The recorded time-intensity profiles during roasting of coffee beans showed differences in flavor formation between the different origins, making possible their discrimination.

The last issue in coffee authenticity is the false declaration of farming regime. Organic and fair trade coffees present an added value and therefore their retail prices are higher than those of regular coffee. Using mass spectral fingerprints and multivariate analysis it was demonstrated that it is possible to discriminate between organic and regular coffee[171]. The study involved the headspace analysis of 110 commercially available coffees by PTR-MS. PLS-DA clearly separated organic coffees (43 samples) from regular ones (67 samples) according to their volatile profiles.

4.2. Food quality

Food quality is of utmost importance for the consumer. First, because the consumer expects the food product to be fresh, good looking and to have a pleasant taste when consuming it; and even more important, because the product has to be safe for consumption. DIMS methods are precise, reliable and allow rapid screening of samples, which allows their use as quality control techniques. Furthermore, they are non-destructive methods which allow the long-term studies to determine the impact of storage conditions in the food product.

Lipid oxidation in food is associated with negative aroma attributes, like rancid, and it has been studied by DIMS in several food products. For example, the accelerated thermal oxidation of New Zealand extra virgin oil was followed by SIFT-MS. Out of the 13 compounds analyzed, propanal, acetone and acetic acid increased their levels with oxidation but it was not possible to correlate sensory rancidity with the volatile profile obtained[172]. In a similar study with Italian extra virgin oils, the evolution of the volatile profile was followed during thermal oxidation. Volatiles correlated with peroxidase activity were identified and mainly comprise aldehydes which were associated to rancid smell[173]. Lipid oxidation aldehydes have been also analyzed in the headspace of tomato that was left at three different temperatures (4, 23 and 37°C) after blending. SIFT-MS measurements revealed that, while the increased concentration of most compounds with temperature was related to the increase in volatility, three compounds (E-2-pentenal, E-2-heptenal and E-2-octenal) were produced at much higher rates at 37°C. Based on those results, the authors proposed a new temperature dependent lipoxygenase pathway in

which some unknown enzymes were activated at 37°C[174]. As a last example, SIFT-MS was compared to SPME-GC-MS for the determination of lipid oxidation products from beef meat packed under a 20% CO_2/80% H_2O atmosphere and refrigerated at 4°C. Measurements were done at 0, 2, 5, 8 and 12 days of storage. Both techniques detected a significant increase of aldehydes with time but SIFT-MS could detect the differences earlier than the GC-MS method[175].

Other studies based on DIMS methods for quality control include the storage stability of meat products[176,177], fish[178], broccoli[179] or fruits[180,181] .

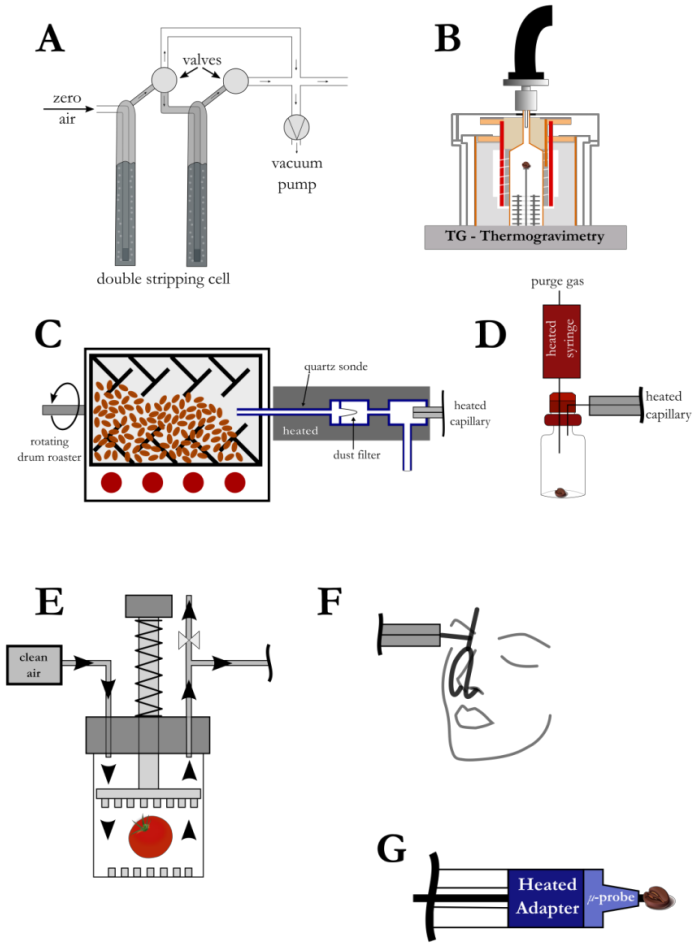

Figure 15. Different set ups used for volatile analysis with direct injection mass spectrometry techniques. A) Double stripping cell used for calculation of partition coefficients in water-air systems using PTR-MS[166]. B) Simultaneous thermal analysis coupled to SPI-MS to study single bean coffee roasting[165]. C) Direct sampling of gases produced during coffee roasting into a SPI-MS[167]. D) Automatic headspace sampling coupled to a PTR-MS[152]. E) Artificial chewing device coupled to PTR-MS to study aroma release during consumption[168]. F) Glass nose-piece for analysis of in-vivo flavor release [169]. G) micro-probe used to monitor volatile formation inside coffee beans during roasting[170].

4.3. Flavor generation.

During food processing and cooking, chemical reactions take place, generating volatile compounds that contribute to the final aroma of the product. Food industries demand fast analytical techniques with high time resolution in order to study those reactions and to optimize the processing parameters leading to the desired final flavor.

One of the most important reactions in aroma generation is the Maillard reaction. It is actually not a single reaction, but a cascade of reactions that starts with the reaction between the carbonyl group of a reducing sugar and the amino group of an amino acid. Maillard reaction leads to the production of compounds that positively affect the sensory quality of food, like flavor compounds (e.g. pyrazines or pyrroles) or colored compounds (e.g. melanoidins). Non-desired compounds are also produced by Maillard reaction, including potential carcinogenic products as hydroxymethylfurfural and acrylamide[182,183].

Maillard reaction has been extensively studied via online DIMS. Acrylamide formation in asparagine/fructose model systems as function of temperature was studied by PTR-MS. High temperatures (>150°C) significantly increased the production of acrylamide. At 170°C acrylamide levels reached a maximum after only 2 minutes of heating, but the high temperature also favored its release, therefore decreasing its concentration rapidly. In the case of potato slices heated at 170°C, acrylamide maximum appeared later (~10 minutes) but the decrease was much slower, with 50% of the maximum concentration still present after 20 minutes[184]. Formation of Maillard products has also been studied using DIMS after thermal treatment of foods including carrot drying[185], dry sausages[175], skim milk powder[186,187], pumpkin seeds roasting[188] or cocoa roasting[189].

Flavor generation during coffee roasting is crucial for the final quality of the product. Several reactions lead to the formation of hundreds of compounds responsible for the roasted coffee aroma (section 1.3). The complexity of coffee chemistry together with its economic importance explains the large amount of research performed on coffee roasting. The first study for online monitoring of coffee roasting with DIMS dates back to 1996[190]. Volatiles produced during the whole roasting process, from green to dark roasted beans, were analyzed on-line with REMPI-MS from the off-gas of a simulated roaster, with a time resolution of 1 Hz. Time intensity profiles during roasting were obtained for 4-vynilguaiacol, guaiacol and indole. The first two were formed during roasting but, while guaiacol increased over the whole roasting time, 4-vynilguaiacol reached a maximum and then decreased, suggesting the degradation of the compound. Indole is a compound found in green beans, and its concentration decreased during roasting. This first work, showed the potential of DIMS for real-time analysis of volatile formation during coffee roasting and opened the door to several other studies using SPI-MS, REMPI-MS and PTR-MS. Coffee roasting has been studied in small sample roasters[167,191], batch roasters[163,192] or even for single beans[165,170]. The online analysis of volatiles in such different conditions needed different sampling setups, some of them shown in Figure 15B, C and G. Figure 15 C shows direct sampling inside a roaster by introducing a quartz tube into the rotating drum. A filter was introduced between the tube and the instrument inlet to avoid solid particles entering the system, and all the system was heated at 250°C to avoid condensation of low volatile compounds and coffee oil[167]. Single bean roasting can be performed directly in a heated vial[21] but some other interesting sampling methods have been reported in literature. The first consists of a μ-probe that was drilled into the coffee bean (Figure 15G) and allowed sampling of the volatiles directly in the place where they are formed (i.e. inside the bean)[170]. A

second one was based in the coupling of a simultaneous thermal analysis and SPI-TOF-MS (Figure 15B). This setup allowed analysis of evolved gasses at the same time that the weight loss of the single bean was monitored over time[165].

4.4. Flavor release.

The last application of DIMS that will be discussed is flavor release. Flavor release consists of the liberation of volatile compounds from the food matrix so they can reach the olfactory epithelium. Therefore, flavor release depends on the partition coefficient of the compound between air and the matrix and it will be affected by any process that alters the matrix structure or composition (e.g. oral processing). Although DIMS techniques have been used to determine volatile release into the headspace in thermodynamic equilibrium (i.e. static headspace), the main advantage of DIMS over traditional techniques (e.g. GC-MS) resides in the possibility of measuring dynamic processes in real-time. An important application of DIMS in flavor release is the on-line monitoring of in-vivo flavor release during food consumption.

4.4.1. Flavor release during consumption.

Release of volatiles during consumption is affected by the food matrix and how it is disrupted during oral processing of food. Therefore, mastication, saliva flow, tongue movements or breathing patterns will impact the release of volatiles into the mouth space and their transport to the odor receptors in the nose. The study of flavor release during consumption is essential for understanding sensory perception of food, and DIMS have proved to be valuable tools for that kind of analysis.

The simplest scenario of food consumption is drinking of beverages. When a liquid is placed in the mouth, the soft palate is closed thus blocking volatiles to pass to the nasal cavity and preventing retro-nasal sensation. During swallowing, volatiles are released and can reach the olfactory epithelium during subsequent breathing. Volatiles reaching the nose cavity can be analyzed by DIMS with different sampling methods (Figure 15F)[117]. The effect of fat content in flavor release was analyzed by spiking different liquids (water and milk with different fat content) with aroma compounds and sampling of the volatiles in-nose with PTR-MS[193]. In all cases, the amount of compound reaching the nose was only a small fraction of the concentration present in the liquids. As expected, lipophilic compounds were retained to a higher extent in the samples containing fat, with differences up to 70% between water and full fat milk. In the case of low lipophilic compounds (2,3-butanedione), no significant difference was found between the different solutions. Fat content of the drink not only impacted the amount of volatiles released to the nose-space but also how long those volatiles are delivered (persistence). Studies using APCI-MS revealed that lipophilic compounds were more persistent in breath after swallowing oil/water emulsions, and persistence was higher when the oil concentration was increased. On the contrary, oil concentration had no effect on non-lipophilic compounds[194]. This oil dependent behavior could be explained by the existence of emulsion residues on the oral tract that kept releasing volatiles over time[195]. Aroma persistence in water solutions depended on the physicochemical properties of the compounds and could be modeled by the quantitative structure property relationship approach (QSPR), taking into account the partition coefficient, the vapor pressure and the ether linkage of the compound[196].

For more rigid food matrices, where mastication of the product is needed, the situation is more complex. The effect of viscosity on flavor release has been analyzed with the use of custard desserts flavored with

aroma compounds. Firmer custards resulted in higher concentrations of volatiles in-nose as measured by PTR-MS. Those custards involved more mouth/tongue movements than the soft ones, which resulted in opening of the soft palate, allowing volatiles to pass to the nasal cavity[197]. Other studies confirmed that differences in oral processing between subjects had a higher effect on the volatiles released than that due to differences in the texture of the food product[198]. By sampling volatiles at different points of the nose and in the nasopharynx after eating soft custards, it was observed that only a small fraction of the volatiles present in the nasopharynx reach the olfactory cleft (20-50%). The decrease was dependent on the hydrophobicity of the compound and could be related to absorption into the watery mucosa of the nose[199]. Therefore physiological parameters also need to be taken into account during in-vivo experiments. For example, the interactions of the aroma compounds with the mouth and nose mucosa can impact the amount of compounds that reach the olfactory receptors, affecting the sensory perception.

In-vivo aroma analysis has also been used in combination with sensory analysis to correlate the volatile content measured in-nose with sensory data. As compounds persist in breath even after swallowing, it is of particularly importance to analyze both the volatile content and the sensations over time. This is the purpose of the Temporal Dominance of Sensations (TDS) method, in which panelists rank different attributes during a defined amount of time. By coupling TDS and on-line nose-space analysis with PTR-MS, correlations have been found between volatiles and sensation for several food products including candy[200], yogurt [201], flavored vodka[202] or coffee[203,204]. In the case of coffee, the effect of crema (coffee foam) was studied. In the presence of crema, the roasted attribute was dominant; this attribute, however, was not perceived in coffees without crema. One compound was also found to be dominant in the volatile profile when crema was present, 2-methylfuran. Although this compound is considered a marker for roasting, it does not contribute to the roasted aroma. Other compounds might be responsible of the roasted attribute but it was not possible to assign them, pointing out the difficulty of relating analytical and sensory data[203]. In another study, two coffees with different roasting degrees were consumed either with or without sugar. Differentiation of coffees related to sugar was clear in the sensory data as the dominant attribute during the first 15 seconds was sweet. On the other hand, analytical data allowed discrimination of coffees according to their roasting degree but not to the sugar content. Some compounds detected in the nose-space could be correlated with sensory as is the case of methyl-pyrrole and acetyl-methyl-pyrrole with burnt notes or pyrazines with roasted flavor [204].

5. Summary of results

5.1. Extraction of volatiles during espresso coffee brewing (papers 1 and 2)

Coffee is not a food product consumed because of its nutritional value but for pleasure. As discussed in section 1, volatiles responsible for coffee aroma are generated during coffee roasting. Figure 16 shows PTR-TOF mass spectra of coffee at different stages: green coffee, roasted ground coffee and coffee brew. The headspace of green coffee contains volatile compounds in low concentration (Figure 16A). Once that coffee is roasted (Figure 16B), the volatile content increases and it is possible to find thermally generated compounds, like pyrazine derivatives. After brewing (Figure 16C), the headspace composition is again altered and presents a different profile of that of roasted ground coffee. The amount of volatiles transferred from the ground coffee to the final brew depend on the brewing method and brewing parameters (i.e. temperature, pressure), used in the preparation of the coffee brew. Aroma is a key factor

in the sensorial experience of drinking coffee; thus, it is quite surprising not to find more studies about how volatile compounds are incorporated into the liquid phase during coffee brewing.

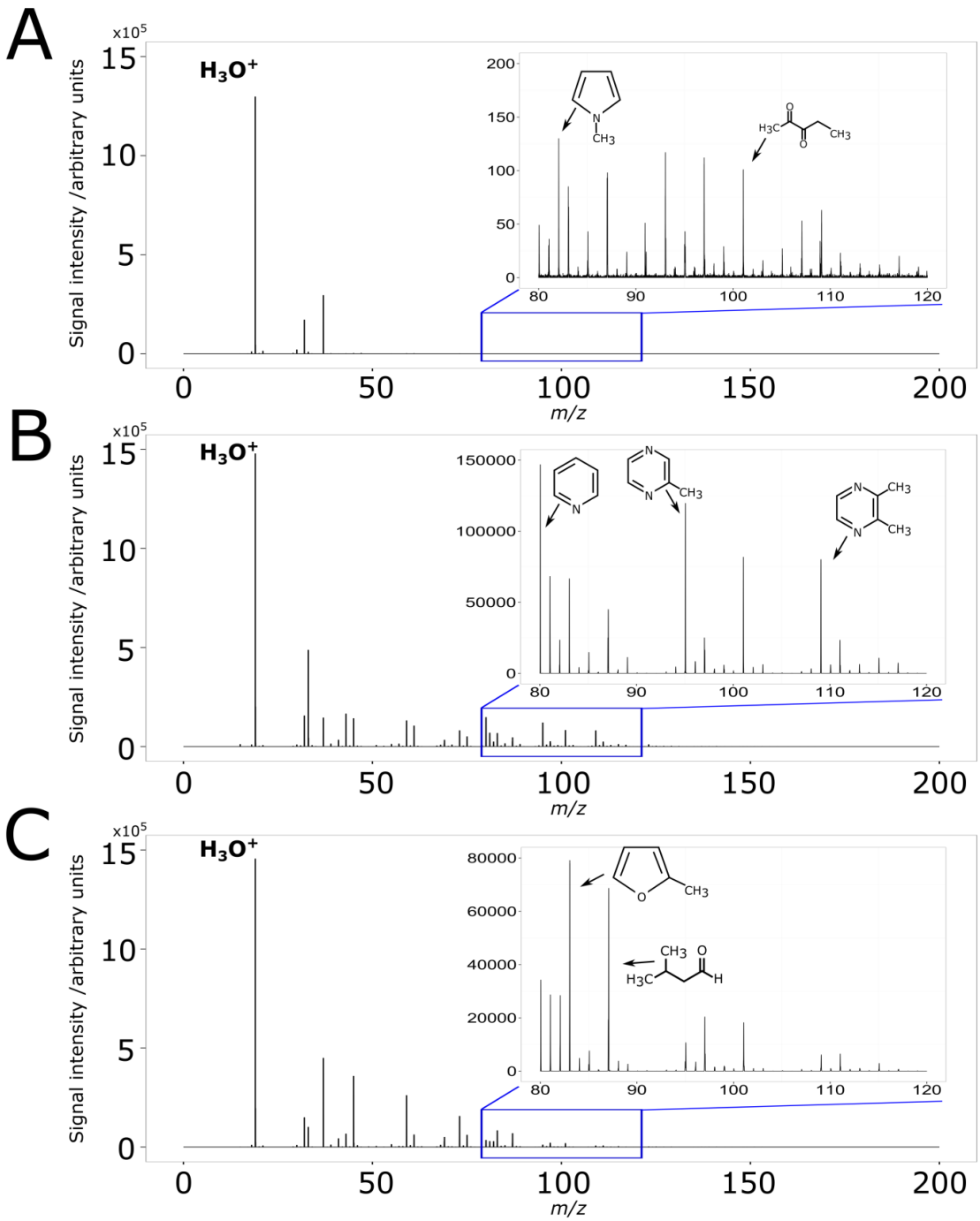

Figure 16. PTR-ToF-MS spectra of A) green coffee, B) roasted ground coffee and C) coffee brew. Tentatively identified compounds are drawn as neutral molecule *M,* although the mass peaks correspond to the protonated compounds $[MH]^+$.

In the case of espresso coffee, brewing is a fast process as the preparation of a full cup takes from 15s (in the case of ristretto) to 40 seconds (for lungo). This short brewing time complicates the sampling of fractions for off-line measurements, which results in large measurement errors. PTR-TOF-MS, as other DIMS methods, has high time resolution and allows recording of a full mass spectra in less than a second, allowing the online monitoring of fast processes as coffee brewing.

The first approach was to monitor volatiles directly from the coffee flow during brewing[205]. For this study, a capsule system was chosen due to its ease of operation and the reproducibility on coffee preparation (extracting time and extracted volume). Direct sampling from the flow in an open atmosphere was not only possible but also highly reproducible. Figure 17 shows the time evolution of the compound tentatively identified as pyridine during the preparation of a lungo espresso.

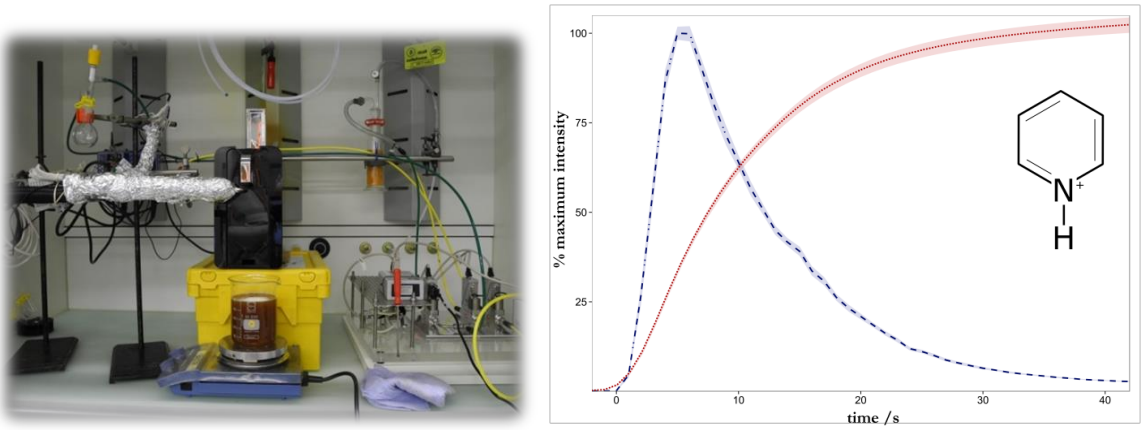

Figure 17. Picture of the set up for on-line monitoring of volatiles in the coffee flow (left). Normalized time intensity profile for pyridine (blue) and the cumulative intensity (red) over the extraction of a lungo capsule. Lines are the average of 10 capsules with ribbons representing the 95% confidence level.

Differences in extraction dynamics were found both between compounds extracted from the same coffee and for the same compound in different coffee capsules. The differences within one capsule could be explained due to the different physicochemical properties of the compounds, mainly polarity, and were in agreement with the previous study of Mestadgh et al. using SPME-GC-MS methods[52]. The main advantage of using PTR-TOF-MS was that we obtained full mass spectra with 1 Hz time resolution over the whole brewing time (i.e. 42s) and we could tentatively identify 47 different compounds. The SPME-GC-MS method, although allowing unambiguous identification of the compounds, was based in the analysis of different aliquots, which added experimental error to the measurements. Furthermore, it required 2 different GC methods (with two columns of different polarity) that took between 60 and 70 minutes to analyze 20 aroma compounds in each sample, and 6 samples to have the profile of the full extraction time.

Differences between coffee capsules could be attributed to different factors as the coffee blend used, roasting degree, particle size or the dose of coffee in the capsule (information that was not available in the commercial product). In order to check if the differences observed in the extraction dynamics were enough to differentiate coffees, we performed PCA and HCA on the area under the curve for all the m/z analyzed at different brewing times. Discrimination of capsules was possible even when only the first 15

seconds of extraction were considered, confirming the usefulness of the dynamic data for classification purposes.

The only drawback of using coffee capsules is that the only variable that allowed manipulation was the capsule type. In a follow-up experiment, the sampling set up was adapted and optimized to be used in a semi-automatic coffee machine where both pressure and temperature could be controlled (Figure 18)[206]. Results showed that, regardless of the conditions used for extraction, volatiles could be clustered by HCA in 5 groups according to their extraction kinetics (Figure 18 right). Compounds in the same group shared physicochemical properties like water solubility, polarity or volatility.

One of the limitations of PTR-TOF-MS is that it is not possible to distinguish between isomers, as they have the same molecular formula. This approach allowed us to discard some possible compound identities as their physicochemical properties did not match the family to which the extraction profile was assigned (e.g. the highly soluble pyridine was grouped in the family with the lowest water solubility).

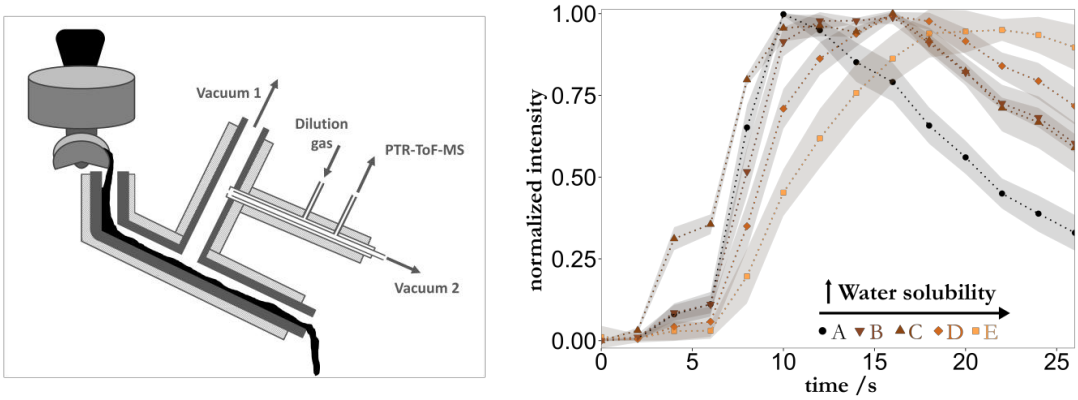

Figure 18. Sampling set up used for sampling volatiles in semi-automatic coffee machine (left). Normalized time-intensity profiles for the 5 differentiated families. Line represents the average of all compounds in the family and the ribbon the 95% confidence interval.

Regarding the impact of brewing parameters, both an increase in pressure or temperature resulted in higher levels of volatiles although the effect was more pronounced for the less soluble compounds, and higher in the case of temperature changes. This result was expected as it has been reported in literature that high temperature extraction leads to higher content of compounds like caffeine, lipids or volatiles[39,45,46]. A similar situation occurred in the case of pressure, with some compounds increasing their concentration when extracted at 9 bar instead of 7 bar; however, pressure did not affect other compounds like caffeine[39,47]. Our work supported the results found in literature (which were measured in the final cup). In addition, and since the whole time profile was obtained, it was possible to determine when the differences between brewing parameters started being significant. In general terms, those differences started being significant after 10 seconds of extraction, thus having a greater effect in the preparation of long cups (i.e. lungo) rather than in short cups (i.e. ristretto).

These two studies in coffee extraction showed the suitability of PTR-TOF-MS for the online analysis of volatiles from a liquid flow in real time, with high sensitivity and high time resolution. They provided new information about extraction of VOCs from ground coffee to water and opened a new line of research in

coffee. Potential applications which can take advantage of this analytical approach include: comparison of coffee machine performance, optimization of full automatic systems (including single dose systems), study of other important parameters in coffee brewing (e.g. water alkalinity), or relating extraction profiles with sensory data, amongst others.

5.2. Volatile profiling of tea leaves and their infusions. Application in authenticity. (paper 3)

The volatile profile of tea is highly dependent on the tea variety, growing conditions, country of origin or post-harvesting conditions. The big differences expected between teas have conditioned tea aroma research and most studies have focused on the identification of the main contributors to aroma for a specific tea. In order to analyze the profiles of a large amount of samples in a fast and reproducible fashion, PTR-TOF-MS was coupled to an autosampler, allowing headspace analysis of a sample in less than 5 minutes. This approach made possible the analysis of 101 commercial teas, both full leaves and infusions, with replicates (909 analyses) in a short time[207].

This profiling study was particularly important for several reasons. First, because it was the first time that the volatile profile of tea had been analyzed for an extensive amount of samples based on static headspace techniques. Wang and co-workers determined the volatile composition of 87 different teas by SDE-GC-MS[208]. As the volatile profile obtained from SDE depends on the solvent used for extraction, our aim was to analyze it by SHS so it better reflects the volatile profile reaching the nose via the orthonasal pathway. Furthermore, this study considered both leaves and infusions. Differences between leaves and infusions had been already reported and some compounds presented higher levels on the infusions, implying that they were formed during tea brewing[60].

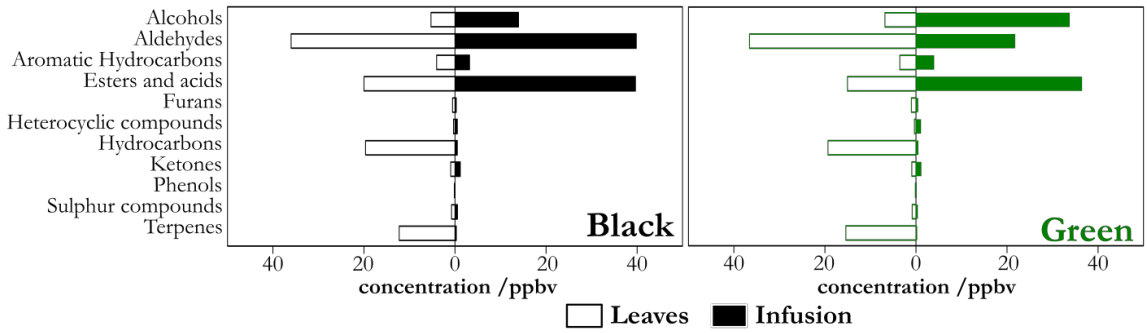

Figure 19. Concentration of volatiles in the headspace of full leaves or brews. Values represent the average of 63 black teas and 38 green teas. VOCs were grouped in different chemical families.

Figure 19 shows the total concentration of volatile compounds present in the headspace of the different samples. Despite the differences between black and green tea, it was possible to observe a clear trend between leaves and infusions. Tea leaves presented high concentration of aldehydes, esters/acids, hydrocarbons and terpenes. While the brews kept similar concentrations of aldehydes, the content of hydrocarbons and terpenes was heavily reduced. This result was expected as non-polar compounds may not be completely extracted during tea brewing. On the other hand, alcohols and esters/acids were found in higher concentration in the infusions. As some tea alcohols are found in the leaves as glycosides,

hydrolysis of the glycosidic precursors may explain why some compounds presented higher levels on the infusions than those on the leaves[60].

The obtained profiles were subjected to statistical analysis with the aim of classifying the samples according to (i) their tea type (black or green) and (ii) the country of origin. Green and black teas could be distinguished by PCA and separation was confirmed with four different classification models and cross-validation. This separation was possible using either the leaves or the infusion data with classification errors <4% and <1% respectively. In the case of the country of origin, classification was more difficult. The classification errors ranged between 30-50%, and most of the confusion was between samples of neighboring countries (e.g. Korea-China). This implies that country of origin is not the best factor to discriminate teas as political borders often split regions with similar climate, growing conditions or processing traditions which will impact the tea aroma and could therefore be better discriminators.

1.1. Extraction of volatiles during tea brewing (paper 4)

The kinetics of extraction of non-volatile soluble compounds from tea leaves has been widely studied [79–89]. Unfortunately, in the case of volatiles, all previous studies have focused on the final cup and not on the dynamics of extraction. The volatile profile of the tea infusions has shown significant differences with that of the leaves, therefore it is important to understand the effect that the brewing process has in the volatile composition of the final infusion. Furthermore, as tea preparation is highly dependent on culture and lifestyle, brewing parameters need to be optimized in order to get a pleasant aroma that guarantees the acceptance of the consumer.

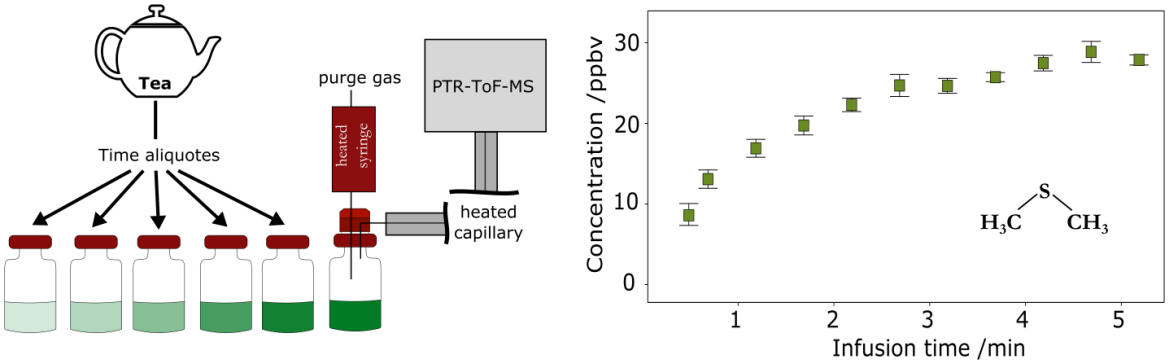

Figure 20. Scheme of the off-line measurement of tea brewing with PTR-TOF-MS (left). Time intensity profile for dimethylsulfide showing the average of three replicates and the standard deviation.

Using PTR-TOF-MS the extraction of volatiles from loose leaves into the final infusion was followed over 5 minutes extraction, at different temperatures, using waters of different mineral composition. As tea brewing is a relatively long process, the initial temperature of water drops several degrees with time. To ensure the monitoring of differences in extraction and not on release (due to the different temperatures), the brewing process was followed off-line by taking fractions every 30 seconds and analyzing them at the same temperature (Figure 20).

Using this approach, the time intensity profiles of 88 m/z (including 34 tentatively identified compounds) were obtained for each of the brewing parameters considered (3 temperatures – 60,70 and 80 °C; 2 leaf sizes – full and broken; and 2 waters – soft and hard). Due to the large amount of samples measured and

the different variables that may impact the extraction, data was subjected to different statistical analysis in order to better interpret the results.

A simple PCA including all analyzed samples allowed differentiation of the effect of the different variables in the volatile profile (Figure 21). HCA using the concentration at all time points for the 88 m/z measured in each sample, allowed discrimination of all temperature and leaf size combinations with exception of broken leaves brewed at 70 or 80°C which were clustered together. The extraction from broken leaves at these temperatures was fast, with no significant differences in volatile levels between broken and full leaves. It was also not possible to discriminate between the two different waters used for extraction within one temperature-size combination.

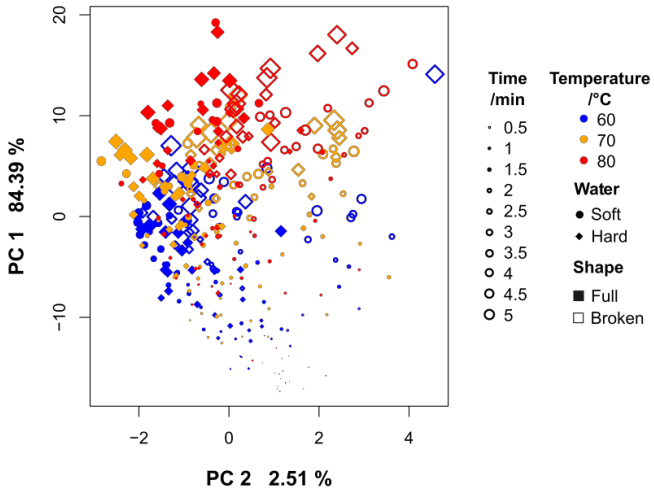

Figure 21. PCA containing all samples analyzed in the study (3 temperatures x 2 leaf sizes x 2 waters x 10 time points x 3 replicates)

HCA was also performed on each individual measurement (10 time points x 3 temperatures x 2 waters x 2 leaf sizes x 3 replicates) revealing the brewing conditions that resulted in equivalent volatile profiles.

In order to compare how volatiles were extracted from leaves, the time intensity profiles were normalized to the intensity at the end of the extraction for each of the measured m/z. HCA on that data resulted in two differentiated groups that corresponded with the two different leaf sizes. This results indicate that temperature plays a role in the total amount of compound being extracted into the infusion (higher levels with higher temperatures), but not on the mechanism by which compounds are extracted; therefore all temperatures had similar extraction profiles. On the other hand, when the leaf size is reduced, the rate of extraction changes, modifying the extraction profile of the compounds.

The combination of PTR-TOF-MS data with multivariate tools proved to be a useful approach to study the tea brewing process and how it is affected by different parameters (temperature, leaf size, brewing time and water hardness).

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7. Annex

I. List of figures

II. List of abbreviations

APCI	Atmospheric Pressure Chemical Ionization
DHS	Dynamic Head Space
DIMS	Direct Injection Mass spectrometry
FAO	Food and Agriculture Organization
GC	Gas Chromatography
GC-O	Gas Chromatography Olfactometry
HC	Hollow Cathode
HCA	Hierarchical Cluster Analysis
HLC	Henry Law Constant
ICO	International Coffee Organization
MS	Mass Spectrometry
OAV	Odor Activity Value
OT	Odor Threshold
PA	Proton Affinity
PCA	Principal Component Analysis
PLS-DA	Partial Least Square Discriminant Analysis
PTR	Proton Transfer Reaction
QSPR	Quantitative Structure Property Relationship
REMPI	Resonant Enhanced Multiple Photon Ionization
SAFE	Solvent Assisted Flavor Extraction
SDE	Simultaneous Distillation Extraction
SHS	Static Head Space
SIFT	Selected Ion Flow Tube
SIM	Single Ion monitoring
SOTA	Self Organizing Three Algorithm
SPI	Single Photon Ionization
SPME	Solid Phase Micro Extraction
TDS	Temporal Dominance of Sensations
TOF	Time of Flight
UK	United Kingdom

UN	United Nations
USD	United States Dollar
VUV	Vacuum Ultraviolet

III. List of publications for thesis work

Paper 1**: José A. Sánchez-López**, Ralf Zimmermann, Chahan Yeretzian. *Insight into the Time-Resolved Extraction of Aroma Compounds during Espresso Coffee Preparation: Online Monitoring by PTR-ToF-MS*, Anal. Chem. 86 (2014) 11696–11704.

Paper 2: **José A. Sánchez López**, Marco Wellinger, Alexia N. Gloess, Ralf Zimmermann, Chahan Yeretzian. *Extraction kinetics of coffee aroma compounds using a semi-automatic machine: On-line analysis by PTR-ToF-MS*, Int. J. Mass Spectrom. 401 (2016) 22-30

Paper 3: Sine Yener, **José A. Sánchez-López**, Pablo M. Granitto, Luca Cappellin, Tilmann D. Märk, Ralf Zimmerman, Günther K. Bonn, Chahan Yeretizian, Franco Biasioli. *Rapid and direct volatile compound profiling of black and green teas (camellia sinensis) from different countries with PTR-ToF-MS*, Talanta 152 (2016) 45-53

Manuscript 1: **José A. Sánchez-López**, Sine Yener, Tilmann D. Märk, Günther Bonn, Ralf Zimmerman, Franco Biasioli , Chahan Yeretizian. *Extraction Dynamics of Tea Volatile Compounds as a Function of Brewing Temperature, Leaf Size and Water Hardness: On-Line Analysis by PTR-ToF-MS*. Submitted to Talanta. 06.04.2016

IV. List of further publications

Paper 4: **José A. Sánchez-López**, Aldo Ziere, Sara I.F.S. Martins, Ralf Zimmermann, Chahan Yeretzian. *Persistence of aroma volatiles in the oral and nasal cavity. Real-time monitoring of decay-rate in air exhaled through the nose and mouth.* Accepted for publication in Journal of Breath Research. 30.05.2016

V. Publications

Publication 1.

Insight into the Time-Resolved Extraction of Aroma Compounds during Espresso Coffee Preparation: Online Monitoring by PTR-TOF-MS

by

José A. Sánchez-López, Ralf Zimmermann, Chahan Yeretzian.

Analytical Chemistry

Volume 86, Issue 23, **2014**, Pages 11696–11704.

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José A. Sánchez-López designed and performed all the experiments. He also performed the data analysis and prepared the manuscript. His work to this publication accounts for approximatelly 90%.

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Insight into the Time-Resolved Extraction of Aroma Compounds during Espresso Coffee Preparation: Online Monitoring by PTR-ToF-MS

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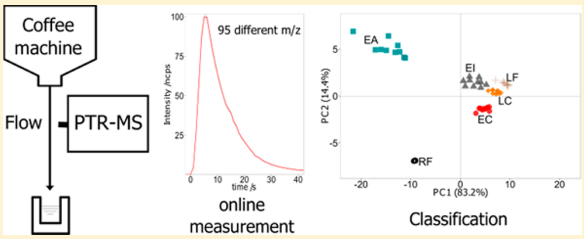
ABSTRACT: Using proton-transfer-reaction time-of-flight mass-spectrometry (PTR-ToF-MS), we investigated the extraction dynamic of 95 ion traces in real time (time resolution = 1 s) during espresso coffee preparation. Fifty-two of these ions were tentatively identified. This was achieved by online sampling of the volatile organic compounds (VOCs) in close vicinity to the coffee flow, at the exit of the extraction hose of the espresso machine (single serve capsules). Ten replicates of six different single serve coffee types were extracted to a final weight between 20−120 g, according to the recommended cup size of the respective coffee capsule (Ristretto, Espresso, and Lungo), and analyzed. The results revealed considerable differences in the extraction kinetics between compounds, which led to a fast evolution of the volatile profiles in the extract flow and consequently to an evolution of the final aroma balance in the cup. Besides exploring the time-resolved extraction dynamics of VOCs, the dynamic data also allowed the coffees types (capsules) to be distinguished from one another. Both hierarchical cluster analysis (HCA) and principal component analysis (PCA) showed full separation between the coffees types. The methodology developed provides a fast and simple means of studying the extraction dynamics of VOCs and differentiating between different coffee types.



Food flavor is a highly complex phenomenon and the strategies and technologies used to elucidate perceived flavors are becoming increasingly sophisticated, requiring multidisciplinary approaches.[1] With more than 7000 flavor compounds reported in food products to date,[2] the unequivocal identification and quantification of these compounds is a crucial step in flavor analysis. The ability to separate compounds by gas chromatography (GC), to identify them by comparison with mass spectral reference libraries and to quantify them using standard compounds makes GC/MS an indispensable technique for flavor scientists. Coupled with olfactory techniques such as GC-olfactometry (GC/O), these approaches allow sensory relevant compounds to be elucidated, and their relative contributions to the flavor of the food product to be estimated.[3,4] While GC/MS is highly suitable for identifying and quantifying flavor-active compounds, it performs less well when it comes to monitoring the temporal evolution of fast dynamic processes and needs to be complemented with other analytical techniques when processes such as flavor generation[5−11] or in vivo release[12−17] need to be monitored. This has led to the introduction of new analytical technologies capable of monitoring volatiles in real-time, including electronic sensors,[18] and direct injection mass spectrometry.[19] Among the various techniques for direct injection techniques, proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) allows volatile organic compounds (VOCs) to be quantified and exhibits low ion fragmentation, high sensitivity, and high time and mass resolution.[20]

In this piece of work, we will focus on the analysis of the dynamic extraction of an espresso coffee using PTR-ToF-MS. Coffee is a food product of great economic relevance and an icon of western life style. The unique and highly appreciated flavor of a cup of coffee is the final expression of a long chain of chemical and physical transformations that link the seed to the cup. The genetic makeup (the variety), agronomic practices, the soil, climatic conditions, and the care given by the farmers set the stage for the later development of the typical coffee flavor. The flavor of unroasted coffee does not bear any resemblance to what is considered the typical flavor of coffee. Roasting generates around 1000 VOCs, although less than 50 might be relevant to the aroma of roasted coffee.[21] Roasting is the most important step for the formation of the coffee aroma, and hence it is also one of the most thoroughly studied

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Table 1. Characterization of the Coffee Capsules

capsule type	blend/origins		powder weight (g)	roast degree (Pt)	particle size		extraction time (s)	extracted weight (g)
	arabica	robusta			$d_{4,3}$ (μm)	$d_{3,2}$ (μm)		
RF	Central America + Africa	India	6.01 ± 0.17	69	345 ± 53	55 ± 18	14.2 ± 0.6	20.06 ± 1.37
EA	Central + South America	India	5.95 ± 0.11	77	330 ± 5	47 ± 8	28.9 ± 0.8	47.21 ± 1.57
EC	Central + South America	India	6.24 ± 0.09	88	346 ± 3	57 ± 1	22.0 ± 0.0	62.10 ± 1.05
EI	Central America + India		6.00 ± 0.15	67	331 ± 32	48 ± 5	23.5 ± 0.8	49.81 ± 1.40
LC	Central+South America+Asia		6.30 ± 0.02	98	343 ± 20	49 ± 6	41.1 ± 0.5	111.64 ± 2.81
LF	Central+South America	Asia	6.04 ± 0.07	73	341 ± 58	56 ± 10	42.0 ± 0.4	117.80 ± 2.27

processing steps.[5−7] However, equally important to the final flavor profile in the cup is the extraction of ground coffee with water. The extraction technique and conditions used for coffee preparation strongly influence the flavor profile in the cup[22] and is often the only parameter that can be influenced by the consumer at home.

Several studies have investigated how the extraction of flavor compounds is affected by the brewing technique,[22,23] temperature,[24−26] pressure,[26,27] water composition,[28,29] and cup length.[30,31] In all of these studies, measurements were carried out on the final extract, but there is a lack of information on how the above-mentioned parameters affect the kinetics of extraction. Few quantitative studies have been published to date on the time-resolved extraction of volatile coffee compounds. By using different volumes of water in the extraction process or taking fractions over the whole extraction time/volume, some authors have published findings on the extraction process of acrylamide, caffeine, and antioxidants.[30,31] To the best of our knowledge, only the recently published work by Mestdagh et al. has reported data on the kinetics of extraction for selected aroma compounds, using solid phase micro extraction (SPME)-GC/MS.[32]

The approach taken here examines whether it is possible to measure VOC release from the coffee flow at the exit of the extraction hose using PTR-ToF-MS. We make the assumption that each compound in the liquid extract is partitioned in the gas phase, so that the gas phase concentration of VOCs at the exit of the hose is proportional to the liquid concentration, with the Henry's Law Constant (HLC) being the proportionality constant.[33,34] Hence the time-evolution in the gas-phase mimics the extract concentration. An analytical approach that is based on online sampling of the volatiles released from the coffee flow was developed and tested for real-time monitoring of the extraction of volatile aroma compounds from single serve coffee capsules.

MATERIALS AND METHODS

Coffee. Six commercial Delizio coffee capsule types (Delica, Birsfelden, Switzerland) were selected: Ristretto Forte (RF), Espresso Intenso (EI), Espresso Alba (EA), Espresso Classico (EC), Lungo Fortissimo (LF), and Lungo Crema (LC). All the capsules of a given type were from the same production batch. To ensure reproducibility among the same types of capsules and to compare different types of capsules, the coffee powder in the capsules was characterized according to (i) total weight in the capsule; (ii) roasting degree, measured with a Colorette 3B instrument (Probat, Emmerich am Rhein, Germany); and (iii) particle size distribution, measured with a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). The results are summarized in Table 1.

Coffee Preparation. Ten different capsules of each coffee type were extracted using a Delizio Compact Automatic coffee machine (Delica, Birsfelden, Switzerland). These were operated according to the factory settings to pump three different volumes of water in an unrestricted mode (no capsule in the brewing unit): 40 mL for Ristretto, 72 mL for Espresso and 131 mL for Lungo. Depending on the coffee inside each capsule type, the actual weight of the extract in the cup (final column in Table 1) showed significant variations, but was very stable for repetitions of the same type. The total time for extraction of the cup and its final weight were measured (Table 1). Note that the expression "espresso" can have two meanings. Either it describes the general fact that coffee was prepared using a pressurized brewing/extraction process and may refer to different extracted volumes (Ristretto, Espresso, and Lungo) or it designates the volume of the extract (here, 50 mL), the context clarifies the meaning. Just before extraction of each capsule, 110 mL of water was passed through the circuit to remove possible residues from the previous extraction and to preheat the circuit. Both for cleaning and extraction, tap water was mixed with filtered water (PURITY 600 Quell ST, BRITA Professional, Taunusstein, Germany) to adjust the extraction water to Alkalinity 4 dH° ± 1° dH, Hardness 6 dH° ± 1° dH (German water hardness).

Sampling Set Up. VOCs released from the coffee flow were measured with the set up shown in Figure 1. Coffee was extracted over an ice-cold water bath to ensure that interference from volatiles from the collected extract were eliminated. The sampling lance was positioned 0.5 cm from the coffee flow and coupled to the inlet of the PTR-ToF-MS. Using a custom built gas dilution system, adapted from Wellinger et al.,[35] we diluted the sampled VOCs 7.5-fold to avoid condensation of VOCs on the tubing and to adjust their concentration to within the dynamic working range of the mass spectrometer. The dilution gas was dry compressed air containing 2-isobutyl-3-methylpyrazine as a standard for mass range calibration. All the sampling and dilution lines were heated to 90 °C and all flows were controlled by mass flow controllers (Bronkhorst, Ruurlo, The Netherlands) and verified using a bubble meter.

PTR-ToF-MS. A commercial PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) was used. The diluted sample was introduced with a flow of 200 sccm into the drift tube, which was operated at 2.2 mbar, 70 °C and 600 V drift voltage. PTR-ToF-MS data were recorded by TOFDAQ v.183 data acquisition software (Tofwerk AG, Thun, Switzerland). Mass spectra were recorded in the mass-to-charge (m/z) range of 0−205 with one mass-spectrum recorded per second. Mass axis calibration was performed on $[H_3{}^{18}O]^+$, $[C_3H_7O]^+$, and $[C_8{}^{13}CH_{15}N_2]^+$.

Data Processing. A PTR-TOF DATA Analyzer software v4.1736 was used for data analysis. Duty cycle corrected signals were normalized to 10^6 H_3O^+ primary ions. During extraction, fluctuations in the flow (mL/s) and the foam

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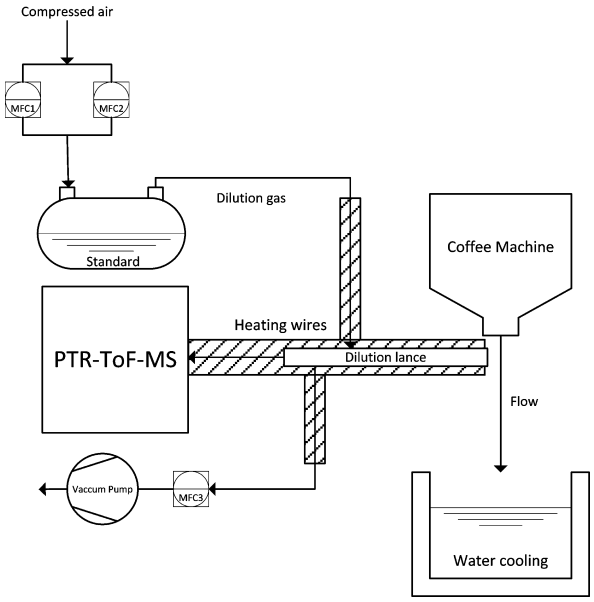

Figure 1. Set up for sampling VOCs from the coffee flow. Volatiles were introduced into the dilution lancet by a flow created with a vacuum pump and were then diluted 7.5 fold using dried compressed air containing a standard for mass calibration.

(different bubble sizes) were observed. To correct for small differences in the absolute intensity and allow for a better comparison between capsules, the intensity of the VOCs was normalized to the maximum intensity of the m/z 69.035 ion trace, before averaging for replicates.

Mass Peaks Selection. Ten replicates for each of the six coffee capsule types (RF, EA, EC, EI, LC, and LF) were analyzed with the set up described in the Sampling Set Up section. Around 300 mass peaks were found in the m/z range recorded, although the exact number was dependent on the capsule type. Only peaks that changed over time and that were present in all samples were included in the subsequent data analysis, yielding a list of 95 ion traces. Out of these, 52 were tentatively identified, based on the literature and were reduced to 47 after removing fragments and isotopologues.[37−39] Each m/z time-intensity profile was characterized using the following parameters: (i) the time at maximum intensity (t_{max}), (ii) the time elapsed between the maximum intensity and the drop to half of the maximum intensity ($t_{1/2}$) and significant differences were calculated using ANOVA and Tukey's test ($p < 0.05$); these numbers are provided in (Table 2). Furthermore, to examine differences in the total amount of extracted VOCs the area under the curve of the time-intensity profiles was also calculated (numerical integration in discrete time-intervals of one second, corresponding to the integration time window during data collection) and subjected to statistical analysis.

Statistical Analysis. Three different areas under the time-intensity profiles for each of the 95 mass traces were calculated: (i) the area under the time intensity curve from t = 0 to 15 s; (ii) the area from t = 0 s to the end of the extraction (total extraction time, which depends on capsule type); and (iii) the area calculated under point ii, normalized/divided by the amount (in grams) of the extracted coffee.

These three sets of 60 samples (10 replicates of 6 different capsule types) with 95 different variables were subsequently subjected to statistical analysis. Hierarchical Cluster Analysis (HCA) was performed by Ward's minimum variance method using half-squared Euclidean distances. Principal Component Analysis (PCA) was performed on mean-centered scaled data. All analysis and graphs were performed with packages and scripts in R (R foundation for statistical computing, Vienna, Austria).

RESULTS AND DISCUSSION

The time-intensity profiles show different extraction dynamics for the VOCs analyzed (Figure 2A). The time at which the maximum intensity was reached ranged from 2 to 24 s, although for 95% of the compounds it was reached in less than 10 s. Once the maximum had been achieved, the intensity fell at different rates, depending on the compound. This decrease of intensity provides information on how the compounds are extracted. A fast decrease implies that the compound is extracted over a relatively short time period while a slow decrease implies that the compound is extracted over a longer period. Using $t_{1/2}$ as a measure of the intensity decrease, we observe a large variability between the different VOCs, encompassing a range of 3 to 25 s for $t_{1/2}$. A few compounds did not fall below 50% of the maximum intensity by the end of the extraction and hence their $t_{1/2}$ could not be determined. Although the extraction of some compounds was relatively slow, 70% of them reached $t_{1/2}$ in less than 10 s and showed intensities lower than 20% of the maximum by the time that the coffee had been prepared (~24 s).

Plotting the integrated intensity of the time-intensity curves for each VOC, we obtained the cumulative concentration of the

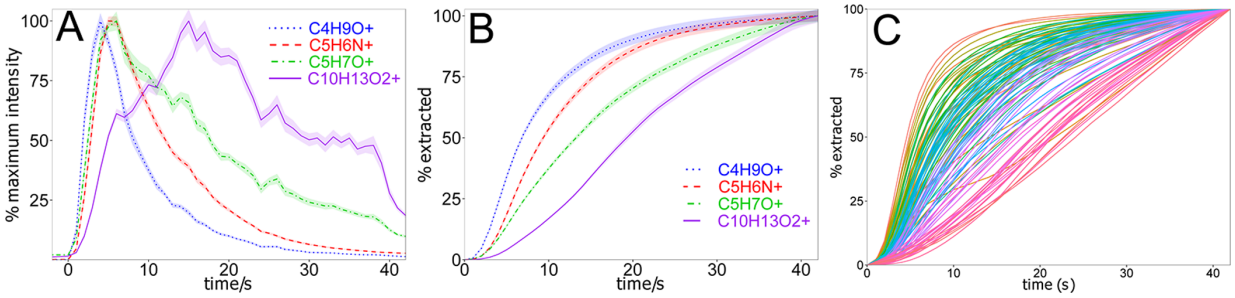

Figure 2. Time intensity profiles in the LC capsule showing differences in extraction. (A) Data normalized to the maximum intensity of four m/z. Integration of the area under the curve at each time point as a percentage of the total area at the end of the extraction for (B) the four selected m/z and (C) for all peaks considered. Shaded ribbons show the 95% confidence interval. Colors in panel C represent different peaks.

Table 2. List of Mass Peaks, Assigned Sum Formulae, and Tentative Compound Identification[a]

				RF		EA		EC		EI		LC		LF	
measured (m/z)	theoretical (m/z)	sum formula	tentative identification	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)
31.018	31.018	CH_3O^+	formaldehyde	3 ± 1^{a}	4 ± 1^{op}	3 ± 0^{a}	6 ± 1^{mp}	3 ± 1^{a}	6 ± 1^{m}	4 ± 1^{a}	3 ± 1^{no}	4 ± 1^{a}	3 ± 1^{n}	4 ± 1^{a}	3 ± 1^{no}
33.033	33.033	CH_5O^+	methanol	3 ± 1^{a}	4 ± 1^{o}	3 ± 0^{a}	4 ± 1^{o}	3 ± 1^{a}	6 ± 1^{m}	4 ± 1^{a}	2 ± 1^{no}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	2 ± 1^{n}
45.034	45.033	$C_2H_5O^+$	acetaldehyde	4 ± 1^{a}	4 ± 1^{op}	4 ± 1^{a}	5 ± 1^{mp}	4 ± 1^{a}	7 ± 1^{m}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	3 ± 1^{no}	4 ± 1^{a}	3 ± 1^{no}
47.013	47.013	$CH_3O_2^+$	formic acid	4 ± 1^{a}	8 ± 1^{m}	4 ± 1^{a}	11 ± 2^{n}	4 ± 1^{a}	9 ± 1^{mn}	4 ± 1^{a}	7 ± 2^{m}	4 ± 1^{a}	3 ± 1^{o}	4 ± 0^{a}	7 ± 1^{m}
55.055	55.054	$C_4H_7^+$	1,3-butadiene	4 ± 1^{ab}	5 ± 1^{no}	6 ± 2^{b}	6 ± 2^{mo}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{ab}	3 ± 1^{n}	4 ± 1^{ab}	4 ± 1^{n}	4 ± 1^{ab}	5 ± 1^{no}
57.035	57.033	$C_3H_5O^+$	2-propenal, prop-1-en-1-one	5 ± 0^{b}	4 ± 1^{no}	5 ± 1^{ab}	5 ± 1^{mo}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{ab}	2 ± 1^{n}	5 ± 1^{ab}	2 ± 1^{n}	5 ± 1^{ab}	2 ± 1^{n}
57.071	57.07	$C_4H_9^+$	1-butene	7 ± 1^{ab}	4 ± 1^{n}	8 ± 3^{b}	14 ± 3^{m}	5 ± 1^{a}	15 ± 2^{m}	5 ± 1^{a}	7 ± 2^{n}	6 ± 1^{a}	13 ± 2^{m}	6 ± 1^{ab}	8 ± 2^{n}
59.05	59.049	$C_3H_7O^+$	acetone, propanal	3 ± 1^{a}	4 ± 1^{o}	3 ± 1^{a}	4 ± 1^{o}	4 ± 1^{a}	6 ± 1^{m}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	2 ± 1^{n}
61.029	61.028	$C_2H_5O_2^+$	acetic acid	3 ± 1^{a}	4 ± 1^{o}	4 ± 1^{a}	4 ± 1^{o}	4 ± 1^{a}	6 ± 1^{m}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	2 ± 1^{n}
63.027	63.026	$C_2H_7S^+$	dimethyl sulfide	6 ± 2^{a}	4 ± 2^{mo}	6 ± 2^{a}	9 ± 2^{n}	4 ± 1^{a}	8 ± 1^{mn}	5 ± 1^{a}	3 ± 2^{o}	4 ± 1^{a}	6 ± 2^{mno}	5 ± 1^{a}	5 ± 2^{mno}
68.051	68.049	$C_4H_6N^+$	pyrrole	6 ± 1^{a}	4 ± 1^{n}	6 ± 2^{a}	6 ± 2^{mn}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}
69.035	69.033	$C_4H_5O^+$	furan	6 ± 1^{a}	4 ± 1^{n}	5 ± 1^{a}	7 ± 1^{m}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	4 ± 1^{n}
70.039	70.04	$C_2H_4N_3^+$	triazole	6 ± 1^{b}	3 ± 1^{n}	6 ± 2^{ab}	6 ± 2^{m}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{ab}	2 ± 1^{n}	5 ± 1^{ab}	3 ± 1^{n}	5 ± 1^{ab}	3 ± 1^{n}
71.051	71.049	$C_4H_7O^+$	methyl-propenal, 3-buten-2-one	6 ± 1^{a}	1 ± 1^{no}	6 ± 2^{a}	6 ± 2^{mo}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{a}	1 ± 1^{n}	5 ± 1^{a}	1 ± 1^{n}	5 ± 1^{a}	1 ± 1^{no}
72.046	72.044	$C_3H_6NO^+$	acrylamide	4 ± 1^{a}	5 ± 1^{mn}	6 ± 2^{b}	5 ± 2^{mn}	4 ± 1^{a}	7 ± 1^{m}	4 ± 0^{a}	3 ± 1^{n}	5 ± 1^{a}	4 ± 1^{n}	4 ± 1^{a}	4 ± 1^{n}
73.066	73.065	$C_4H_9O^+$	methyl propanal	4 ± 1^{ab}	5 ± 1^{o}	6 ± 2^{b}	5 ± 2^{mo}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{ab}	2 ± 1^{n}	5 ± 1^{ab}	3 ± 1^{no}	5 ± 1^{ab}	3 ± 1^{no}
75.045	75.044	$C_3H_7O_2^+$	propanoic acid, ethyl acetate	4 ± 1^{a}	4 ± 1^{o}	4 ± 1^{a}	5 ± 1^{mo}	4 ± 1^{a}	6 ± 1^{m}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	2 ± 1^{n}	4 ± 1^{a}	3 ± 1^{n}
80.052	80.049	$C_5H_6N^+$	pyridine	7 ± 1^{b}	5 ± 1^{n}	9 ± 1^{c}	9 ± 2^{mo}	5 ± 1^{a}	10 ± 1^{m}	6 ± 1^{a}	5 ± 2^{no}	5 ± 1^{a}	6 ± 1^{no}	6 ± 1^{ab}	6 ± 1^{no}
82.068	82.065	$C_5H_8N^+$	methyl pyrrole	7 ± 1^{b}	3 ± 1^{no}	9 ± 2^{c}	7 ± 2^{mn}	4 ± 1^{a}	10 ± 1^{m}	5 ± 1^{ab}	5 ± 2^{mn}	5 ± 1^{a}	2 ± 1^{o}	6 ± 1^{ab}	5 ± 1^{no}
83.052	83.049	$C_5H_7O^+$	methyl furan	7 ± 1^{a}	3 ± 1^{o}	8 ± 2^{a}	14 ± 2^{m}	8 ± 3^{a}	14 ± 3^{m}	6 ± 2^{a}	14 ± 2^{m}	6 ± 1^{a}	10 ± 2^{n}	7 ± 2^{a}	13 ± 2^{mn}
85.067	85.065	$C_5H_9O^+$	methylbutenal	6 ± 1^{a}	4 ± 1^{n}	6 ± 2^{a}	7 ± 2^{m}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{a}	2 ± 2^{n}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	4 ± 1^{n}
87.046	87.044	$C_4H_7O_2^+$	2,3-butanedione	5 ± 1^{b}	3 ± 1^{no}	5 ± 1^{ab}	4 ± 1^{o}	4 ± 1^{a}	6 ± 1^{m}	5 ± 1^{ab}	2 ± 1^{n}	4 ± 1^{ab}	2 ± 1^{n}	4 ± 1^{ab}	2 ± 1^{n}
87.083	87.08	$C_5H_{11}O^+$	methylbutanal	7 ± 1^{bc}	3 ± 1^{o}	10 ± 1^{d}	5 ± 2^{no}	4 ± 1^{a}	10 ± 2^{m}	5 ± 1^{ab}	8 ± 2^{mn}	6 ± 1^{abc}	8 ± 2^{mn}	7 ± 2^{c}	5 ± 2^{no}
89.062	89.06	$C_4H_9O_2^+$	methylpropanoate	5 ± 1^{a}	4 ± 1^{no}	5 ± 1^{a}	6 ± 1^{mo}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}
95.06	95.06	$C_5H_7N_2^+$	methylpyrazine	6 ± 1^{b}	4 ± 1^{n}	6 ± 2^{ab}	7 ± 2^{m}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{ab}	3 ± 1^{n}	5 ± 1^{ab}	4 ± 1^{n}	5 ± 1^{ab}	4 ± 1^{n}
97.032	97.028	$C_5H_5O_2^+$	furfural	6 ± 1^{a}	4 ± 1^{no}	6 ± 2^{a}	6 ± 2^{mo}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{a}	2 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{no}
99.045	99.044	$C_5H_7O_2^+$	furfuryl alcohol	6 ± 1^{b}	4 ± 1^{n}	6 ± 2^{ab}	6 ± 2^{m}	4 ± 1^{a}	8 ± 1^{m}	5 ± 1^{ab}	2 ± 1^{n}	5 ± 1^{ab}	4 ± 1^{n}	5 ± 1^{ab}	4 ± 1^{n}
101.062	101.06	$C_5H_9O_2^+$	pentanedione	6 ± 1^{a}	4 ± 1^{no}	6 ± 2^{a}	6 ± 2^{mo}	4 ± 1^{a}	7 ± 1^{m}	5 ± 1^{a}	2 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{no}
103.078	103.075	$C_5H_{11}O_2^+$	hidroxypentanone, methyl butanoic acid	6 ± 1^{b}	4 ± 1^{n}	5 ± 1^{ab}	6 ± 1^{o}	4 ± 1^{a}	8 ± 1^{m}	5 ± 1^{ab}	3 ± 1^{n}	5 ± 1^{ab}	4 ± 1^{n}	5 ± 1^{ab}	3 ± 1^{n}
107.056	107.06	$C_6H_7N_2^+$	ethenylpyrazine	3 ± 1^{b}	2 ± 1^{o}	9 ± 2^{c}	12 ± 2^{n}	6 ± 1^{a}	13 ± 2^{mn}	5 ± 1^{a}	7 ± 2^{m}	6 ± 1^{a}	9 ± 1^{mn}	6 ± 2^{a}	7 ± 2^{m}
109.079	109.076	$C_6H_9N_2^+$	dimethylpyrazine, ethylpyrazine	7 ± 1^{bc}	4 ± 1^{n}	8 ± 2^{c}	8 ± 2^{mo}	5 ± 1^{a}	9 ± 1^{m}	6 ± 1^{ab}	4 ± 2^{no}	5 ± 1^{ab}	5 ± 1^{no}	6 ± 1^{ab}	6 ± 1^{no}
110.06	110.06	$C_6H_8NO^+$	acetylpyrrole, methyl-pyrrolyl ketone	7 ± 1^{b}	5 ± 1^{n}	9 ± 1^{c}	8 ± 2^{mo}	5 ± 1^{a}	9 ± 1^{m}	6 ± 1^{ab}	4 ± 2^{n}	6 ± 1^{ab}	5 ± 1^{n}	6 ± 1^{ab}	5 ± 1^{n}
111.045	111.044	$C_6H_7O_2^+$	acetylfuran	7 ± 1^{b}	3 ± 1^{n}	7 ± 2^{b}	8 ± 2^{mo}	5 ± 1^{a}	9 ± 1^{m}	5 ± 1^{ab}	3 ± 2^{n}	5 ± 1^{ab}	4 ± 1^{no}	5 ± 1^{ab}	4 ± 1^{n}
113.063	113.06	$C_6H_9O_2^+$	methylfurfuryl alcohol, dimethylfuranone	6 ± 1^{bc}	4 ± 1^{n}	8 ± 2^{c}	8 ± 2^{m}	4 ± 1^{a}	9 ± 1^{m}	5 ± 1^{ab}	3 ± 1^{n}	5 ± 1^{ab}	5 ± 1^{n}	6 ± 1^{ab}	4 ± 1^{n}
115.078	115.075	$C_6H_{11}O_2^+$	4-methyltetrahydro-2*H*-pyran-2-one	7 ± 1^{b}	3 ± 1^{o}	7 ± 3^{b}	9 ± 3^{mn}	4 ± 1^{a}	12 ± 2^{m}	5 ± 1^{ab}	4 ± 2^{no}	5 ± 1^{ab}	7 ± 2^{mo}	6 ± 1^{ab}	6 ± 1^{no}
117.052	117.055	$C_5H_9O_3^+$	2-oxopropyl acetate, acetol acetate	6 ± 1^{a}	4 ± 1^{n}	6 ± 2^{a}	7 ± 2^{m}	4 ± 1^{a}	8 ± 1^{m}	5 ± 1^{a}	3 ± 1^{n}	5 ± 1^{a}	3 ± 1^{n}	6 ± 1^{a}	2 ± 1^{n}

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Table 2. continued

measured (*m/z*)	theoretical (*m/z*)	sum formula	tentative identification	RF		EA		EC		EI		LC		LF	
				t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)	t_{max} (s)	$t_{1/2}$ (s)
121.075	121.076	$C_7H_9N_2^+$	ethenylmethyl-pyrazine	8 ± 1^{ab}	5 ± 1^{n}	12 ± 3^{b}	14 ± 3^{m}	7 ± 3^{a}	15 ± 3^{m}	11 ± 6^{ab}	11 ± 3^{m}	7 ± 2^{a}	12 ± 3^{m}	8 ± 2^{ab}	13 ± 3^{m}
123.092	123.092	$C_7H_{11}N_2^+$	ethyl-methylpyrazine, trimethylpyrazine	8 ± 1^{a}	5 ± 1^{n}	10 ± 2^{b}	14 ± 2^{m}	7 ± 2^{a}	13 ± 2^{n}	7 ± 2^{a}	11 ± 3^{m}	6 ± 1^{a}	9 ± 2^{m}	7 ± 1^{a}	9 ± 2^{m}
124.083	124.076	$C_7H_{10}NO^+$	acetylmethylpyrrole	7 ± 1^{b}	4 ± 1^{n}	9 ± 2^{c}	13 ± 2^{m}	6 ± 1^{a}	14 ± 3^{m}	6 ± 1^{a}	9 ± 2^{m}	6 ± 1^{ab}	9 ± 2^{m}	6 ± 1^{ab}	9 ± 2^{mn}
125.065	125.06	$C_7H_9O_2^+$	guaiacol, methylbenzenediol	7 ± 1^{a}	4 ± 1^{n}	9 ± 2^{b}	11 ± 2mo	5 ± 1^{a}	12 ± 2^{m}	5 ± 1^{a}	7 ± 2no	6 ± 1^{a}	8 ± 2no	7 ± 1^{a}	7 ± 2no
127.038	127.039	$C_6H_7O_3^+$	maltol, methylfuroate	3 ± 1^{a}	3 ± 1^{n}	5 ± 1^{b}	4 ± 2^{n}	4 ± 1^{a}	8 ± 1^{m}	4 ± 1^{ab}	2 ± 1^{n}	5 ± 1^{ab}	9 ± 2^{m}	4 ± 1^{ab}	3 ± 2^{n}
127.08	127.075	$C_7H_{11}O_2^+$	ethylbenzenediol, ethylcyclopentanedione	7 ± 1^{bc}	4 ± 1^{n}	9 ± 2^{c}	9 ± 2no	5 ± 1^{a}	12 ± 2^{m}	6 ± 1^{a}	6 ± 2no	6 ± 1^{ab}	7 ± 2no	6 ± 2^{ab}	7 ± 2no
131.074	131.07	$C_6H_{11}O_3^+$	ethyl acetoacetate	6 ± 1^{ab}	4 ± 1^{n}	7 ± 2^{b}	8 ± 2mo	4 ± 1^{a}	9 ± 1^{m}	5 ± 1^{ab}	3 ± 2^{n}	5 ± 1^{ab}	6 ± 2no	6 ± 1^{ab}	4 ± 1^{n}
135.096	135.092	$C_9H_{11}N_2^+$	ethylvinylcyclopentapyrazine	11 ± 2^{a}		15 ± 5^{bc}		11 ± 4^{ab}		17 ± 5^{c}		11 ± 4^{ab}	14 ± 3^{m}	11 ± 3^{ab}	23 ± 3^{o}
137.067	137.071	$C_7H_9N_2O^+$	methyl-pyrazinylethanone	7 ± 2^{a}	6 ± 2^{n}	10 ± 3^{b}	17 ± 3^{m}	6 ± 1^{a}		6 ± 2^{a}	16 ± 2^{m}	7 ± 1^{a}	17 ± 2^{m}	7 ± 2^{a}	17 ± 2^{m}
149.113	149.107	$C_9H_{13}N_2^+$	dihydro-dimethyl cyclopentapyrazine	9 ± 1^{c}		22 ± 3^{b}		15 ± 4^{a}		20 ± 2^{b}		15 ± 3^{a}	13 ± 4^{m}	17 ± 3^{ab}	10 ± 4mo
165.1	165.091	$C_{10}H_{13}O_2^+$	allylguaiacol	10 ± 1^{d}		24 ± 3^{c}		17 ± 3^{a}		21 ± 1^{bc}		17 ± 3^{a}	22 ± 3^{o}	19 ± 4^{ab}	22 ± 2^{o}

[a]For each coffee variety the time at maximum intensity (t_{max}) and time elapsed until the intensity drops to half of maximum intensity ($t_{1/2}$) is shown. Data followed by different letters (a−d for t_{max} and m−p for $t_{1/2}$) are significantly different within the capsule tipes according to ANOVA ($p < 0.05$).

VOC released from the flow at each time-point (Figure 2B). The slope of these curves reflects the extraction rate. Normalizing this data to the total amount of compound extracted (intensity at the end of the extraction time was set to 100%), it is possible to compare the extraction behavior/rate of the different compounds within a coffee capsule. For each compound, and as a function of time, these curves represent the extracted fraction with respect to the total amount in the final cup. We can observe that, as a consequence of the different extraction behavior of the different compounds over time, the VOC profiles and ratios of aroma compounds in the samples differ at each time point.

Extraction of single serve capsules is similar to espresso extraction, where hot water at high pressure passes through the ground coffee bed and results in an extract containing dissolved compounds, suspended solid particles and emulsified oil and foam. The high pressure at which the water is pumped through the coffee makes espresso extraction much faster than other coffee brew techniques (e.g., compared to filter coffee extraction by gravitational force). A simple visual inspection of the coffee flow out of an espresso machine, shows that the color of the extract becomes progressively lighter with extraction time. This indicates that most of the colored compounds are extracted at the beginning of the extraction, in the first few seconds (first few milliliters). The same happens with the VOCs, although it is expected that VOC extraction is even faster and occurs more quickly than the colored, higher molecular weight compounds. Extraction of VOCs mostly occurs at the very beginning of the espresso extraction, resulting in an intense signal at the start of the time intensity profile, which is expressed as a steep slope on the integrated curve. Our results agree with those of Mestdagh et al.,[32] who extracted *Nespresso* coffee capsules stepwise with increasing volumes of extracts, from 10 mL up to 150 mL, and quantified 20 flavor active VOCs using GC-MS and isotopically labeled standards. Despite the variance associated with the use of different capsules for each volume point and the low time resolution (six points for 150 mL), they were able to describe the kinetics of extraction for 20 compounds and found some correlation between the polarity of the compound and extraction efficiency: more polar compounds were extracted faster. The same behavior was observed by Ludwig et al.[30] for nonvolatile compounds, such as caffeine, 3-, 4-, and 5-caffeoylquinic acids. They found that 70% of these compounds were extracted in the first 8 s, while only 50% of the total 3,4-,3,5- and 4,5-dicaffeoylquinic acids were extracted in the same 8 s time window, showing slower rates during the whole process of making an espresso coffee. Diccaffeoylquinic acids are less polar than monocaffeolyquinic acids and have stronger chemical interactions with melanoidins, due to potential esterification. This suggests that not only polarity but also possible interactions with other polymers present in the coffee powder modulate the rate of extraction of the different compounds.

Besides the differences in the extraction dynamics between the VOCs for each coffee, differences in individual compounds for different capsule types were also apparent. Figure 3 shows the integrated time-intensity profiles of two selected compounds as an example for this observation, methylbutanal and pyridine. Both the slopes and the final intensities are different for each capsule type with only pyridine exhibiting the same profile for EC and LC.

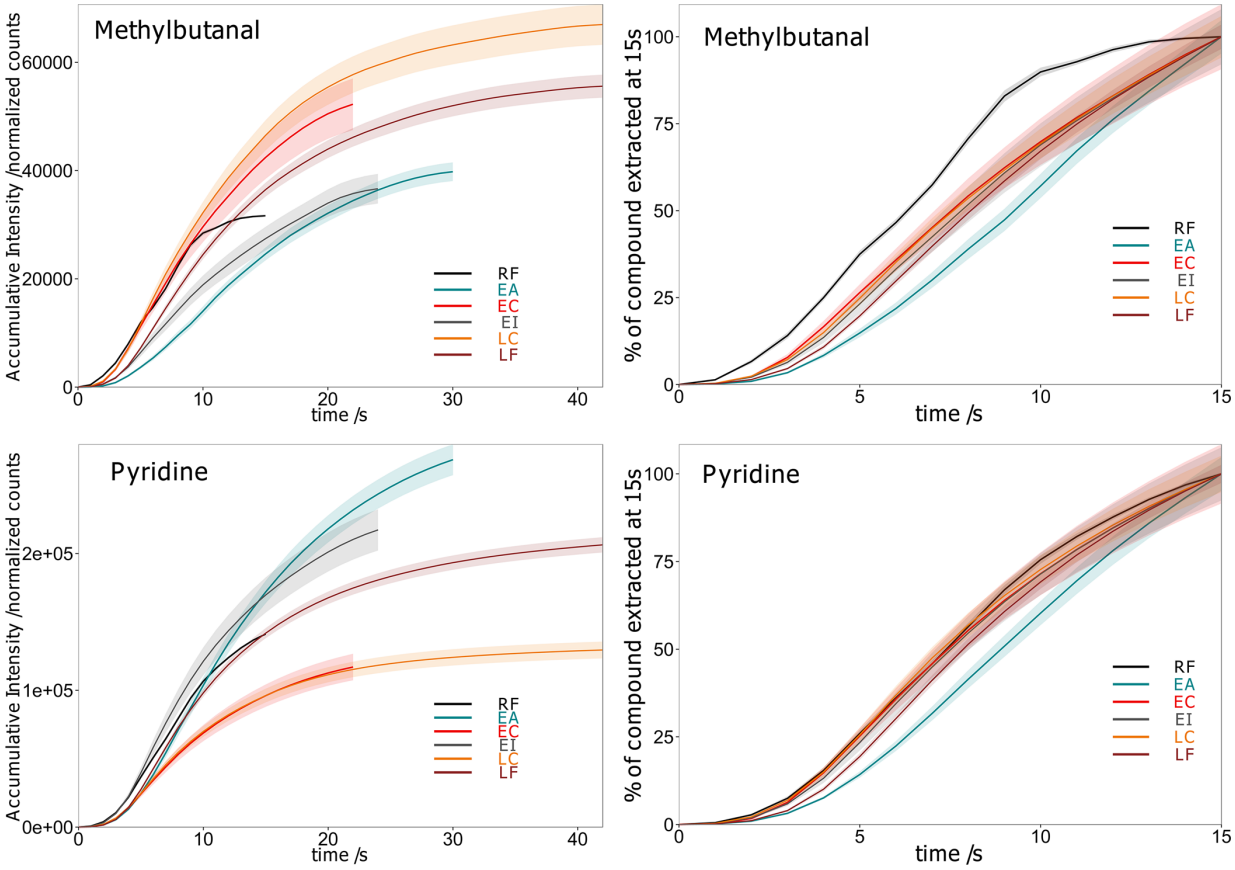

Figure 3. Integrated intensity over time for pyridine and methylbutanal for the six coffee types analyzed. The two upper graphs show the accumulative intensity over the whole extraction time. The lower graphs show the dynamics of extraction during the first 15 s: the accumulated intensity for each coffee at 15 s is considered 100%. Shaded ribbons represent the 95% confidence interval.

Espresso extraction is affected by two different sets of parameters, those related to the water, such as temperature, pressure, and mineralization content—and those related to the coffee bed—such as dose, particle size distribution, compressing force, blend, and roast. In this case, parameters regarding the water flowing through the coffee were kept constant. Thus, observed differences can be linked to differences in the coffee powder inside the capsules. The particle size distribution and the amount of coffee in the samples were measured for all capsules; they turned out to be very similar for all capsules and capsule types. Hence, this could not account for any of the observed differences. The coffees extracted were blends from different origins and species/varieties and were roasted to different roasting degrees, which leads to the formation of different amounts and profiles of VOCs, depending on the blends and the roasting condition. Consequently, it is expected that the differences observed between the various types of capsules, were related to different initial concentrations of compounds in the coffee powder. To corroborate this hypothesis, we checked how the compounds were extracted in the first 15 s (~30 mL), before the compounds are exhausted in the coffee powder. To allow for the comparison between capsules of different concentrations (different blends and roast degrees), we normalized the compound extracted to its accumulated value after 15 s (Figure 3). We can clearly observe that the slopes for the different coffee types are very similar for both methylbutanal and pyridine. Nevertheless, some differences can be observed, such as for methylbutanal in RF. In this case, methylbutanal reached the plateau before 15 s, suggesting that most of the methylbutanal in the powder had already been extracted by that time. By normalizing the integrated intensities at 10 s (instead of 15 s), the slope of RF becomes the same as for the other capsule types (data not shown), showing the same rate of extraction for all the capsules, relative to the total amount of extracted VOCs in that time window. These data suggest that, when comparing the extraction kinetics of different coffee types for selected VOCs, these extraction kinetics are essentially identical for all capsule types, as long as the VOC has not been depleted from the roast and ground coffee bed.

The dynamic time-intensity data discussed above provide insights into the extraction rates of the different compounds in the coffee capsules. However, besides exploring the extraction dynamics, the data were also used to distinguish between coffee types, by means of statistical analysis. Three approaches were used: (i) integration up to 15 s, (ii) integration over the whole extraction time, and (iii) integration over the whole extraction time, but normalized to the amount of coffee extracted (division by the total weight of the final cup). For each approach, HCA and PCA were performed.

Fifteen Seconds. The shortest to prepare coffee included in this study was RF (30 mL) with a total extraction time of

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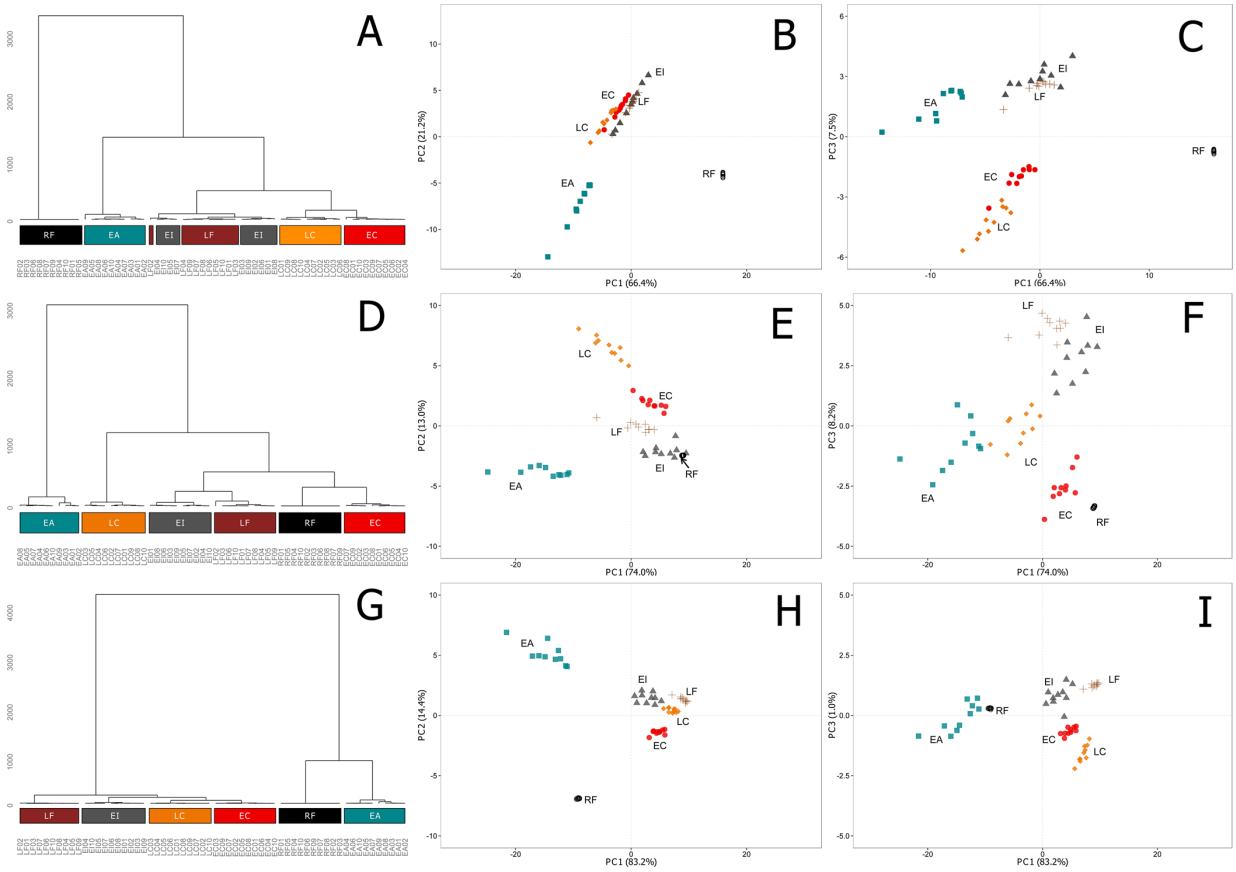

Figure 4. Hierarchical clustering and score plots for the first three dimensions of PCA of the six capsule varieties using the integrated area at 15 s (A, B, C), full extraction time (D, E, F), and full time corrected by weight of extracted coffee (G, H, I).

15 s. We selected this specific time window for the first comparison between capsules since all the capsule types were extracted for at least 15 s, allowing direct comparison of the time-intensity profiles. HCA (Figure 4A) showed good separation for four out of the six capsules (RF, EA, EC, and LC); each of these four clusters exclusively contain the ten repetitions for each capsule type. Only EI and LF could not be separated into individual clusters. PCA provided similar information. The first principal component (66.4% of the total variance) could only separate RF from the rest, but better separation was obtained for the second and third components. The PCA showed that, except for EI and LF, all the other capsule types could be separated on the plots for the first three principle components. It also showed that EC and LC are close to each other on the plots for the three first components of the PCA.

Since all the capsules were extracted for at least for 15 s, differences between types of capsules in HCA and PCA are indicative of differences between the coffees (i.e., coffee varieties, blend, roasting degree) used to manufacture each of the capsule types. Our results suggest that the coffees used for EI and FL are similar and therefore appear close on the PCA plots. A similar situation is observed for EC and LC. Checking the capsule characteristics, it was possible to see that both EI and FL contained more coffee powder (around 6.25 g) than the others (around 6.00 g). Furthermore, together with RF, they had the darkest roasting degrees of those included in this study. EC and LC showed the lightest roasting of all the coffees. Roasting is one of the key factors that affect the final coffee aroma profile and is most probably responsible for the observed clustering, although it is not the only factor at play. The blend used for each coffee also impacts the compounds formed during roasting, as the aroma precursors differ. Since the origins of the coffees used for each blend are only known based on the manufacturer's general descriptions, observed similarities cannot be related to the specific composition of the blends.

Full Time Extraction. When the time-intensity profiles are integrated over the full extraction time (which varies between the different coffee types), HCA is able to separate all replicates of each coffee type into six individual clusters (Figure 4D). PCA analysis also shows total separation of the six capsule types on the plots for the first three principle components (Figures 4E and 4F). Integration over the full extraction time allowed the EI and LF coffees to be separated, which was not possible when integrating over the first 15 s.

By integrating the whole area under the time intensity profiles, it was possible to obtain a value proportional to the total amount of the extracted compound in the cup. The extraction of a Lungo takes approximately 20 s longer than for an Espresso, and during that extra time some compounds are still in the process of being extracted, resulting in better separation in the HCA and PCA plots. Although the main driving force for separation is the difference in extraction time, compounds with identical extraction times can also be

separated from each other. These results indicate that, when extracted according to manufacturer recommendations, the amount and ratio of the VOCs in the final product is different for all six different capsule types.

Final Concentration. Full extraction−time integration reflects the total amount of each compound extracted, but does not account for the dilution factor due to different cup volumes. Hence it is a measure of the total amount *in* the cup, but does not reflect the volatile profiles *above* the cup (the headspace). As shown, the majority of the compounds are extracted during the first seconds of the coffee extraction process. As extraction evolves, the remaining amounts of the compounds in the coffee bed decrease, and their concentrations in the extract decrease as well. To get data that is closer to the concentration in the final cup (and to the HS), the results for the amount of coffee extracted were normalized. One of the advantages of PTR-MS is that the signal is proportional to the measured concentration,[40] and therefore, the data could be easily corrected for dilution by dividing the total amount of compound extracted (integrated area over the total time of extraction) by the weight of the final coffee extract. This result reflects the concentration of each compound in the final coffee and is comparable to headspace measurements for the final cup. Both HCA and PCA showed good clustering for all the capsules when the data are corrected for dilution. Therefore, we can conclude that the aroma profile of the extracts and consequently the HS profiles are clearly different for the six capsule types investigated here.

■ CONCLUSIONS

We have presented a novel, high time-resolution methodology for monitoring the extraction dynamics of espresso coffee and applied it to six different capsules types. The results presented in this work show the suitability of PTR-ToF-MS for monitoring changes in the volatile composition of a liquid flow in an open atmosphere. Online analysis of coffee extraction revealed the kinetics of extraction for different VOCs and highlighted the differences between commercial coffee capsules over the whole extraction time. The presented method overcomes the problems of previous GC-based approaches: (i) it increases temporal information, from a few data points over the whole extraction time to a one second resolution, (ii) it reduces sources of variability, as the time-evolution of each VOC is monitored online in a single extraction process and is not a combination of multiple different extracts. The simplicity, high sensitivity and time resolution of the method makes it a perfect approach for investigating the impact of different parameters that affect extraction dynamics of flavor compounds. On the basis of such data, the process can be fine-tuned in order to achieve the desired aroma balance in the final cup.

The methodology also allows the user to differentiate between coffee types, by applying HCA and PCA on the cumulated intensities of VOCs over specific time windows.

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All authors have given approval to the final version of the manuscript

Notes

The authors declare no competing financial interest.

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Publication 2.

Extraction kinetics of coffee aroma compounds using a semi-automatic machine:

On-line analysis by PTR-ToF-MS

by

José A. Sánchez-López, Marco Wellinger, Alexia N. Gloess, Ralf Zimmermann, Chahan Yeretzian.

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Extraction kinetics of coffee aroma compounds using a semi-automatic machine: On-line analysis by PTR-ToF-MS

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ABSTRACT

The hot-water extraction process used to make an espresso coffee is affected by a large number of factors. A proper understanding of how these factors impact the profile of the final cup is important to the quality of an espresso coffee. This work examines the effect of water temperature and pressure on the extraction kinetics of volatile organic compounds (VOCs) in coffee. This was achieved by on-line monitoring of the volatiles directly from the coffee flow, using proton-transfer-reaction time-of-flight mass-spectrometry (PTR-ToF-MS). Using hierarchical cluster analysis (HCA), tentatively identified compounds were grouped into 5 families according to their time–intensity profiles. VOCs grouped into each family had similar physicochemical properties while polarity was found to be one of the main forces driving VOC extraction kinetics. The effect of pressure was studied by extracting espresso coffees at 7, 9 and 11 bar. A pressure of 11 bar resulted in an increased extraction of volatiles over the entire extraction time (25 s). To study the effect of temperature, espresso coffees were extracted at 82, 92 and 96 °C. An increase in temperature produced a significant increase in the extraction of VOCs, especially during the last part of the extraction. The effect of temperature on extractability was more pronounced for the less polar compounds.

1. Introduction

Coffee is one of the most widely consumed beverages [1]. The beverage is made from coffee beans that are first harvested and processed, then roasted and ground before finally being extracted. Each and every single transformation step, from the seed to the cup, must be mastered and performed with great care in order to deliver the best quality in the cup [2–9]. Here, we focused on the last and crucial transformation step, the extraction – more specifically, espresso coffee extraction using a semi-automatic coffee machine.

During extraction, soluble compounds are dissolved and, depending on the extraction technique, non-soluble compounds are washed away with the extraction water, ending up in the extract as dissolved or suspended solids [10–15]. Many different extraction techniques have been introduced over the past centuries, which vary according to factors such as geography, culture and social context, as well as personal preferences; these different factors can result in vastly different flavor profiles in the extract. Of all coffee brewing methods, espresso brewing is among the most popular techniques.

Starting with whole roasted coffee beans, the preparation of the "perfect" espresso is as much a science as an art. It is the result of the interplay between several parameters that must be carefully controlled. These parameters include the particle size distribution of the ground coffee, the water-to-coffee ratio, the final volume of the brew in the cup and the temperature and pressure of the extracting water. An espresso is defined as a 25–35 ml beverage prepared from 7 to 9 g of coffee, through which clean, 92–95 °C water has been forced at 9–10 atmospheres of pressure, and where the grind of the coffee is such that the brewing 'flow' time is approximately 20–30 s [11].

An increase in the extraction temperature, for example, leads to higher quantities of non-volatiles (i.e. total solids, caffeine, lipids) as well as higher quantities of some volatiles, such as pyrazines, in the final cup [16–18]. This may result in over-extraction and a coffee cup with negative flavor notes, such as woody, burnt or acrid flavors. Increasing pressure up to 11 bar also resulted in coffees with higher odor intensity and lower consumer acceptance than coffees

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extracted at 7 or 9 bar [19]. In these previous studies, the authors focused on the composition and sensorial attributes of the final cup in order to assess the impact of water temperature and pressure on espresso preparation. In a complementary line of research, some scientists have been exploring the extraction kinetics of the volatiles that contribute to the aroma of coffee. Two main methods have been previously used to determine the extraction kinetics of coffee volatiles: off-line analysis of fractions using GC–MS [20] and on-line analysis of the volatiles released by the coffee flow using Proton Transfer Reaction Time of Flight Mass Spectrometry (PTR-ToF-MS) [21,22]. In both cases, the methods were applied to single-serve capsule systems in which both temperature and pressure were kept constant. The objective of the research presented here was to investigate the effect of temperature and pressure on the extraction kinetics of coffee aroma compounds by applying on-line analysis by PTR-ToF-MS. We focused on 46 VOCs in particular and explored the link between extraction kinetics and their physicochemical properties.

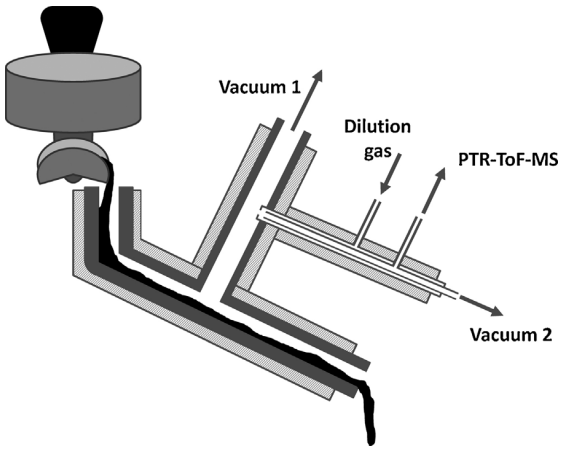

Fig. 1. Setup used for the online monitoring of volatiles during coffee extraction.

2. Materials and methods

2.1. Coffee extraction

The yellow bourbon variety of *Coffea Arabica* L. from Mogiana, Brazil (Roaster: Kaffeepur, Switzerland, "Yellow Sun"), roasted to a medium roast degree of 95 Pt (Colorette 3b, Probat, Germany), was used for all of the extraction experiments. The coffee was frozen two weeks after roasting and defrosted 12 h prior to the experiments to ensure a constant and equal freshness of the coffee for all of the experiments. Less than one month elapsed from roasting to extraction. The beans were ground using a Compak K10 grinder (Barcelona, Spain) using position 47 on a scale from 0 (fine ground, Turkish coffee) to 60 (coarse ground, French Press pot). 18 g of the ground coffee were weighed into a double porta-filter basket, tapered by hand and extracted for 25 s using a semi-automatic coffee machine (Dalla Corte Mini, Dalla Corte, Italy). The water used for the extractions was commercially available Volvic mineral water (total mineralization 130 mg/L; HCO_3^-: 71 mg/L; SO_4^{2-}: 8.1 mg/L; Na^+: 11.6 mg/L; Ca^{2+}: 11.5 mg/L; Mg^{2+}: 8 mg/L). Extractions were performed with five different combinations of water pressure and temperature, one within the recommendations provided by the Specialty Coffee Association of America (center point conditions) and the others with values that exceed or were lower than recommended: (9 bar/92 °C: center point; 7 bar/92 °C; 11 bar/92 °C; 9 bar/82 °C; and 9 bar/96 °C). Although all the coffees were prepared by an experienced barista, variations resulting from the manual preparation process were expected. To reduce this variability, we performed 8 extractions for each set of conditions and selected the 5 replicates for which the final weight of the extract was closest to 30 g, ending up with coffee weights in the range of 31.5 g ± 2 g.

2.2. Sampling setup

Volatiles were sampled using a previously used setup [22], with certain modifications (Fig. 1). The extracted coffee flowed into a custom built system that was heated to 96 °C to avoid condensation. Volatiles were drawn out using a vacuum pump and diluted 10-fold with dry compressed air to reduce their concentration to the dynamic range of the PTR-ToF-MS being used.

2.3. PTR-ToF-MS

A commercial PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) was used for all measurements. The diluted sample was introduced via a 90 °C heated sampling line into the drift tube operated at 2.3 mbar, 90 °C and 600 V drift tube voltage, resulting in an E/N value (electric field strength/gas number density) of 140 Townsend (Td, 1 Td = 10^{-17} cm^2/V s). PTR-ToF-MS data were recorded by TOFDAQ v.183 data acquisition software (Tofwerk AG, Thun, Switzerland). Mass spectra were recorded in the mass-to-charge (m/z) range of 0–300 with one mass-spectrum recorded every 2 s.

2.4. Data processing

Dead time correction, mass calibration, peak extraction and integration were performed using PTR-TOF DATA Analyzer software (v4.17) [23]. Duty cycle corrected signals were normalized to 10^6 H_3O^+ primary ions and the concentration in parts per billion by volume (ppbv) was estimated using 2×10^{-9} cm^3 s^{-1} as a reaction rate constant coefficient [24].

More than 500 mass peaks were detected in the range 0–300 m/z. Ions not related to the sample (O_2^+, NO^+ and water clusters) were eliminated, the background was subtracted and a concentration threshold of 1 ppb was set for further peak selection. This resulted in a reduction to 120 mass peaks that were present in all of the samples. From these peaks, 46 compounds (Table 1) were tentatively identified by comparing them to the literature [25,26].

2.5. Statistical analysis

The areas under the time–intensity curves were calculated for the 120 mass traces of each of the five replicates of the five different extraction conditions. Principal Component Analysis (PCA) was performed using mean centered and scaled areas. Analysis of Variance (ANOVA) was applied to assess the effect of the different extraction variables on the total area of the selected compounds using Tukey's Honest Significant Difference (HSD) post hoc test ($p < 0.01$). In order to identify compounds with similar dynamic behavior, first the time–intensity profiles of all the 120 m/z were normalized to their maximum intensity before performing self-organizing tree algorithm (SOTA). Subsequently the same SOTA analysis was performed on only the tentatively identified 46 VOCs. In addition the normalized time–intensity profiles of the 46 tentatively identified compounds were subjected to Hierarchical Cluster Analysis (HCA) using Ward's minimum variance method and half-squared Euclidean distances. All analyses were performed and all graphs were created using existing packages (clValid, multcomp, and ggplot2) and scripts developed in R [27].

Table 1
List of tentatively identified mass peaks, assigned sum formula and physic-chemical properties. Compounds are grouped in families according to the results obtained from Hierarchical Cluster Analysis (HCA).

Compound number	Measured *m*/*z*	Theoretical *m*/*z*	Sum formula	Tentative identification	Boiling point/°C	log K_{aw}	Vapor pressure/ KPa at 25 °C	Water solubility/g L^{-1}	log K_{ow}
Family A									
1	31.019	31.018	CH_3O^+	Formaldehyde	−19	−4.861	518	400	0.35
2	33.034	33.033	CH_5O^+	Methanol	65	−3.730	16.9	1000	−0.77
3	45.034	45.033	$C_2H_5O^+$	Acetaldehyde	20	−2.564	120	1000	−0.34
4	59.047	59.049	$C_3H_7O^+$	Acetone	56	−2.790	30.9	1000	−0.24
				Propanal	48	−2.523	42.3	306	0.59
5	61.029	61.028	$C_2H_5O_2^+$	Acetic acid	118	−5.388	2.09	1000	−0.17
6	75.045	75.044	$C_3H_7O_2^+$	Propanoic acid	141	−4.740	0.471	1000	0.33
				Ethyl acetate	77	−2.261	12.4	80	0.73
7	87.046	87.044	$C_4H_7O_2^+$	2,3-Butanedione	88	−3.265	7.57	200	−1.34
				Butyrolactone	204	−5.667	0.060	1000	−0.64
Family B									
8	47.014	47.013	$CH_3O_2^+$	Formic acid	101	−5.166	7.01	1000	−0.54
9	57.036	57.033	$C_3H_5O^+$	2-Propenal	53	−2.302	36.5	212	−0.01
10	71.051	71.049	$C_4H_7O^+$	2-Methylpropenal	68	−2.023	20.7	50	0.74
				3-Buten-2-one	81	−2.721	12.2	60.63	0.41
11	85.066	85.065	$C_5H_9O^+$	Methylbutenal	117	−2.444	2.39	25	1.15
12	89.061	89.060	$C_4H_9O_2^+$	Methylpropanoate	80	−2.148	11.2	62.4	0.84
13	101.061	101.060	$C_5H_9O_2^+$	Pentanedione	138	−4.017	0.495	166	0.4
				γ-Valerolactone	192	−2.255	0.073	93.81	0.11
Family C									
14	55.057	55.054	$C_4H_7^+$	1,3-Butadiene	−4	0.478	281	0.735	1.99
15	63.03	63.026	$C_2H_7S^+$	Dimethyl sulfide	37	−1.182	66.9	22	0.92
16	73.064	73.065	$C_4H_9O^+$	Butyraldehyde	75	−2.328	14.8	71	0.88
				2-Methylpropanal	65	−2.133	23.1	89	0.74
				Butanone	80	−2.633	12.1	223	0.29
Family D									
17	68.051	68.049	$C_4H_6N^+$	Pyrrole	130	−3.133	1.11	45	0.75
18	69.036	69.033	$C_4H_5O^+$	Furan	31	−0.656	80.0	10	1.34
19	70.041	70.040	$C_2H_4N_3^+$	Triazole	203	−4.212	0.080	240	−0.29
20	82.064	82.065	$C_5H_8N^+$	Methylpyrrole	112	−3.388	2.85	10	1.21
21	95.062	95.060	$C_5H_7N_2^+$	Methylpyrazine	135	−4.046	1.29	80	0.21
22	97.031	97.028	$C_5H_5O_2^+$	Furfural	162	−3.861	0.295	77	0.41
23	99.046	99.044	$C_5H_7O_2^+$	Furfuryl alcohol	171	−5.493	0.081	1000	0.28
24	103.077	103.075	$C_5H_{11}O_2^+$	Hydroxypentanone	179	−3.255	0.033	48.868	0.2
				1-Methyl-2-butanoic acid	177	−4.468	0.065	45	1.18
25	109.078	109.076	$C_6H_9N_2^+$	Dimethylpyrazine	156	−3.838	0.365	38.16	0.54
				Ethylpyrazine	153	−3.999	0.476	28.41	0.69
26	111.046	111.044	$C_6H_7O_2^+$	Acetylfuran	175	−3.398	0.126	39.1	0.52
				5-Methylfurfural	187	−3.218	0.091	29.11	0.67
27	113.062	113.060	$C_6H_9O_2^+$	5-Methylfurfuryl alcohol	191	−5.010	0.017	49.18	0.99
28	115.075	115.075	$C_6H_{11}O_2^+$	4-Methyltetrahydro-2H-pyran-2-one	213	−2.131	0.025	32.19	0.6
29	117.057	117.055	$C_5H_9O_3^+$	2-Oxopropyl acetate	171	−4.467	0.199	151.9	−0.19
30	131.073	131.070	$C_6H_{11}O_3^+$	Ethyl acetoacetate	181	−4.309	0.104	110	0.25
Family E									
31	80.052	80.049	$C_5H_6N^+$	Pyridine	115	−3.347	2.77	1000	0.65
32	87.082	87.080	$C_5H_{11}O^+$	Methylbutanal	94	−2.187	1.39	10	1.23
33	105.068	105.070	$C_8H_9^+$	Vinylbenzene	145	−0.949	0.853	0.310	2.95
34	107.06	107.060	$C_6H_7N_2^+$	Ethenylpyrazine	167	−4.121	0.240	21.38	0.84
35	110.064	110.060	$C_6H_8NO^+$	2-Acetylpyrrole	220	−6.171	0.004	17.59	0.93
36	121.075	121.076	$C_7H_9N_2^+$	Ethenylmethyl-pyrazine	188	−3.762	0.072	7.284	1.33
37	123.093	123.092	$C_7H_{11}N_2^+$	2-Ethyl-5-methylpyrazine	169	−3.715	0.081	1.903	1.53
				Trimethylpyrazine	171	−3.795	0.193	15.21	0.95
38	125.063	125.060	$C_7H_9O_2^+$	Guaiacol	205	−4.309	0.014	18.7	1.32
				Methylbenzenediol	241	−8.580	4.6×10^{-4}	16.48	1.58
39	127.075	127.075	$C_7H_{11}O_2^+$	Ethylbenzenediol	265	−8.456	1.0×10^{-4}	5.52	2.07
				Ethylcyclopentanedione	225	−5.081	0.017	104.8	−0.05
40	135.091	135.092	$C_8H_{11}N_2^+$	5-Methyl-6,7-dihydro-5H-cyclopentapyrazine	201	−3.948	0.019	2.416	1.83
41	138.087	138.091	$C_8H_{12}NO^+$	2-Acetyl-1-ethylpyrrole	209	−4.718	0.022	3.716	1.6
				3-Acetyl-2,4-dimethylpyrrole	247	−6.085	3.1×10^{-4}	3.327	1.65
42	148.077	148.076	$C_9H_{10}NO^+$	1-Furfurylpyrrole	292	−3.195	0.011	0.568	2.5
43	149.112	149.107	$C_9H_{13}N_2^+$	Dihydro-dimethyl cyclopentapyrazine	237	−3.985	0.004	0.557	2.51
44	165.093	165.091	$C_{10}H_{13}O_2^+$	Allylguaiacol	264	−5.706	3.1×10^{-4}	0.305	2.73
Separate compounds									
45	57.072	57.070	$C_4H_9^+$	1-Butene	−1	0.978	300	0.2	2.4
46	83.051	83.049	$C_5H_7O^+$	Methylfuran	65	−0.615	20.8	3	1.85

3. Results and discussion

3.1. Dynamics of extraction

During the 25 s coffee extraction, the intensity over time of the volatile compounds in the coffee was monitored and analyzed in detail for the 46 tentatively identified compounds. The focus was put on these 46 compounds, in contrast to the complete list of 120 compounds, as this allowed to link the identified VOC to their physical properties. Hierarchical Clustering Analysis (HCA) was firstly applied to the normalized time–intensity profiles of the center point experiment (9 bar/92 °C) resulting in the classification of 44 compounds into five different families that shared similar time–intensity profiles (Table 1 and Fig. 2). Two compounds did not fit into any of the five main families: 1-butene ($C_4H_9^+$) and methylfuran ($C_5H_7O^+$). In the case of 1-butene, the intensity showed an irregular profile during extraction, with high standard deviation between the replicates. The *m*/*z* attributed to 1-butene (57.073) has also been reported as an alcohol fragment [28]. This suggests *m*/*z* 57.073 does not correspond only to 1-butene but rather to a superposition of ion intensities from additional compounds and/or fragments with the same mass-to-charge ratio, which interfered with the 1-butene signal. In the case of methylfuran, the signal rose to its maximum value at 4–6 s and then remained constant until the end of the extraction process. This behavior was not observed for any other compound. It should also be noted that the time–intensity profiles recorded for methylfuran did not show any significant differences for any of the extraction conditions analyzed.

HCA was subsequently applied to the time–intensity profiles of all the extraction conditions together, to check if the VOC families observed for the center point were independent of the extraction conditions. Essentially the same five families were reproduced when considering all conditions, although a few compounds clustered differently for the lowest pressure and the highest temperature conditions, relative to the center pint extraction: (i) for extraction at 7 bar, three compounds from family D (methylpyrazine, furfural and acetol acetate) were classified as members of family B; (ii) for extraction at 96 °C, all three compounds from family C (butadiene, dimethylsulfide and methylpropanal) appeared in family B, and four compounds from family E (ethenyl pyrazine, pyridine, acetylpyrrole and ethylbencenediol) appeared in family D.

Clustering was performed only on the 46 tentatively identified compounds. In order to confirm that those compounds are representative of all the measured *m*/*z*, we used the Self Organizing Tree Algorithm (SOTA) on a data set composed of the 3000 intensity profiles recorded (5 brewing conditions × 5 replicates × 120 *m*/*z*). Six clusters were obtained of which five were identical to the main families and a sixth one containing only 49 profiles corresponding to mass traces that did not fit with the other families (Supplementary material 1). This corroborates the robustness of the five main families of volatile, and of their corresponding classification according to different extraction dynamics during espresso brewing.

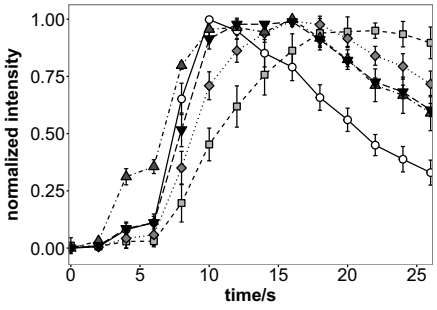

Fig. 2. Normalized time–intensity profiles of the different families of compounds extracted at 92 °C and 9 bar. Lines represent the mean and the error bars represent the standard deviation of all the compounds in family A (●), B (▼), C (▲), D (♦), E (■).

Fig. 2 shows the time–intensity profiles of the center point experiment for each of the five families. Time zero corresponds to the moment at which the pump of the coffee machine started to run, however, coffee did not start to flow out of the portafilter until 5–6 s later. After 25 s, the pump was stopped and 1 s later the coffee flow also stopped. This figure shows the distinguishing features found between the different families.

Family A was characterized by a fast rise in intensity 6 s after the coffee machine started to pump water, reaching maximum intensity at 10 s. From that point until the end of the extraction, this family exhibited the fastest decrease in intensity of all of the families, with final values lower than 40% of the maximum intensity. Independent of the extraction conditions, the same seven compounds were always clustered into this family.

Family B's profile was similar to that of A during the first 10 s, after which and in contrast to compounds of family A the intensity did not change (i.e. decrease) significantly, resulting in a plateau of maximum intensity until second 16. At this point it started to decrease, before finally reaching 60–70% of the maximum of the intensity at the end of the extraction process.

Family C exhibited a local maximum in intensity (visible as a peak shoulder) at 4 s. This shoulder was also present in the other families, although much less pronounced. From that point on, the intensity rose rapidly until it reached a maximum at 10 s, after which the profile was almost identical to family B, with a plateau until 16 s and a subsequent decrease. This high similarity in profiles, in particular for the second half of the extraction time, meant that families B and C were clustered together for the extraction at 96 °C. Despite not being separated by HCA at the highest extraction temperature, the profiles for family C could still be visually differentiated by the characteristic shoulder at 4 s.

In **Family D**, the intensity started to rise after 6 s and reached a maximum at between 14 and 16 s. After that, the intensity decreased to 70–80% of the maximum. For extraction at 7 bar, three compounds from this family were grouped together with those in family B. As will be discussed in Section 3.2, extraction at 7 bar showed a slower increase in intensity and lower maximum intensities than the center point experiment (9 bar/92 °C) for compounds belonging to family B, making the profiles of this family more similar to those of D and hence affecting the classification into families.

Family E showed the slowest increase in intensity from 6 s to a maximum at 20 s. The intensity decreased slightly from the maximum until the end of the extraction, with values that were approximately 80–95% of the maximum intensity. When the extraction was performed at the highest temperature, the characteristic decrease in intensity during the last part of the extraction was not observed for some compounds in family D. In fact the intensity increased until the end of the extraction for some compounds in family E (Fig. 5). This effect resulted in similar profiles to family D, meaning that four compounds from family E were grouped with those of family D for the 96 °C extraction.

3.2. Classification of families

The extraction of aroma compounds from the coffee beans by water is mainly driven by polarity [20]. For the 46 compounds that were tentatively identified, values for $\log K_{ow}$ (partition coefficient between octanol and water), $\log K_{aw}$ (partition coefficient between air and water), water solubility, boiling point and vapor pressure are provided in Table 1. Apart from a few exceptions, water solubility decreases and $\log K_{ow}$ increases as one moves from family A

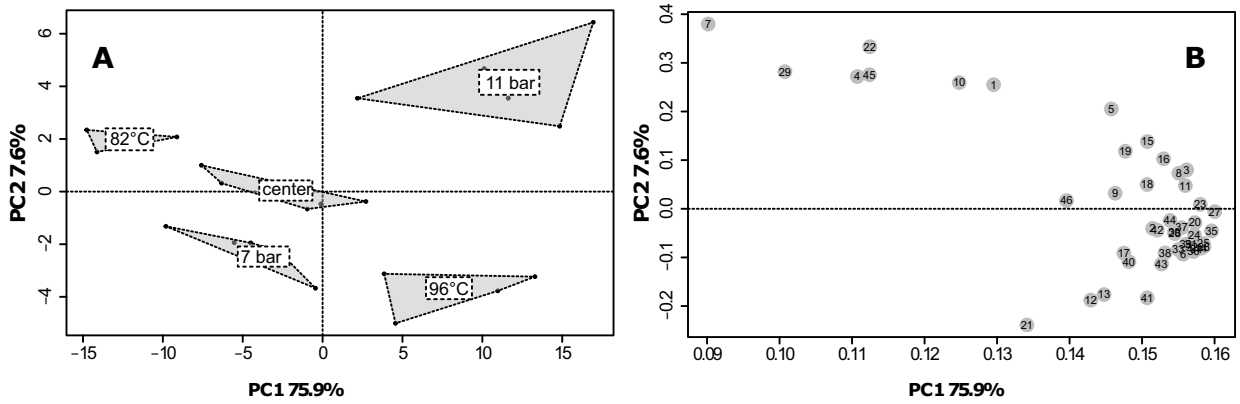

Fig. 3. Score-plot (A) and loading-plot (B) of the first two components detected in the PCA, performed on the area under the time–intensity profiles for the 120 *m*/*z*. Data points on the score plot represent the five different extractions performed for each set of conditions. Numbers on the loading plot correspond to the compound list in Table 1. Non-identified compounds have been omitted from the loading plot for the purposes of clarity. A plot showing the loadings of all 120 *m*/*z* can be found in the supplementary materials.

to E, indicating that the most polar compounds belong to family A and the least polar ones to family E. Compounds in family A – with water solubility of between 80 and 1000 g L^{-1} and log K_{ow} between 1.34 and 0.73 – were extracted quickly, within the first seconds of extraction, and their concentration levels decreased significantly at the later stage of the extraction. Polarity and water solubility of the compounds in family B are slightly lower than in family A, meaning that the intensity also increased quickly at the beginning, but their extraction lasted longer. In family C, the compounds have a lower water solubility (0.7–223 g L^{-1}) and lower polarity (log K_{ow} 0.29–1.99) than those of families A and B. This would imply that these compounds are extracted more slowly from the coffee bed. However, their intensity did increase rapidly during the first 6 s. This fast increase in signal can be attributed to the high volatility of the compounds in this family (vapor pressure: 12–281 KPa and log K_{aw}: −2.64 to 0.48) that favored their release to the gas phase. This could also explain the characteristic shoulder at 4 s, time at which the coffee had not started to flow, but the coffee powder had already been wetted by hot water and, as a consequence, compounds were released to the gas phase. Families D and E contain the least polar, water soluble and volatile compounds. Therefore compound transfer from the ground coffee particles to water was slow, with an important fraction of these compounds still being extracted after 25 s.

Grouping of dynamic data into families can also be used to improve compound identification. Generally, tentative identification of compounds using PTR-ToF-MS and other direct mass spectrometry techniques is performed by assigning a sum formula to the measured mass and comparing it with compounds previously reported in the literature. However, this can potentially lead to errors in compound assignment. When clustering all the compounds according to their dynamic behavior, it is expected that compounds in the same group will have similar physicochemical characteristics and those which differ might have been miss-identified. In Table 1, some compounds can be singled out as possibly having been miss-identified. For example, compound number 6 ($C_3H_7O_2^+$) has been reported in the coffee literature as both propanoic acid and ethyl acetate. The polarity and water solubility of ethyl acetate are lower than those of the other compounds in family A, suggesting that the measured compound was most probably propanoic acid. Another potential miss-assignment is compound 31 ($C_5H_6N^+$), which was reported as pyridine and clustered in family E. However, the physicochemical characteristics of pyridine are closer to those of family A or B than to those of family E, indicating we may have detected a fragment of a less polar compound containing a pyridine ring instead of pyridine itself.

3.3. Factors affecting extraction

To evaluate the impact of temperature and pressure on the extraction, a PCA was performed using the total area under the time–intensity curves of the selected 120 mass traces (Fig. 3). The first two principal components explained 82.5% of the total variability in the data, and the graphical representation of the scores for these two components allowed differentiation into five different groups corresponding to the different conditions used for extraction (Fig. 3A). More information can be drawn from the loading plot (Fig. 3B). Loadings for the 46 tentatively identified compounds were all positive for PC1, indicating an increase in total area under the curve for higher temperatures and pressures. For PC2, 18 tentatively identified compounds had positive scores and are related to high pressure extraction. All but four of the compounds (18, 19, 22, 29) belong to the most polar groups A, B or C. Compounds with negative scores for PC2 belonged to families D and E, together with compounds number 2, 6, 12, 13 and 14 from the other families. Negative PC2 scores are related to an increased area under the curve for higher extraction temperatures, indicating that increases in temperature had a greater effect on the less polar compounds.

To obtain additional information on how pressure and temperature affected the extraction, the respective time–intensity profiles and the time evolution of the area under the curve were compared for one compound from each family (Fig. 4 for pressure and Fig. 5 for temperature effect). As discussed in the Section 3.1, the grouping of the different time–intensity profiles hardly varied between the different extraction parameters and so we, therefore, assumed that one compound would be representative of the whole family.

3.3.1. Effect of pressure

The time–intensity profiles of family A showed no significant differences based on extraction pressure for the representative compounds (Fig. 4). For $C_5H_9O^+$ and $C_4H_9O^+$, representatives of families B and C respectively, extraction at 7 bar resulted in lower intensities, but only during the middle phase of the extraction (8–16 s). The highest effect of pressure on the time–intensity profiles was observed for families D and E, where there were no differences between 7 and 9 bar. However, extraction at 11 bar resulted in significantly higher intensities during the last 10 s of extraction. Since the differences in intensities at each point in time were small, but could accumulate and have high impact in the final cup, we also calculated and plotted the total area under the curve up to each point in time (second column Fig. 4). Boxplots showed no significant differences between 7 and 9 bar for any of the families, but extraction at 11 bar always resulted in significantly higher

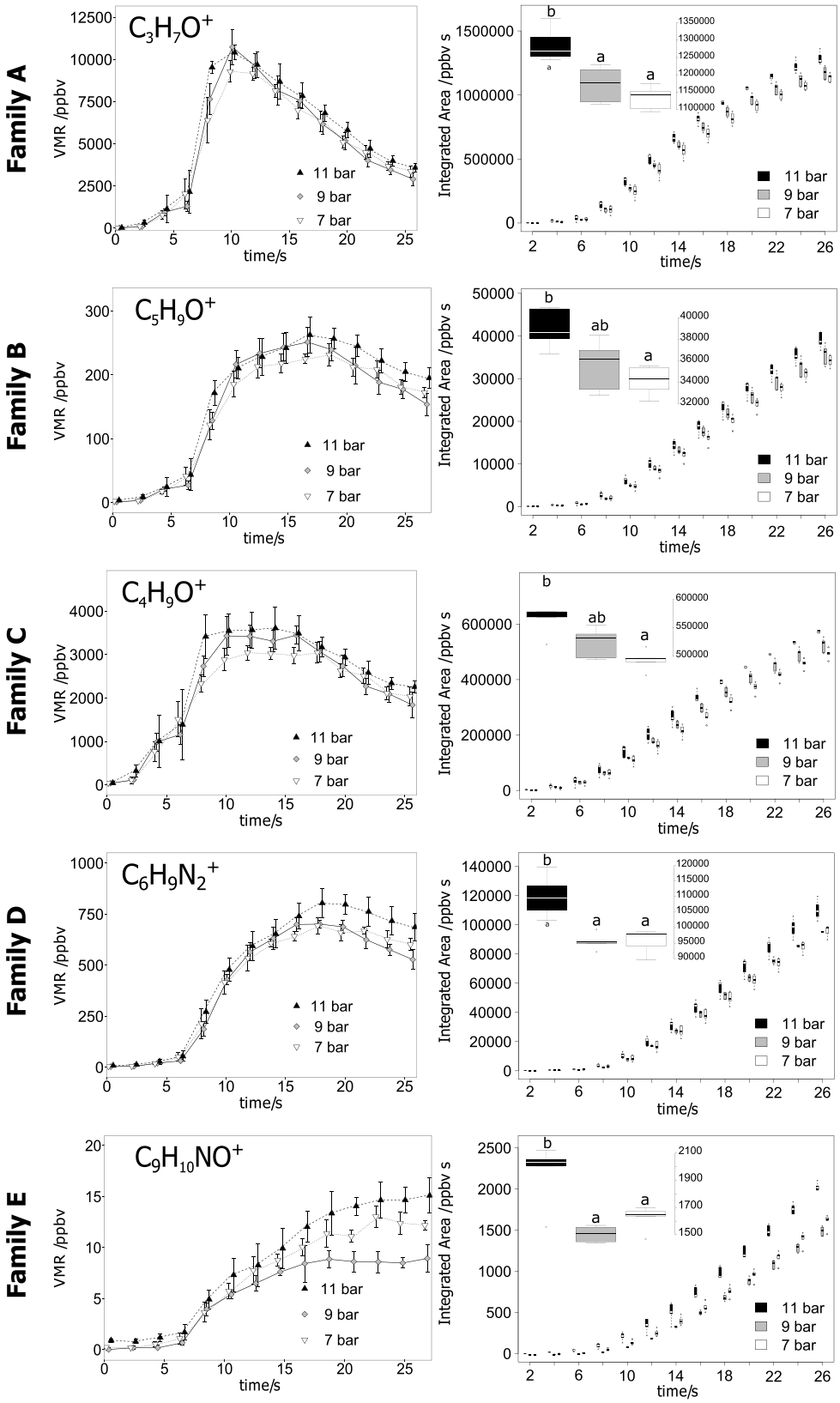

Fig. 4. Time–intensity profiles of compounds chosen as single representatives of each family during coffee extraction at three different pressures. Points represent the mean and the error bars represent the standard deviation of the replicates. Boxplots represent the area under the curve at that point in time and the insert in the left corner is a magnification of the last point (26 s), with letters representing measurements that differ significantly for the different extraction parameters (Tukey's test, $p < 0.01$).

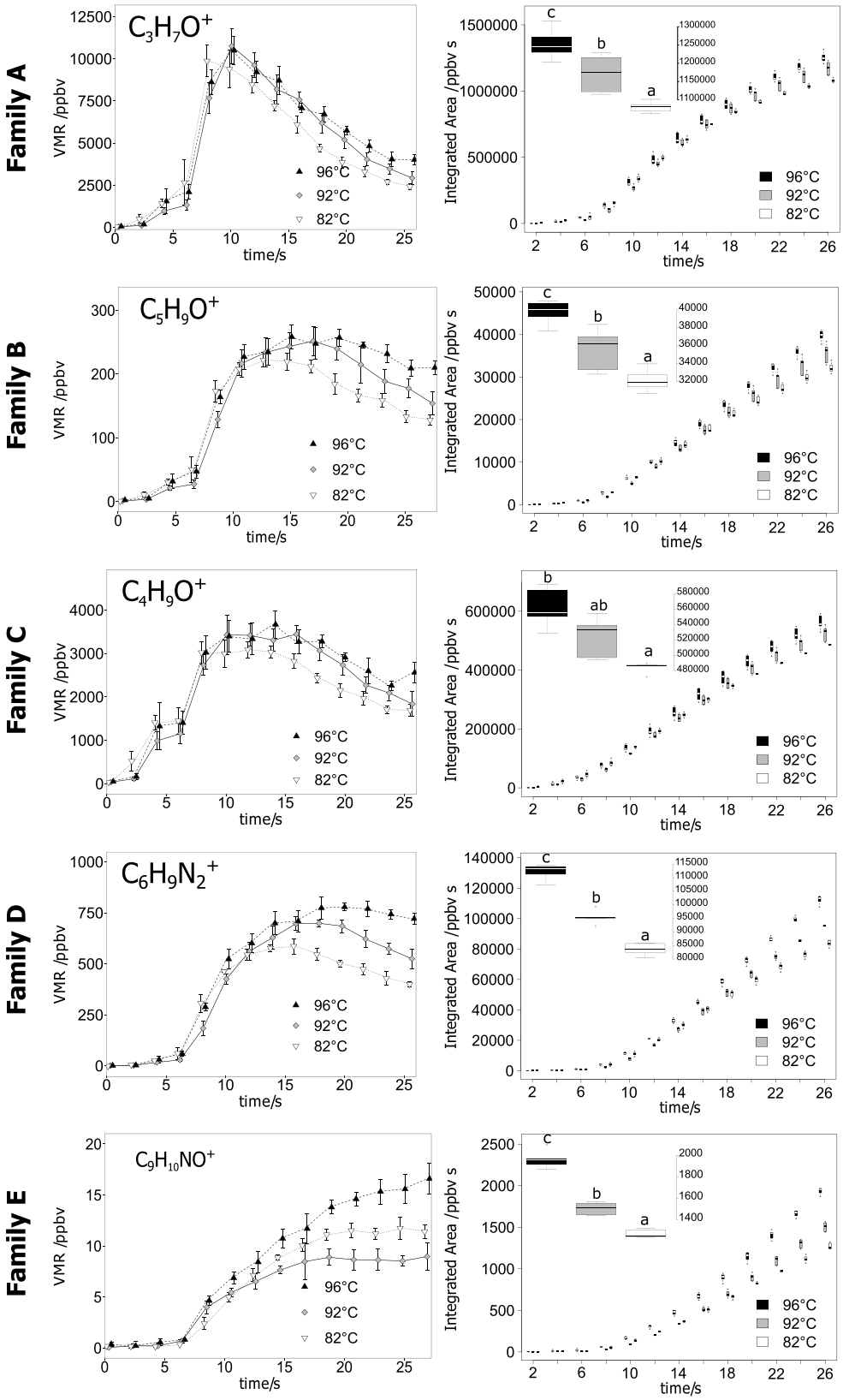

Fig. 5. Time–intensity profiles of one representative compound of each family during coffee extraction at three different temperatures. Points represent the mean and the error bars represent the standard deviation of the replicates. Boxplots represent the area under the curve at that point in time and the insert in the left corner is a magnification of the last point (26 s) with letters representing measurements that differ significantly for the different extraction parameters (Tukey's test, $p < 0.01$).

concentrations over the entire extraction time, compared to the extraction at 7 bar.

Pressure is the driving force required to produce a flow of water through the compacted coffee cake, assisting the extraction of compounds trapped inside the coffee particle structure, and also helping to transfer solid particles and oil droplets from the ground beans to the cup [15]. Some authors have reported an increase in chlorogenic acids, lipids, coffee oil, diterpenes and aroma compounds when increasing pressure from 7 to 9 bar [19,29] although no differences in caffeine or total solids were observed. A further pressure increase to 11 bar had either no effect or produced a decrease in the above compounds. The negative effect on extraction efficiency of high pressures has been attributed to a decrease in flow [19,29]. In our case, the extraction at 11 bar did not significantly change the average flow of the coffee. This might be the reason why the extraction efficiency of aroma compounds did not decrease and extraction at 11 bar resulted in the highest intensity of volatile compounds. This general increase in concentrations of volatile compounds at 11 bar also correlates with the highest ranking for odor intensity reported by Andueza et al. when extracting at this pressure [19].

3.3.2. *Effect of temperature*

Increases in temperature resulted in an increase in the measured intensity of VOCs, as shown on the time–intensity profiles (Fig. 5). This was especially visible in the second half of the extraction ($t > 14$ s); however, the effect was different for each compound family.

For family A, no significant difference was observed between extractions at 92 and 96 °C, but the extraction at 82 °C resulted in lower intensities. Boxplots of the evolution of the area under the curve showed differences at the two extreme temperatures (82 °C and 96 °C) after 20 s of extraction, with all three temperatures resulting in significantly different areas under the curve at the end of the extraction. Family C displayed similar behavior, but there was only a statistical difference between 82 °C and 96 °C at the end of the extraction. For family B, differences were evident on the time–intensity profiles after 16 s, although only in the last 2 s of the extraction was the area under the curve significantly different for all three temperatures.

The greatest effect of temperature was observed for families D and E. Significant differences in the area under the curve for the two extreme temperatures appeared earlier than for the other families (starting at 16 and 12 s for family D and E, respectively), and increased with increasing extraction time. In the case of dimethylpyrazine, the representative of family D, we observed that at 96 °C the intensity reached a maximum at 16 s and then remained constant until the end of the extraction, while for the other extraction temperatures the intensity started to decrease once the maximum had been reached. In the case of the representative compound of family E (furfurylpyrrole), the increase of extraction efficiency at 96 °C was even more evident. The time–intensity curve increased until the end of the extraction, suggesting that the maximum had not been reached within the extraction time of 25 s.

In general, an increase in water temperature results in higher water solubility for some compounds. The use of water at high temperatures for brewing espressos has been related to increases in extraction yield, caffeine, diterpenes, coffee oil and lipids [15–17,29]. The more efficient extraction of coffee oil and lipids at higher extraction temperatures may, in turn, favor the extraction of more lipophilic compounds. This could explain the greater effect of temperature on the extraction of volatiles from families D and E, which contain lower polarity compounds.

4. Conclusions

On-line PTR-ToF-MS analysis of volatile coffee compounds released from the coffee flow during extraction has revealed itself to be a very powerful approach for studying the kinetics of coffee aroma extraction for various pressure and temperature parameters using a semi-automatic coffee machine. The time–intensity profiles showed large differences in the extraction kinetics between different volatile compounds and allowed compounds to be grouped into five families with similar physicochemical characteristics. It was shown that the polarity of the volatile compounds was the main driving force for their extraction. Extraction profiles of the aroma compounds changed with the different brewing parameters used: increases in both pressure and temperature resulted in higher extraction of VOCs, with the least polar compounds being the most affected, mainly impacting the aroma balance in the last stage of the extraction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijms.2016.02.015.

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Publication 3.

Rapid and direct volatile compound profiling of black and green teas (camellia sinensis) from different countries with PTR-ToF-MS

by

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José A. Sánchez-López was involved in the design and execution of the experiments. He collaborated on the data analysis and the preparation of the manuscript. His work to this publication accounts for approximatelly 40%.

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Rapid and direct volatile compound profiling of black and green teas (*Camellia sinensis*) from different countries with PTR-ToF-MS

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ABSTRACT

Volatile profiles of 63 black and 38 green teas from different countries were analysed with Proton Transfer Reaction-Time of Flight-Mass Spectrometry (PTR-ToF-MS) both for tea leaves and tea infusion. The headspace volatile fingerprints were collected and the tea classes and geographical origins were tracked with pattern recognition techniques. The high mass resolution achieved by ToF mass analyser provided determination of sum formula and tentative identifications of the mass peaks. The results provided successful separation of the black and green teas based on their headspace volatile emissions both from the dry tea leaves and their infusions. The volatile fingerprints were then used to build different classification models for discrimination of black and green teas according to their geographical origins. Two different cross validation methods were applied and their effectiveness for origin discrimination was discussed. The classification models showed a separation of black and green teas according to geographical origins the errors being mostly between neighbouring countries.

1. Introduction

In tea production, the leaves of the tea plant *Camellia sinensis* are used as the same starting material but the differences in the processing techniques result in a wide range of characteristic teas with distinct sensory properties. According to the way of processing, teas are usually classified into three big groups based on their fermentation degrees: non-fermented (green and white), semi-fermented (oolong) and fully fermented (black tea including puerh tea) [1]. There are several tea producing countries in the world. The main five tea producing countries are China, India, Kenya, Sri Lanka and Turkey [2]. Each country has different regions with their own climate and tea processing methods which characterize colour, appearance and flavour of the final product. For this reason, most tea products are marketed with the indication of the production region for product authentication and valorization.

Aroma compounds play an important role for consumer preferences and perception of tea. Starting with the fresh tea leaves, which have a greenish and unripe odour, the characteristic tea aroma is developed during tea leaves processing. The most investigated volatile compounds (VOCs) in tea mainly consist of non-terpenoid and terpenoid components; the former are products of fatty acid degradation and provide the fresh green flavour, the latter are mostly monoterpene alcohols which give a floral sweet aroma [3,4].

Various studies have been conducted in the field of tea aroma research as recently reviewed by Yang et al. (2013) [5]. In short, gas chromatography-mass spectrometry (GC–MS) is generally used as a reference method in order to identify and quantify VOCs. The odour characteristics of volatiles have been detected with aroma dilution and GC-olfactometry; and recently electronic nose techniques have been used for fast analysis of tea aroma. These methods have allowed analysing the volatile profiles of teas at

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different fermentation degrees and also to classify green, black and oolong teas according to their geographical origins [6–11]. Among them, GC–MS has turned out to be the most accurate and effective method for identification, separation and quantification of volatile compounds; however it requires capturing volatiles by various extraction methods which are generally time consuming and their efficiency depends on the characteristics and limitations of the analytical approach (e.g. the absorption and desorption of volatiles from a specific material in the case of SPME) [12].

To link sensory perception of tea with instrumental data, direct and non-destructive instrumental analysis of volatiles can be considered to be the most appropriate approach because it provides a direct estimation of the VOCs released of from tea and that reach the human olfactory system. In this regard, proton transfer reaction-mass spectrometry, PTR-MS, provides an efficient approach as a direct injection, soft chemical ionization method for the analysis of VOCs at trace levels. The direct injection method requires no sample pre-treatment which allows real-time monitoring of VOCs [13, 14] without making any changes in the volatile composition of samples. The technique uses H_3O^+ ions for protonation of VOCs with proton affinities higher than that of water which can be further analysed by a quadruple or a time-of-flight (ToF) mass analyser [15]. ToF mass analysers provide high sensitivity that leads to detection of volatiles at ppt levels and high mass resolution which allows, in most cases, the identification of the sum formula of the observed peaks [13].

PTR-MS allows collecting the overall mass spectral fingerprints of the samples which can be further processed with advanced data analysis tools for successful discrimination and classification of the food products [16]. To the best of our knowledge, neither a study has been conducted on the analysis of volatile compounds emitted from various tea types by PTR-MS nor was this method applied for discrimination of teas from different geographical origins.

With this study, we aim to apply PTR-ToF-MS, for the first time, for aroma profiling of black and green tea samples, both leaves and brew, from different countries and to investigate the possibility of origin tracing on the basis of their geographical origins with the aid of chemometric tools.

2. Materials and methods

2.1. Tea samples

In total, 101 commercially available pure tea samples, without addition of flavouring agents, from 16 different countries (Table 1) were purchased from the market; 63 black teas and 38 green teas. The samples were stored in their original bags at room temperature before analysis. Trademarks and producers were kept confidential but the commercial names, origins and other characteristics of the tea samples are provided in Supplementary material S1.

Table 1
Distribution of tea samples according to tea types and countries of origin.

Country (code)	Tea types	
	Black	*Green*
Argentina (ARG)	1	–
China (CHI)	13	15
India (IND)	25	4
Indonesia (INDO)	3	–
Japan (JAP)	–	9
Kenya (KEN)	2	–
Korea (KOR)	1	4
Nepal (NEP)	4	1
Rwanda (RWA)	–	1
Sri Lanka (SRI)	8	1
Tanzania (TNZ)	1	1
Turkey (TUR)	3	–
Vietnam (VIE)	1	2
Zimbabwe (ZIM)	1	–
(Total)	**63**	**38**

2.2. Analysis of tea volatiles by PTR-ToF-MS

The volatile compounds of dry tea leaves and their infusions were analysed with PTR-ToF-MS by direct injection headspace analysis without destructing the original samples. For the analysis of dry tea leaves, 500 mg tea leaves were weighted into 22-ml glass vials (Supelco, Bellefonte, PA) and 3 replicates were prepared for each tea sample. Tea brewing was performed by applying a 3 min fixed infusion time for all tea samples. Deionized hot water (25 ml, 85 °C) was used for brewing of tea leaves (400 mg) in 40 ml amber vials (Supelco, Bellefonte, PA). The liquid infusion was taken right after brewing by a micropipette and 2 ml of aliquots were transferred into 22-ml glass vials. Each tea sample was brewed 3 times and each brew was analysed in duplicate.

The headspace measurements were performed by using a commercial PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria). The instrumental conditions in the drift tube were set as following: drift voltage 550 V, drift temperature 110 °C, drift pressure 2.33 mbar affording an *E*/*N* value (electric field strength/gas number density) of 140 Townsend (Td, 1 Td = 10^{-17} V cm^2). All the vials containing samples and blank vials (air for tea leaves and hot water for tea brews) were incubated at 37 °C for 30 min before headspace analysis. The headspace mixture was directly injected into PTR-MS drift tube with a flow rate of 40 sscm via a PEEK tube at 110 °C. Sample injection was performed with a multipurpose autosampler (Gerstel GmbH, Mulheim am Ruhr, Germany). A different sample was analysed every 5 min. Each sample was measured for 30 s, at an acquisition rate of one spectrum per second. The measurement order was randomized while measuring the volatile emissions of tea leaves and tea brews.

2.3. Data processing and analysis

2.3.1. Treatment of mass spectrometric data

Data processing of ToF spectra included dead time correction, internal calibration and peak extraction steps performed according to a procedure described elsewhere [17] to reach a mass accuracy ($\geq$ 0.001 Th) which is sufficient for sum formula determination. The baseline of the mass spectra was removed after averaging the whole measurement and peak detection and peak area extraction was performed by using modified Gaussian to fit the data [18]. Whenever a peak was detected, the volatile concentrations were calculated directly via the amount of detected ions in ppbv (part per billion by volume) levels according to the formulas described by Lindinger et al. [13] by assuming a constant reaction rate coefficient ($k_R = 2 \times 10^{-9}$ cm^3/s). For H_3O^+ as a primary ion, this introduces a systematic error for the absolute concentration for each compound that is in most cases below 30% and can be accounted for if the actual rate constant is available [19].

2.3.2. Selection of mass peaks

The direct injection headspace analysis of tea (leaves and infusion) samples resulted in identifying 455 mass peaks in the range 15–300 *m/z*. After eliminating the interfering ions (O_2^+, NO^+ and water clusters) and their isotopologues, 438 mass peaks remained for further analysis. The signals belonging to blank vials were subtracted from the whole data set (air from tea leaf emissions, water from infusion emissions). A concentration threshold of 0.1 ppb was set for further reduction of noise in the data

matrices. After this step 257 mass peaks × 303 (i.e. 101 samples, three biological replicates) data points were left to build the matrix containing tea leaf emissions; 162 mass peaks × 606 (i.e. 101 samples, three infusions, two analytical replicates) data points were left for tea infusion data matrix. These final data matrices were used for univariate and multivariate data analysis methods.

After mass peak selection and extraction, tentative peak identification was performed by using an in-house library developed by the authors where the peak annotations were done automatically with the scripts developed under R programming language [20].

2.3.3. Statistical analyses

The significant differences between tea types were calculated using ANOVA (99% confidence level) and the pairwise comparison was performed with Tukey's test to highlight these differences with letter annotations.

As a first step, the final data matrices were subjected to principal component analysis (PCA). Secondly, Random Forests (RF), Penalized Discriminant Analysis (PDA), Support Vector Machines (SVM) and Discriminant Partial Least Squares (dPLS) classification methods were applied for sample discrimination [21] and their classification power was compared.

Two types of validation methods were tested for each classification method: a simple 6-fold cross validation and Leave-Group-Out (LGO) cross-validation. The six-fold cross-validation was performed by randomly dividing the whole data set into 6 folds. One of the folds was removed at each time and used as a test set where the rest of the data (the train set) was used to build the discriminant method and predict the origins of samples. Using this cross validation method, analytical or biological replicates of the same tea sample can be at the same time in both the train and test sets. With the highly flexible classification methods used in this work, this can easily leads to overfitting the data and to produce biased estimates of classification errors. This effect was verified in preliminary experiments (not shown) and the method was discarded. In the case of the more elaborated LGO cross-validation, the analytical and biological replicates of each tea sample were considered as a group when discriminating tea types and geographical origin. Each time, one group was removed from the full dataset and used as a test set. Mean classification errors and confusion matrices were used to evaluate the performance of each classification method. All the multivariate data analyses were performed by using the scripts and packages developed under R programming language [20].

3. Results and discussion

3.1. Volatile profiling of black and green teas and discrimination based on tea type

One-way ANOVA of the mass peaks extracted in black and green tea headspace, showed 135 mass peaks significantly different ($p < 0.01$ with Bonferroni correction) between emissions of black and green tea leaves and 125 mass peaks between their infusions. Among the mass peaks extracted, 62 of them were tentatively identified as one or more volatile compounds based on their presence in dry tea leaves and brews reported in literature. The details of the tentatively identified mass peaks are shown in Table 2 with their average concentrations in black and green tea leaves and infusions.

The leaves of different tea types showed greater volatile emissions as compared with infusions. Various terpenes and their fragments dominated the volatile emission of tea leaves, followed by esters/acids and aldehydes/ketones. In particular, green tea leaves emitted more terpenes and sulphur compounds than black teas. The most abundant volatile compounds in the headspace of green tea infusions were sulphur compounds, aldehydes/ketones and terpenes. The headspace of black tea infusions contained aldehydes/ketones the highest; sulphur compounds, terpenes and alcohols were other most abundant chemical groups.

Some distinct differences and similarities can be pointed out between black and green teas: the most abundant sulphur compound detected in both tea infusions was tentatively identified as dimethyl sulphide. It has been reported that this sulphur compound improves the flavour of green teas harvested in spring [3]. The information about the season when the green teas were picked was not available for all the tea samples but for some of the black teas. Interestingly, we observed that the black teas that had the highest dimethyl sulphide contents were indeed picked during spring (e.g. sample no 102, 110, and 116 in Supplementary file 1).

We observed that the percentage of total monoterpenes and their fragments in the headspace of black tea infusions (~20%) was higher than the amount emitted from green tea infusion (~12%). Terpenes, especially monoterpenes, are responsible for the characteristic floral odour of tea [22]. Important aroma compounds derived from breakdown of carotenoids during black tea processing like linalool, geraniol, linalool oxide and ionone [3] were also higher in the headspace of black teas and their infusions than in green teas. Most of the monoterpenes and derived compounds were significantly lost during tea brewing; in particular linalool oxide (*m/z* 171.133) in green tea infusions.

Vanillin was previously reported to be one of the compounds of highest flavour dilution factor (FD) in black tea infusion [23]. In our study, the peak corresponding to vanillin was negligible in green tea infusions, but clearly observable in black teas with little effect of brewing.

When PCA was performed, the first three principal components provided a good separation of black teas from green teas based on the volatile emissions both from dry leaves and infusions (Fig. 1a and b). The first PCs explain 53.2 and 54.7% variances for the dry tea leaves and infusions, respectively. This reflects the high variance between black and green tea volatile emissions as well as within each tea type (black or green) depending on the different production methods and origins. The release mechanisms of volatiles might be influenced by matrix characteristics (*i.e.* leave shape and size) as teas can be produced in various shapes. For example, the green teas can be shaped like needle, twisted, flat, round, compressed shape or even as ground powder as a results of fixing and drying methods. Besides, leaf disruption also occurs in cutting and rolling steps of black tea production that leads to grading of black teas according to leaf size [22].

Fig. 2a and b show score plots of the first two PCs of tea leaves and tea infusions (loadings of the first two components of tea leaves and infusions are provided in Supplementary file S2). According to these Fig. 2a and b, some black tea samples with broken leaves (sample numbers 1, 10, 30, 43, 112, 146-148) were closely located and separated from others. These samples were characterized by the mass peaks at *m/z* 59.049, 85.065, 97.065, 99.081, 111.081, 113.096, 115.074, 115.112, 139.113, 141.127 and 143.144 which were mostly attributed to aldehydes and ketones; mass peaks at *m/z* 101.096 and 87.080 to alcohols and mass peak at *m/z* 169.126 to geranic acid in the headspace of dry tea leaves. In addition, mass peaks; *m/z* 71.049 (butenal), 77.058 (propandiol), 129.099 (hexenyl formate), 127.112 (methylheptenone) had high loadings in the headspace of tea infusions with broken leaf shape. Broken and smashed tea may release more catechins than firmly pressed tea leaves and they may undergo heavier oxidation [24]. Broken leaves also provide a larger surface area during fermentation favouring enzymatic (*i.e.* glycosidases, fatty acid hydroperoxide lyase) activity for production of volatile aldehydes [25]. These findings indicate the importance of leaf shape on volatile compound generation and their extraction during the infusion process.

Table 2
The average concentrations (ppb) of tentatively identified mass peaks in the headspace of black and green tea leaves and infusions. Peaks were selected on the basis of one-way ANOVA and the relative *p*-values are listed in the right columns.

Measured mass	Theoretical mass	Sum formula	Chemical class	Tentative identification	Average concentration ± standart deviation (ppbv)		*p*-Value	Average concentration ± standart deviation (ppbv)		*p*-Value	Reference
					Black tea leaves	Green tea leaves		Black tea infusion	Green tea infusion		
33.0336	33.033	CH_4OH+	Alcohols	Methanol	11,756 ± 3992	9555 ± 3826	< 0.001	170 ± 82	120 ± 63	< 0.001	[28]
45.0333	45.034	$C_2H_4OH^+$	Aldehydes	Acetaldehyde	804 ± 608	265 ± 260	< 0.001	416 ± 158	108 ± 58	< 0.001	[28]
47.0491	47.049	$C_2H_5OH^+$	Alcohols	Ethanol	138 ± 152	110 ± 360	0.345	6 ± 14	11 ± 18	< 0.001	[28]
49.0110	49.011	CH_4SH^+	Sulphur compounds	Methanetiol	0.9 ± 0.5	0.5 ± 0.3	< 0.001	0.4 ± 0.5	1.2 ± 1.5	< 0.001	[28]
59.0488	59.049	$C_3H_6OH^+$	Aldeydes/ketones	Propanal/acetone	340 ± 479	265 ± 784	0.293	115 ± 99	92 ± 85	0.002	[28]
61.0280	61.028	$C_2H_4O_2H^+$	Esters and acids	Acetic acid	804 ± 605	390 ± 848	< 0.001	2.3 ± 4.8	6.34 ± 17	< 0.001	[29]
63.0260	63.026	$C_2H_6SH^+$	Sulphur compounds	Dimethylsulfide	10 ± 8	12 ± 12	0.191	264 ± 289	275 ± 299	0.675	[28]
69.0333	69.034	$C_4H_4OH^+$	Furans	Furan fragment	7.0 ± 7.4	4.7 ± 6.1	0.004	7.2 ± 7.8	2.0 ± 3.0	< 0.001	[30]
69.0697	69.070	$C_5H_8H^+$	Terpene fragment	Isoprene	183 ± 145	220 ± 217	0.067	145 ± 76	35 ± 21	< 0.001	n.a.
71.0489	71.049	$C_4H_6OH^+$	Aldehydes	Butenal	19 ± 17	12 ± 8	< 0.001	4.6 ± 4.0	2.2 ± 2.3	< 0.001	[31]
73.0646	73.065	$C_4H_8OH^+$	Aldehydes	Methylpropanal	206 ± 143	195 ± 565	0.811	292 ± 159	43 ± 26	< 0.001	[29]
75.0438	75.044	$C_3H_6O_2H^+$	Esters and acids	Propionic acid	96 ± 103	42 ± 42	< 0.001	9.7 ± 6.1	4.5 ± 2.6	< 0.001	[28]
79.0536	79.054	$C_6H_6H^+$	Aromatic hydrocarbons	Benzene	40 ± 18	26 ± 22	< 0.001	5.3 ± 7.3	4.1 ± 7.7	0.043	[29]
81.0697	81.070	$C_6H_8H^+$	Terpene fragment	Cyclohexadiene (Terpene fragment)	612 ± 905	423 ± 461	0.035	29 ± 24	9 ± 12	< 0.001	[28]
83.0854	83.086	$C_6H_{10}H^+$	Terpene fragment	Cyclohexene (Terpene fragment)	175 ± 166	60 ± 70	< 0.001	71 ± 63	19 ± 23	< 0.001	[28]
85.0646	85.065	$C_5H_8OH^+$	Aldehydes/Ketones	Pentenal/pentenone	32 ± 33	37 ± 37	0.229	12 ± 9	7.5 ± 7.6	< 0.001	[24,29,32]
87.0431	87.044	$C_4H_6O_2H^+$	Ketones	Butanedione	19 ± 16	6.4 ± 4.6	< 0.001	2.7 ± 3.1	1.3 ± 0.7	< 0.001	[29]
87.0802	87.080	$C_5H_{10}OH^+$	Alcohols	Pentenol	55 ± 37	62 ± 157	0.549	165 ± 89	23 ± 14	< 0.001	[24,29]
91.0559	91.058	$C_4H_{10}SH^+$	Sulphur compounds	Diethylsulphide/butanethiol (fragment)	7.9 ± 3.6	4.4 ± 2.7	< 0.001	0.9 ± 0.9	0.3 ± 0.5	< 0.001	[33]
93.0365	93.037	$C_3H_8OSH^+$	Sulphur compounds	Methylsulfanylethanol	8.6 ± 3.7	5.6 ± 2.6	< 0.001	1.7 ± 1.2	0.4 ± 0.6	< 0.001	n.a.
93.0698	93.070	$C_7H_8H^+$	Aromatic hydrocarbons	Toluene	55 ± 88	34 ± 34	0.014	5.9 ± 7.5	2.2 ± 2.3	< 0.001	[28,29,32]
95.0173	95.016	$C_2H_6O_2SH^+$	Sulphur compounds	Dimethyl sulfone (methylsulfonylmethane)	2.4 ± 0.9	1.3 ± 0.7	< 0.001	0.2 ± 0.3	n.d.	< 0.001	n.a.
95.0478	95.049	$C_6H_6OH^+$	Phenols	Phenol	6.5 ± 4.8	3.8 ± 2.1	< 0.001	0.3 ± 0.4	0.4 ± 0.4	0.188	[34]
95.0854	95.086	$C_7H_{10}H^+$	Terpenes	Methylcyclohexadiene (α-terpinene fragment)	87 ± 121	64 ± 61	0.051	4.2 ± 3.2	2.6 ± 2.2	< 0.001	[33]
96.0814	96.081	$C_6H_9NH^+$	Heterocyclic compounds	Ethylpyrrole	7.1 ± 8.2	5.9 ± 4.4	0.144	2.1 ± 1.8	2.5 ± 1.6	0.002	[32]
97.0282	97.028	$C_5H_4O_2H^+$	Aldehydes	Furfural	13 ± 12	2.9 ± 1.9	< 0.001	1.8 ± 2.7	0.4 ± 0.7	< 0.001	[32,33]
97.0647	97.065	$C_6H_8OH^+$	Aldehydes/Furans	Hexadienal/ethylfuran	19 ± 23	19 ± 24	0.797	25 ± 26	8.3 ± 8.6	< 0.001	[24,32]
99.0803	99.080	$C_6H_{10}OH^+$	Aldehydes	Hexenal/methylpentenone	19 ± 16	15 ± 9	0.009	21 ± 22	4.6 ± 2.8	< 0.001	[24,29]
101.0960	101.096	$C_6H_{12}OH^+$	Alcohols	Hexenol	24 ± 28	10 ± 13	< 0.001	13 ± 11	3.7 ± 4.5	< 0.001	[32,33]
103.0755	103.075	$C_5H_{10}O_2H^+$	Esters and acids	Methylbutanoic acid	12 ± 9	11 ± 11	0.414	1.1 ± 1.4	0.7 ± 1.1	0.002	[23]
105.0343	105.037	$C_4H_8OSH^+$	Sulphur compounds	Methional	2.0 ± 0.8	0.4 ± 0.4	< 0.001	0.2 ± 0.2	n.d.	< 0.001	[30]
105.0689	105.070	$C_8H_8H^+$	Aromatic hydrocarbons	Styrene/ethylbenzene/vinylbenzene	13 ± 5	1.4 ± 0.8	< 0.001	1.0 ± 0.7	0.1 ± 0.3	< 0.001	[9]
107.0488	107.049	$C_7H_6OH^+$	Aldehydes	Benzaldehyde	33 ± 15	13 ± 9	< 0.001	9.4 ± 5.9	1.8 ± 1.8	< 0.001	[24,29,33,35]
107.0855	107.086	$C_8H_{10}H^+$	Aromatic hydrocarbons	Xylene/ethylbenzene	58 ± 43	43 ± 37	0.002	9 ± 16	8 ± 15	0.398	[24,35]
109.0658	109.065	$C_7H_8OH^+$	Phenols	Benzyl alcohol (cresol)	3.2 ± 1.9	1.8 ± 1.3	< 0.001	0.5 ± 0.3	0.2 ± 0.2	< 0.001	[24,29,33]
109.1013	109.101	$C_8H_{12}H^+$	Hydrocarbons	Cyclooctadiene	25 ± 29	21 ± 17	0.154	4.0 ± 3.5	2.3 ± 2.2	< 0.001	[29]
111.0466	111.044	$C_6H_6O_2H^+$	Furans	Acetyl furan	3.8 ± 3.5	2.2 ± 1.6	< 0.001	0.3 ± 0.7	0.2 ± 0.4	0.028	[24,33]

111.0805	111.080	$C_7H_{10}OH^+$	Aldehydes	Heptadienal	17 ± 20	20 ± 24	0.213	7.3 ± 7.2	6.4 ± 7.4	0.120	[24,36]
113.0960	113.096	$C_7H_{12}OH^+$	Aldehydes	Heptenal	3.3 ± 2.5	2.6 ± 2.2	0.010	1.6 ± 1.3	0.7 ± 0.7	< 0.001	[24,29,32]
115.0738	115.074	$C_6H_{10}O_2H^+$	Ketones	Caprolactone	2.5 ± 1.7	1.2 ± 1.0	< 0.001	0.2 ± 0.1	0.1 ± 0.1	< 0.001	[29]
115.1119	115.112	$C_7H_{14}OH^+$	Ketones	Heptanone	10 ± 10	5.5 ± 5.9	< 0.001	3.6 ± 2.9	1.2 ± 1.1	< 0.001	[28]
121.0291	121.028	$C_7H_4O_2H^+$	Terpenes	cyclohexadienone (fragment)	0.8 ± 0.4	n.d.	< 0.001	0.2 ± 0.3	n.d.	< 0.001	[24,29,33]
121.0648	121.065	$C_8H_8OH^+$	Aldehydes	Methylbenzaldehyde-coumaran	2.5 ± 1.2	1.1 ± 0.6	< 0.001	2.3 ± 1.3	0.5 ± 0.6	< 0.001	[24,32,35]
121.1004	121.101	$C_9H_{12}H^+$	Aromatic hydrocarbons	Methylethylbenzene	8.7 ± 6.9	7.6 ± 6.1	0.134	0.8 ± 0.4	0.5 ± 0.5	< 0.001	[29]
123.1170	123.117	$C_9H_{14}H^+$	Terpenes	Santene	5.6 ± 2.3	5.9 ± 2.3	0.242	1.2 ± 0.5	0.7 ± 0.4	< 0.001	n.a.
127.1116	127.112	$C_8H_{14}OH^+$	Ketones	Octenone/methylheptenone	9 ± 11	7.3 ± 6.2	0.030	2.6 ± 2.3	1.2 ± 1.1	< 0.001	[24,29]
129.0901	129.091	$C_7H_{12}O_2H^+$	Esters and acids	Hexenyl formate	1.7 ± 1.1	0.8 ± 1.1	< 0.001	0.4 ± 0.3	n.d.	< 0.001	[32]
129.1276	129.127	$C_8H_{16}OH^+$	Ketones	Octanone/Dimethylcyclohexanol	2.9 ± 2.7	2.1 ± 1.8	0.002	1.2 ± 1.1	0.4 ± 0.3	< 0.001	[32,33]
131.1069	131.107	$C_7H_{14}O_2H^+$	Esters and acids	Heptanoic acid/hexyl formate	9.9 ± 9.4	8.2 ± 11.0	0.138	2.3 ± 2.1	1.1 ± 1.7	< 0.001	[24,32,33]
135.1170	135.117	$C_{10}H_{14}H^+$	Aromatic hydrocarbons	Methylpropylbenzene	12 ± 14	6.2 ± 4.9	< 0.001	1.0 ± 1.0	0.4 ± 0.4	< 0.001	[29]
136.1212	136.112	$C_9H_{13}NH^+$	Heterocyclic compounds	Butyl-pyridine/ethyl-propylpyridine	3.7 ± 4.9	2.3 ± 2.3	0.005	0.2 ± 0.2	n.d.	< 0.001	n.a.
137.1321	137.133	$C_{10}H_{16}H^+$	Terpenes	Various monoterpenes	368 ± 548	252 ± 277	0.033	13 ± 14	5.6 ± 7.5	< 0.001	[29,32,33,36]
139.1124	139.112	$C_9H_{14}OH^+$	Aldehydes	Nonadienal	27 ± 16	19 ± 12	< 0.001	2.8 ± 1.5	1.3 ± 0.9	< 0.001	[29,33,36]
141.1271	141.127	$C_9H_{16}OH^+$	Aldehydes	Nonenal	3.2 ± 1.5	2.5 ± 1.2	< 0.001	0.9 ± 0.6	0.5 ± 0.3	< 0.001	[29,36]
143.1435	143.143	$C_9H_{18}OH^+$	Ketones/Aldehydes	Nonanone/nonanal	0.9 ± 0.9	0.6 ± 0.4	0.002	0.8 ± 0.8	0.3 ± 0.3	< 0.001	[24,29]
151.1114	151.112	$C_{10}H_{14}OH^+$	Terpenes	Carvacrol/safranal	1.0 ± 0.4	0.9 ± 0.5	0.365	0.3 ± 0.13	0.2 ± 0.1	< 0.001	[24,33,36]
153.0550	153.055	$C_8H_8O_3H^+$	Aldehydes	Vanillin, methyl salicylate	6.1 ± 4.0	n.d	< 0.001	4.2 ± 3.4	n.d.	< 0.001	[33,36,37]
153.1272	153.127	$C_{10}H_{16}OH^+$	Aldehydes	Decadienal	29 ± 26	4.4 ± 2.9	< 0.001	2.9 ± 1.9	0.6 ± 0.5	< 0.001	[36]
155.1430	155.143	$C_{10}H_{18}OH^+$	Alcohols	Linalool/geraniol	2.1 ± 1.5	1.1 ± 1.5	< 0.001	0.6 ± 0.4	0.2 ± 0.3	< 0.001	[29,33,35,36]
171.1332	171.138	$C_{10}H_{18}O_2H^+$	Terpenes	Linalool oxide	2.9 ± 2.6	0.2 ± 0.1	< 0.001	0.2 ± 0.2	n.d.	< 0.001	[24,29,36]
193.1587	193.159	$C_{13}H_{20}OH^+$	Terpenes	B-ionone	0.4 ± 0.2	0.3 ± 0.2	0.141	0.2 ± 0.2	0.2 ± 0.2	0.626	[29,33,35,36]
195.0879	195.088	$C_8H_{10}N_4O_2H^+$	Ketones	Caffeine	n.d.	0.2 ± 0.1	< 0.001	0.2 ± 0.1	n.d.	< 0.001	[29]

n.a.: Not available, n.d.: Not detected.

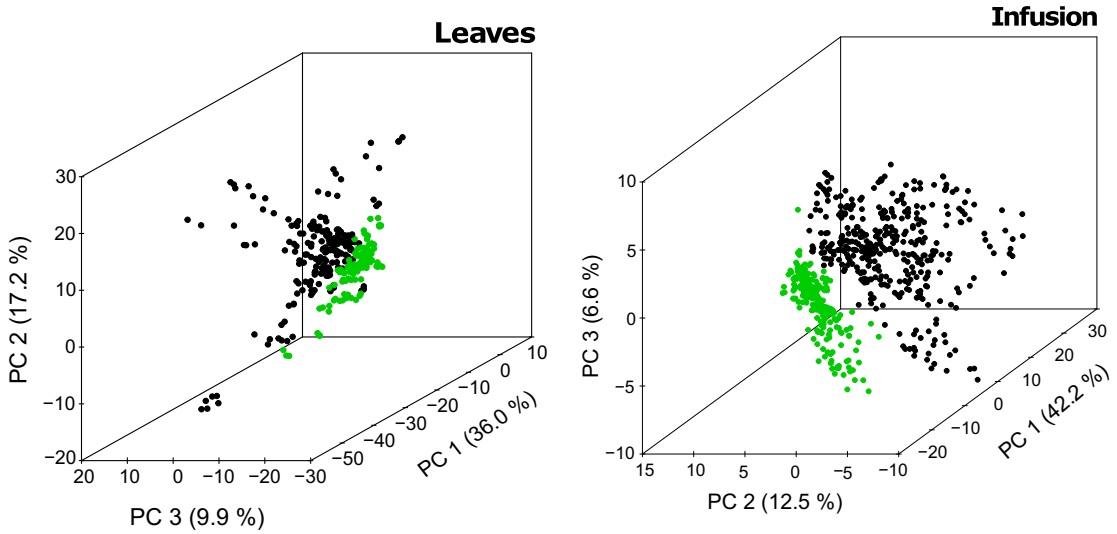

Fig. 1. 3D PCA score plots of black and green tea leaves (a) and tea infusions (b). Black and green colours represent black and green teas, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

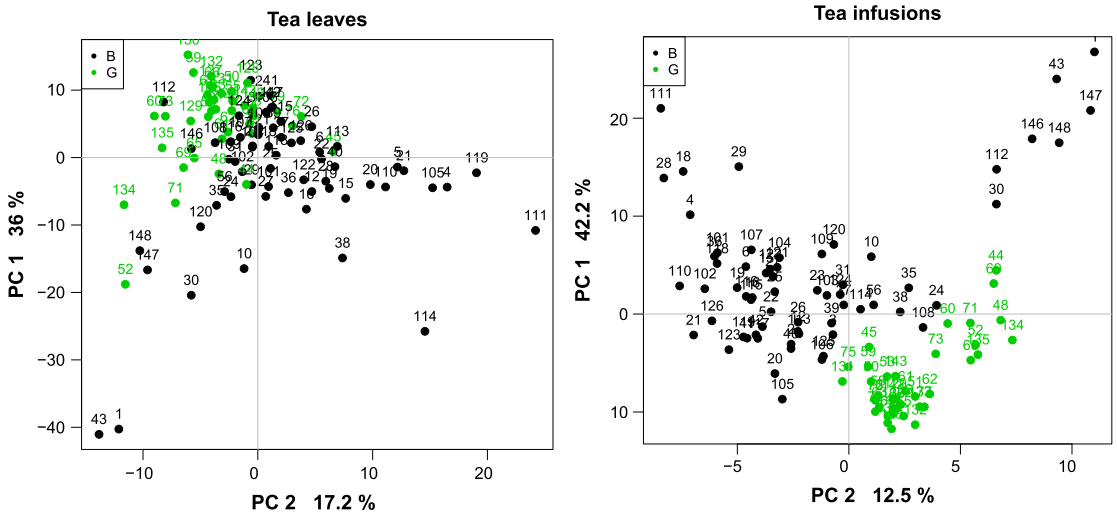

Fig. 2. 2D PCA score plots of black and green tea leaves and infusions. Black and green colours represent black and green teas, respectively. Due to the good repeatability of the analytical replicates, PCA was built via averaging the replicates. This improved the visualization of each sample. The numbers on the points indicate the sample codes given in Supplementary file S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PCA showed a relatively good separation of black and green teas by using three principal components (Fig. 1a–b, a–b). To be able to assess the performance of discrimination, we applied 4 classification models by using two different cross validation methods for discrimination of black and green teas. According to LGO cross validation the average errors for classification black and green tea leaves were 2.6%, 3.9%, 1.3% and 3.6%; the average errors for classification black and green tea infusions were 0.6%, 0.2%, 0.0% and 0.5% obtained by RF, PDA, SVM and dPLS classification models, respectively. In general, all the classification techniques showed very good classification efficiency with an average error rate less than 4.0% for differentiating black and green tea volatile profiles emitted from leaves and infusions. In all cases, the classification errors were lower for tea infusions than tea leaves.

3.2. Geographical origin discrimination with supervised classification methods

The results described above highlighted significant differences between black and green tea aroma profiles and successful separation of large number of tea samples according to tea type. However it would more relevant to demonstrate that the volatile composition of tea might be related to its geographical origin, as well. For this reason, we applied supervised classification methods on the black and green tea volatile profiles in order to differentiate them according to their origins.

To get a more representative data set for classification studies, we selected origins (countries) represented by at least 4 different teas. Black teas from China, India, Sri Lanka and Nepal (50 samples) were included for classification of black teas; China, India, Japan and Korea (32 samples) were selected for classification of green teas. Each classification algorithm ended up with an average classification error and a confusion matrix where the original tea origins were compared with the origins assigned by the classification method. The classification methods were applied on normalized volatile concentrations with LGO cross validation tests. The normalized concentrations were obtained by normalizing each mass spectrum to unit area as described in [26].

The classification performances obtained by using emissions of the tea leaves and tea infusions were similar and they provided

Table 3
Confusion matrices showing the origin separation of black and green tea infusions for leave-group-out cross validation obtained by random forests (RF), penalized discriminant analysis (PDA), support vector machine (SVM) and discriminant partial least squares (dPLS) classification models.

Classification method	Black tea					Average error rate (%)	Green tea					Average error rate (%)
		Chi	Ind	Sri	Nep			Chi	Ind	Jap	Kor	
RF												
	Chi	**58**	19	0	1	32.4	**Chi**	**74**	6	4	0	39.6
	Ind	9	**146**	0	1		**Ind**	15	**0**	9	0	
	Sri	0	24	**0**	0		**Jap**	10	3	**42**	5	
	Nep	12	35	0	**1**		**Kor**	16	0	8	**0**	
PDA												
	Chi	**55**	11	4	8	36.5	**Chi**	**69**	2	10	3	45.3
	Ind	2	**108**	30	16		**Ind**	5	**5**	14	0	
	Sri	0	19	**5**	0		**Jap**	17	0	**28**	15	
	Nep	0	24	0	**24**		**Kor**	5	0	16	**3**	
SVM												
	Chi	**57**	16	1	4	37.5	**Chi**	**62**	5	14	3	42.2
	Ind	4	**105**	22	35		**Ind**	7	**14**	3	0	
	Sri	0	18	**6**	0		**Jap**	17	0	**31**	12	
	Nep	1	26	0	**21**		**Kor**	8	0	12	**4**	
dPLS												
	Chi	**44**	12	7	15	43.3	**Chi**	**67**	0	12	5	48.4
	Ind	2	**101**	29	24		**Ind**	7	**4**	13	0	
	Sri	0	19	**5**	0		**Jap**	17	0	**24**	19	
	Nep	0	27	0	**21**		**Kor**	6	0	14	**4**	

relatively good separations which were between 30-50%. Due to the fact that, the tea infusions are the final consumed products, in the following discussion, we will focus on the classification models tested for black and green tea infusions.

Table 3 shows confusion matrices and the performances of the classification models applied on black and green tea infusions. Among the 50 black teas from 4 countries tested, the lowest prediction error was around 32% obtained by RF; the same method also provided the highest classification performance for green tea infusions prepared by 32 samples from 4 countries. Among the black teas, teas from Sri Lanka were classified with lowest errors followed by India, China and Nepal. In the case of green teas, Chinese teas had the lowest error followed by Japan, India and Korea. The confused tea samples were mostly from the neighbouring countries. For instance, Korean green teas were confused with Chinese and Japanese green teas but not with Indian green teas with RF method. This finding is not surprising because political borders are not likely to affect tea quality while climate, growing conditions, picking method and processing traditions [3,22,27] are the key factors for differentiating tea classes and their characteristics. Unfortunately we were not able to find better geographical indications for many samples.

Similar cases have been reported in literature with various classification performances when different tea samples were discriminated according to geographical origin based on their volatile profiles. Togari, Kobayashi and Aishima (1995) [10] performed the first study on the geographical origin determination of different tea categories based on their volatile profiles. The study included GC–MS analysis of 44 tea samples where tea volatiles were extracted by simultaneous dilution and extraction (SDE) method by mixing the tea samples with hot water. Black teas from India (8), Sri Lanka (4) and Japan (1) were successfully classified by supervised pattern recognition techniques, however neither oolong (China (10) and Taiwan (4)) nor green teas (15, from different regions of Japan) could be classified according to origin. Kovács et al., [8] applied electronic nose technology with electronic tongue and sensory assessment for geographical origin discrimination of five Sri Lankan teas. When electronic tongue responses of tea infusion headspace was treated with linear discriminant analysis, 100% correct classification was obtained for middle and low elevation regions (n=3) however two samples from high elevation showed overlapping. Ye, Zhang and Gu [11] analysed volatile profiles of 23 green tea samples produced in two different regions of China with SPME/GC–MS via extracting the volatiles from tea powder. They could classify the production areas of tea samples. Lee et al. [9] analysed 24 green tea samples from 8 different countries (China (7), India (1), Japan (6), Kenya (2), Korea (4), Sri Lanka (2), Tanzania (1) and Vietnam (1)) with GC-SPME method nevertheless no relationship has been found between country of origin and aroma where specific information about the samples other than origin was not known for the tested tea samples. In another study, 38 tea samples from China (2 oolong, 2 green, 3 black), Japan (5 green, 3 black, 2 oolong), India (5 black), Sri-Lanka (5 black), and Chinese Taipei (6 oolong, 2 black) were analysed by GC–MS and they were classified according to their origins by clustering methods [6]. Lastly, four varieties of oolong teas were analysed by olfaction and gustation sensing systems, the samples were classified according to producing regions by using the information each sensing system provided. When all information was merged with data fusion techniques, the discrimination power increased compared to individual classification performances suggesting the possibility to use these systems with multivariate methods for discriminating and classifying tea samples [7].

When our results and the literature were considered together, different tea types from various countries can be discriminated to some extent according to geographical origin based on their volatile emissions from dry tea leaves or tea infusions. Moreover fermented tea products are better classified than non-fermented and semi-fermented teas which was also observed from our results when we compare the classification efficiencies of black and green teas.

When black tea infusions from India (Assam (9) and Darjeeling (12)), China (Anhui (3) and Yunnan (7)), Sri Lanka (all country) and Nepal (all country) were classified according to tea producing

regions a significant improvement on the classification performance was observed providing 15% average error rate (confusion matrices not shown). The results indicated 4 classes: China Anhui (class 1); Sri Lanka and India Assam (class 2) Nepal and Darjeeling (class 3) and China Yunnan (class 4) by showing the geographically close regions in the same group.

Overall, these findings point out that the regions might be better differentiators instead of the country and the regions closely located to each other share more similar properties and they are likely to create a group. Besides, there might be other factors affecting the volatile composition of different types of tea in addition to geographical location such as the age of the tea plant, plucking (fine or coarse), plucking season, tea processing, packaging of tea, conditions during storage and storage time, which should be taken into consideration.

4. Conclusions

In this study, for the first time, the volatile profiles of black and green teas from 12 different geographical origins were analysed by PTR-ToF-MS. The volatile compounds of a large sample set (101 samples, with replicates, both leaves and infusions) were analysed by direct injection of the headspace without altering the original tea components and destructing the original sample. The high mass resolution and sensitivity achieved by the mass analyser enabled annotation of sum formulas to the detected mass peaks. Tentative identifications lead defining important aroma compounds in black and green tea volatile emissions and pointed out the differences among them.

Black and green teas were correctly classified by the volatile compounds emitted from tea leaves and their infusions independent from their geographical origins. Classification models were built to predict the geographical origins of black and green teas. Results provided a good separation of tea origins; however countries geographically close to each other were most likely to be confused. Preliminary analysis indicated that a better discrimination of tea samples might have been achieved if teas were classified according to production region rather than just country of origin. This was not feasible here, since information about production region was available only for a limited number of samples.

Our results showed that PTR-ToF-MS fingerprints combined with multivariate statistical techniques provided successful evaluation of tea products. Considering the very promising results obtained so far, in discriminating for processing and country, it seems highly warranted to collect significantly more detailed information about the individual tea samples, for future studies. This may include e.g. information on production region, producer, harvesting season, post-harvest treatment and age of the product. It may also be significant to investigate the effect of tea leave shape and infusion conditions. Finally, it is also important to direct our interest towards the consumer, by analysing the volatile compounds release from the nosespace and analysed by PTR-ToF-MS, when a tea product is being consumed, and conducting sensory profiling as well. Combining such a large spectrum of different data sets might currently seem to be a veritably challenging task; we believe this will need to be approached in steps towards a more complete understanding of the factors affecting tea aroma profiles.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.05.063.

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Publication 4.

Extraction Dynamics of Tea Volatile Compounds as a Function of Brewing Temperature, Leaf Size and Water Hardness: On-Line Analysis by PTR-ToF-MS.

by

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José A. Sánchez-López was involved in the design and execution of the experiments. He performed data analysis and prepared the manuscript. His work to this publication accounts for approximatelly 70%.

Title: Extraction Dynamics of Tea Volatile Compounds as a Function of Brewing Temperature, Leaf Size and Water Hardness: On-Line Analysis by PTR-ToF-MS

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Highlights

- Extraction of volatiles into tea infusion was analyzed by PTR-TOF-MS
- The impact of temperature, leaf size, water hardness and brewing time was determined
- Samples with similar volatile profiles were identified and grouped
- Grouping of profiles revealed differences in extraction dynamics between conditions

Abstract

Changes in the volatile profile of tea infusion during brewing were determined by analyzing the headspace of aliquots taken every 30 seconds with PTR-ToF-MS (Proton Transfer Reaction Time-of-Flight Mass Spectrometry) coupled with Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). The effect of three different brewing temperatures (60, 70 and 80 °C), two leaf sizes (broken and full leaves) and two water mineralizations (soft and hard) on the concentration of volatile compounds in the head-space of tea was studied. An increase in brewing temperature resulted in increased volatile content, with the differences on extraction efficiency becoming more pronounced at longer brewing times. Leaf size had also a big impact on the extraction of volatile compounds, but mainly during the beginning of the brewing. Water mineralization had low impact in the volatile content. Furthermore, samples prepared with different combinations of brewing parameters but resulting in analogous volatile profiles could be identified using HCA

Key words

PTR-MS, tea aroma, volatile extraction, tea infusion

1 INTRODUCTION

Tea, one of the world most popular beverages in the world, is obtained from the hot water infusion of *Camellia sinensis* leaves. Tea leaves are classified in three main classes according to the way they have been processed: green tea (unfermented), oolong tea (semifermented) and black tea (fermented). Green tea is mainly produced and consumed in Asia, however its sensory characteristics and health promoting properties [209–211] have contributed to its growing popularity and increasing consumption in the Western world as well.

Green tea brewing is considered an art in some cultures and recommendations on brewing temperature, brewing time, water-to-tea ratio or the number of repeated infusions (re-steeping) depend on the tea variety, region and individual preferences. Extensive research has been performed on the effects of brewing parameters on extraction kinetics of health-related green tea compounds such as flavanols, flavonols, catechines or caffeine [77,79,81,84,212]. In the case of volatile aroma compounds, previous studies have focused on the analysis of either the dry leaves or the final infusion with the aim of characterizing the aroma profile of different tea varieties[58,72,76,213,214], discrimination of tea according to variety[215], grade[69] , fermentation [67] or origin[207], and on the impact of some parameters such as the temperature on the final volatile composition of the infusion[116]. To the best of our knowledge, no work has been performed on the extraction dynamics of tea volatiles and how different brewing parameters can influence the volatile profile of the tea infusion over the brewing time.

In this work, we investigated the effect of three different parameters - water temperature, leaf size and water mineralization - on the extraction profiles of green tea volatiles by analyzing the headspace of aliquots taken over the brewing time. Volatile compound concentration in the head-space of tea samples was measured by Proton Transfer Reaction Time of Flight Mass Spectrometry (PTR-ToF-MS). The use of this fast and highly sensitive direct injection mass spectrometry technique allowed the direct sampling

and monitoring of the headspace of tea infusions without the need for any pre-concentration step, which could otherwise change the volatile composition of the samples[128,216]. PTR-ToF-MS allows not only the recording of the overall mass spectral fingerprint of each sample, but, due to its high mass resolution, also the assignment of a sum formula to the detected peaks and the tentative identification of compounds[133]. Although our approach consisted of studying the effect of brewing parameters on the whole volatile profile by using Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA), changes in concentration over time are discussed for dimethyl sulfide, one of the key aroma compounds found in the headspace of green tea infusions[207].

2 MATERIALS and METHODS

2.1 Tea samples

Full leaves of Gunpowder Chinese green tea were purchased in a local tea shop and stored in its original bag at room temperature before analysis. A portion of the full leaves was chopped with a kitchen blender in order to obtain broken leaves. Three grams of tea leaves (either full or broken) were infused in 150 mL water at three different temperatures (60, 70 and 80 °C) using two commercial waters of different hardness: Aqua Panna (AP – solid residue: 139 mgL^{-1}; HCO_3^-: 103 mgL^{-1}; SO_4^{2-}: 22 mgL^{-1}; NO_3^-: 2.9 mgL^{-1}; Cl^-: 8.5 mgL^{-1}; Na^+: 6.4 mgL^{-1}; Ca^{2+}: 32 mgL^{-1}; Mg^{2+}: 6.2 mgL^{-1}. Total hardness: 105 mgL^{-1} of $CaCO_3$) and San Benedetto (SB – solid residue: 265 mgL^{-1}; HCO_3^-: 313 mgL^{-1}; SO_4^{2-}: 3.7 mgL^{-1}; NO_3^-: 9 mgL^{-1}; Cl^-: 2.2 mgL^{-1}; Na^+: 6 mgL^{-1}; Ca^{2+}: 50.3 mgL^{-1}; Mg^{2+}: 30.8 mgL^{-1}. Total hardness: 252 mgL^{-1} of $CaCO_3$). Samples were gently agitated during infusion with a magnetic stirrer. Each infusion was performed in triplicate.

2.2 Analysis of tea volatiles by PTR-ToF-MS

The volatile compounds of the tea infusions were analyzed by PTR-ToF-MS using direct injection headspace analysis. During the first five minutes of each tea infusion, 1 mL aliquots were taken by a micropipette every 30 seconds and transferred into 22-ml glass vials (Supelco, Bellefonte, PA). The vials were placed into a cooling tray at 4 °C until measurement. The sampling order was randomized to prevent memory effects.

The headspace measurements were performed by using a commercial PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria). The instrumental conditions in the drift tube were set as following: drift voltage 550 V, drift temperature 110 °C, drift pressure 2.33 mbar affording an E/N value (electric field strength/gas number density) of 140 Townsend (Td, 1 Td = 10^{-21} $V \cdot m^2$). All the vials containing samples and blank vials (hot water) were incubated at 37 °C for 30 min before headspace analysis. The headspace mixture was directly injected into PTR-MS drift tube with a flow rate of 40 sscm via a PEEK tube. Sample injection was performed with a multipurpose autosampler (Gerstel GmbH, Mulheim am Ruhr, Germany). The sampling order was randomized and 1 sample was analyzed every 5 min. Each sample was measured for 30 s, at an acquisition rate of one spectrum per second.

2.3 Data processing and analysis

2.3.1 Treatment of mass spectrometric data

Raw mass spectrometric data was corrected for dead time, calibrated and peaks were extracted according to a procedure described elsewhere[217], leading to a mass accuracy ≥ 0.001 Th which allowed sum formula determination. After background subtraction and peak detection, finally peak area extraction was performed by using a modified Gaussian to fit the data[218]. The volatile concentrations in ppbv for each peak detected were calculated from the amount of ions detected according to the formulas described by Lindinger et al. [216] assuming a constant reaction rate coefficient ($k_R = 2\times10^{-9}$ cm^3/s).

2.3.2 Selection of mass peaks

The direct injection headspace analysis of tea infusions resulted in the monitoring of 447 mass peaks in the range 15-250 m/z. After eliminating the interfering ions (O_2^+, NO^+ and water clusters) and their isotopologues, 430 mass peaks remained for further analysis. The signals belonging to blank vials were subtracted from the whole data set and only signals higher in intensity than 0.1 ppb were included. After this step, 88 mass peaks x 360 data points (3 temperatures, 2 sizes, 2 waters, 10 time points and 3 replicates) were left for further analysis.

After mass peak selection and extraction, tentative peak identification was performed using an in-house library developed by the authors where the peak annotations were done automatically with the scripts developed under R programming language [219], and reducing the list to 34 tentatively identified compounds. The concentrations (ppb) of gunpowder tea infusions after 5 minutes of brewing, for the 34 VOC tentatively identified VOC, are listed in Table 1, for all the different brewing conditions investigated here. The table also includes information on statistically significant differences, according to ANOVA ($p<0.01$).

2.3.3 Statistical analyses

Principal Component Analysis (PCA) was performed on mean centered and scaled intensities of the 88 mass peaks considered. To identify conditions leading to similar volatile profiles, both the raw intensity and the intensity normalized to the intensity at 5 minutes (last time point) were subjected to Hierarchical Cluster Analysis (HCA) using Ward's minimum variance method and half-squared Euclidean distances. Significant differences between headspace concentration were calculated using ANOVA and Tukey's test ($p<0.01$). All the statistical analyses were performed by using the scripts and packages developed under R programming language [219].

3 RESULTS and DISCUSSION

3.1. Principal Component Analysis of the tea brewing process.

Principal component analysis of the 120 samples analyzed allowed visualization of the different parameters influencing the aroma profile during tea preparation (Figure 1). Time, as well as temperature, both showed a clear and consistent impact on the extraction of VOCs. The first principal component

separated samples according to the brewing time. All PC1 loadings were positive (data not shown), thereby indicating that an increase in extraction time resulted in higher concentration of the volatile compounds measured. The same effect was observed with increasing temperature, which also illustrated differences along the first principal component axis, with higher scores corresponding to the highest temperature. The second principal component is related to the size of the leaves and, although this component carried only 2.5% of the variance, it allowed differentiation between the infusions prepared from full leaves and those from broken leaves. While a PCA with all samples provides an overall insight and information about the evolution of the volatile profile, in order to better visualize how the different brewing parameters affect the volatile profile over time, data was split and presented as 4 individual PCAs for increasing brewing times: 0.5, 2, 3.5 and 5 minutes (Figure1). After 30 seconds of extraction, only infusions from full and broken leaves can separated on the PCA, resulting in a separation along the first component on the score plot. In contrast, at this early stage of the extraction process, no separation for brewing temperature or mineral content is observed. As brewing time increases (2 minutes), the effect of temperature becomes more evident. Samples with the same leaf size but prepared at different temperatures are separated in the space of the two first components. Samples infused at the same temperature but with different sizes could also be distinguished. On the other hand, some infusions from different leaves sizes at different temperatures (i.e. broken leaves at 60°C and full leaves at 80°C) did not show separation on the PCA. By observing individual leaf size and temperature combinations, samples infused with different waters were partially separated by the second component. This was the only time point at which the effect of water type could be perceived on the PCA. After 3.5 minutes of infusion, the different temperatures within one leaf size were separated along the first principal component axis. Different leaf sizes within one temperature could be differentiated, although the differences between sizes at 80 °C became minimal. At the end of the infusion (5 minutes), the first component allowed only separation of infusions from the same leaf size at different temperatures, and it was the second component that allowed separation based on leaf size. Reviewing the PCAs for the individual extraction time, it appears that an extraction time of two minutes led to the finest differentiation of VOC profiles for changing extraction conditions; all three parameters, extraction temperature, leaf size and mineral content of the water led to some degree of separation along the principle components axes. While two minute extraction time is essentially the only time at which the mineral content of the water seem to have an impact, at shorter times, no differentiation with extraction temperature is observed, while at longer than two minutes no differentiation with leave size is observed.

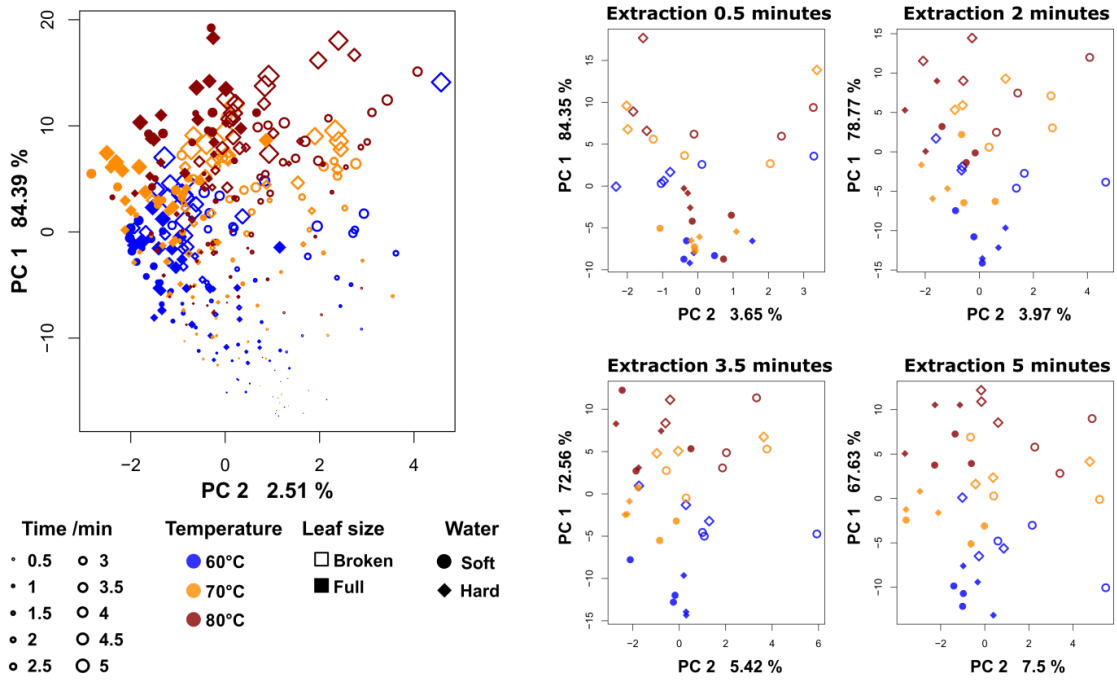

Figure 1. PCA score-plots of the (left) 180 analyzed samples (3 temperatures x 2 leaf sizes x 2 waters x 10 time points x 3 replicates) and (right) individual PCA score-plots at four different infusion times (0.5, 2, 3.5 and 5 minutes).

3.2. Identification of conditions resulting in similar volatile profile.

In addition to differences in extraction rate and profile as a function of different extractions conditions, we also examined which set of different extraction conditions lead to similar profiles. Hence, to gain some deeper insight into the infusion and extraction process, samples were grouped by Hierarchical Cluster Analysis (HCA) using two different approaches. The first approach entailed the clustering of all the time points measured for each of the conditions, using the mean value of the three replicates performed. Two HCA were performed using either all the 88 m/z or only the 34 tentatively identified compounds (Table 1). Both HCA resulted in the same five main clusters (Table 2). This grouping of samples according to their volatile content allowed the identification of which infusion conditions resulted in similar headspace composition. Cluster 1 contained 13 samples, all but one belonging to infusions from full leaves. In this cluster we found samples that had been infused at 60 °C (from 0.5 to 1.5 minutes), at 70 °C (0.5 and 1 minutes) and at 80 °C (0.5 minutes). These results reflect the effect of temperature on the extraction of aroma compounds from the leaves. At the lower temperature of 60 °C, the extraction is slow, with minor differences in the aroma profile during the first 1.5 minutes of infusion. At the other extreme, the infusion at 80 °C reached in only 30 seconds extraction time the same volatile composition as an infusion at 60 °C after 1.5 minutes or at 70 °C after 1 minute. With the exception of the sample infused at 60 °C in hard water for 30 seconds (B60SB_0.5), no other infusion of broken

leaves was found in the first cluster, reflecting the differences in extraction speed between full and broken leaves. Overall, this cluster can be characterized as one with an overall low extraction yield on volatiles. The first samples prepared from broken leaves could be found in Cluster 2. If we check at the infusion at 60 °C we find samples between 2 and 3.5 minutes in the second cluster for full leaves while for broken leaves the longest time is 2 minutes. Analyzing all the clusters, we can identify a general trend. For the same size of leaf, the higher the temperature used for the infusion, the lower the time needed to achieve similar extraction of volatiles. Within the same temperature, aroma compounds are extracted faster from broken leaves and therefore less time is needed to obtain comparable composition than infusions made from full leaves.

Table 1. Concentration (ppb) of tentatively identified mass peaks in the headspace of gunpowder tea infusions after 5 minutes of brewing in different conditions. Data followed by different letters are significantly different according to ANOVA ($p<0.01$)

Measured *m/z*	Theoretical *m/z*	Sum formula	Tentative identification	Broken leaves						Full leaves					
				Soft water			Hard water			Soft water			Hard water		
				60 °C	70 °C	80 °C	60 °C	70 °C	80 °C	60 °C	70 °C	80 °C	60 °C	70 °C	80 °C
33.0327	33.033	CH_4OH^+	Methanol	40.79 ± 7.34^{a}	45.37 ± 4.76^{ab}	44.76 ± 0.69^{ab}	48.76 ± 1.62^{bc}	47.54 ± 4.27^{ab}	55.50 ± 4.14^{cd}	48.52 ± 1.71^{bc}	51.20 ± 1.89^{b}	55.53 ± 0.63^{cd}	47.14 ± 1.17^{ab}	55.90 ± 2.62^{d}	56.83 ± 1.60^{d}
45.0334	45.034	$C_2H_4OH^+$	Acetaldehyde	77.39 ± 6.61^{ab}	111.59 ± 5.56^{cd}	151.61 ± 1.21^{f}	96.45 ± 9.47^{c}	130.50 ± 6.39^{e}	174.99 ± 5.69^{g}	68.35 ± 2.64^{a}	93.69 ± 8.09^{bc}	129.78 ± 9.36^{de}	75.60 ± 8.88^{ab}	108.59 ± 10.26^{c}	151.26 ± 14.03^{f}
49.0108	49.011	CH_4SH^+	Methanetiol	0.82 ± 0.21^{bce}	0.91 ± 0.36^{de}	1.15 ± 0.32^{e}	0.39 ± 0.08^{ac}	0.59 ± 0.14^{acd}	0.74 ± 0.28^{bce}	0.49 ± 0.08^{acd}	0.58 ± 0.07^{acd}	0.83 ± 0.33^{ce}	0.24 ± 0.04^{a}	0.36 ± 0.05^{ab}	0.53 ± 0.05^{acd}
55.054	55.054	$C_4H_7^+$	1,3-Butadiene	12.18 ± 0.75^{bc}	15.16 ± 1.01^{e}	16.84 ± 1.68^{ef}	12.28 ± 0.71^{bc}	14.70 ± 1.35^{de}	17.92 ± 0.52^{f}	9.44 ± 0.54^{a}	12.04 ± 0.57^{bc}	15.61 ± 0.55^{e}	10.65 ± 0.98^{ab}	12.96 ± 0.53^{cd}	16.85 ± 1.27^{ef}
63.026	63.026	$C_2H_6SH^+$	Dimethyl sulfide	22.00 ± 3.23^{a}	28.32 ± 2.41^{bd}	34.22 ± 1.63^{ef}	25.32 ± 2.55^{abc}	30.59 ± 0.77^{cde}	38.43 ± 1.14^{fg}	21.00 ± 1.69^{a}	28.12 ± 2.71^{bd}	34.35 ± 3.63^{eg}	24.31 ± 1.20^{ab}	31.31 ± 2.72^{de}	39.81 ± 3.45^{g}
69.0327	69.034	$C_4H_4OH^+$	Furan fragment	1.44 ± 0.20^{ab}	1.68 ± 0.19^{bcd}	1.54 ± 0.08^{ac}	1.51 ± 0.11^{ac}	1.63 ± 0.03^{ad}	1.69 ± 0.07^{bcd}	1.40 ± 0.13^{a}	1.65 ± 0.10^{ad}	1.73 ± 0.11^{cd}	1.46 ± 0.13^{ac}	1.72 ± 0.07^{cd}	1.85 ± 0.09^{d}
69.0696	69.070	$C_5H_8H^+$	Isoprene	14.49 ± 1.54^{ab}	17.06 ± 1.1^{bd}	19.03 ± 2.77^{d}	14.55 ± 0.75^{ab}	16.8 ± 1.75^{bd}	18.79 ± 1.51^{d}	11.74 ± 0.76^{a}	14.19 ± 0.89^{ab}	17.27 ± 1.54^{bd}	13.03 ± 1.57^{a}	15.08 ± 0.73^{abc}	18.35 ± 1.68^{cd}
71.0488	71.049	$C_4H_6OH^+$	Butenal	0.86 ± 0.06^{ac}	1.08 ± 0.01^{de}	1.30 ± 0.04^{fg}	0.91 ± 0.07^{ac}	1.15 ± 0.14^{ef}	1.35 ± 0.05^{g}	0.76 ± 0.09^{a}	0.95 ± 0.03^{bcd}	1.27 ± 0.09^{fg}	0.78 ± 0.08^{ab}	0.98 ± 0.07^{cd}	1.37 ± 0.08^{g}
73.0644	73.065	$C_4H_8OH^+$	Methylpropanal	25.34 ± 2.82^{ab}	30.35 ± 1.91^{cde}	32.27 ± 0.87^{de}	27.56 ± 2.07^{bc}	31.03 ± 1.04^{cde}	34.59 ± 0.79^{e}	22.81 ± 1.42^{a}	28.53 ± 2.43^{bd}	33.74 ± 1.93^{e}	24.13 ± 3.09^{ab}	30.23 ± 2.11^{cde}	34.15 ± 1.89^{ef}
75.0436	75.044	$C_3H_6O_2H^+$	Propionic acid	1.68 ± 0.05^{a}	2.03 ± 0.23^{a}	1.74 ± 0.16^{a}	1.77 ± 0.10^{a}	1.76 ± 0.13^{a}	1.98 ± 0.16^{a}	1.80 ± 0.33^{a}	1.86 ± 0.23^{a}	2.09 ± 0.26^{a}	1.66 ± 0.10^{a}	1.91 ± 0.19^{a}	2.00 ± 0.15^{a}
81.0694	81.070	$C_6H_8H^+$	Cyclohexadiene (Terpene fragment)	2.97 ± 0.41^{ac}	3.60 ± 0.70^{cd}	3.62 ± 0.33^{cd}	2.95 ± 0.17^{ac}	3.38 ± 0.26^{bcd}	4.12 ± 0.53^{d}	2.64 ± 0.21^{ab}	3.04 ± 0.11^{ac}	3.45 ± 0.16^{cd}	2.32 ± 0.10^{a}	3.08 ± 0.15^{bc}	3.65 ± 0.27^{cd}
83.0485	83.049	$C_5H_7O^+$	Methylfuran	2.69 ± 0.22^{a}	3.12 ± 0.24^{bcd}	3.11 ± 0.20^{bcd}	2.78 ± 0.29^{ab}	3.16 ± 0.08^{bcd}	3.27 ± 0.18^{cd}	2.61 ± 0.01^{a}	2.98 ± 0.17^{ac}	3.31 ± 0.16^{cd}	2.65 ± 0.21^{a}	3.20 ± 0.11^{cd}	3.48 ± 0.10^{d}
83.0853	83.086	$C_6H_{10}H^+$	Cyclohexene (Terpene fragment)	5.43 ± 0.28^{a}	6.73 ± 0.21^{ef}	7.45 ± 0.94^{ef}	5.48 ± 0.46^{bc}	6.58 ± 0.61^{cde}	7.79 ± 0.54^{f}	4.03 ± 0.12^{a}	5.16 ± 0.32^{ab}	6.74 ± 0.51^{ef}	4.81 ± 0.58^{ab}	5.79 ± 0.22^{bd}	7.20 ± 0.61^{ef}
85.0643	85.065	$C_5H_8OH^+$	Pentenal Pentenone	2.01 ± 0.14^{a}	3.06 ± 0.26^{b}	4.17 ± 0.10^{cd}	2.22 ± 0.22^{a}	3.14 ± 0.11^{b}	4.83 ± 0.12^{d}	1.85 ± 0.07^{a}	2.84 ± 0.13^{b}	4.19 ± 0.19^{c}	1.94 ± 0.17^{a}	3.14 ± 0.23^{b}	4.95 ± 0.14^{d}
87.0432	87.044	$C_4H_6O_2H^+$	Butanedione	1.07 ± 0.03^{ab}	1.34 ± 0.07^{cd}	1.4 ± 0.09^{de}	1.24 ± 0.09^{bcd}	1.38 ± 0.11^{de}	1.57 ± 0.12^{e}	1.01 ± 0.08^{a}	1.14 ± 0.02^{ac}	1.32 ± 0.11^{cd}	0.95 ± 0.15^{a}	1.16 ± 0.03^{ac}	1.33 ± 0.14^{cd}
87.0799	87.080	$C_5H_{10}OH^+$	Pentenol	9.51 ± 0.92^{ac}	11.41 ± 0.52^{def}	11.89 ± 0.33^{ef}	10.22 ± 0.74^{bcd}	11.35 ± 0.62^{def}	12.51 ± 0.34^{f}	8.31 ± 0.54^{a}	10.14 ± 0.81^{bcd}	12.14 ± 0.62^{ef}	8.74 ± 1.26^{ab}	10.78 ± 0.57^{ce}	12.13 ± 0.48^{ef}

95.0851	95.086	$C_7H_{10}H^+$	Methylcyclohexadiene (Terpene fragment)	1.14 ± 0.11^{bc}	1.45 ± 0.17^{e}	1.59 ± 0.08^{ef}	1.16 ± 0.10^{bc}	1.39 ± 0.07^{de}	1.70 ± 0.06^{f}	0.95 ± 0.07^{ab}	1.19 ± 0.01^{cd}	1.54 ± 0.14^{ef}	0.90 ± 0.06^{a}	1.22 ± 0.05^{cd}	1.52 ± 0.06^{ef}
96.0815	96.081	$C_6H_9NH^+$	Ethylpyrrole	0.64 ± 0.09^{ac}	0.76 ± 0.04^{cd}	0.72 ± 0.04^{bcd}	0.67 ± 0.09^{ad}	0.73 ± 0.03^{cde}	0.75 ± 0.01^{cd}	0.57 ± 0.06^{a}	0.64 ± 0.07^{ac}	0.75 ± 0.09^{cd}	0.58 ± 0.09^{ab}	0.72 ± 0.04^{cd}	0.79 ± 0.03^{d}
97.0641	97.065	$C_6H_8OH^+$	Hexadienal Ethylfuran	4.55 ± 0.57^{ac}	5.45 ± 0.65^{ce}	5.15 ± 0.27^{bce}	4.79 ± 0.48^{acd}	5.38 ± 0.18^{ce}	5.41 ± 0.16^{ce}	4.06 ± 0.22^{a}	4.78 ± 0.39^{acd}	5.52 ± 0.32^{de}	4.27 ± 0.53^{ab}	5.25 ± 0.41^{ce}	5.75 ± 0.17^{e}
99.0800	99.080	$C_6H_{10}OH^+$	Hexenal Methylpentenone	2.02 ± 0.20^{ab}	2.33 ± 0.14^{cf}	2.43 ± 0.06^{def}	2.15 ± 0.19^{bcd}	2.31 ± 0.02^{bcf}	2.56 ± 0.13^{f}	1.84 ± 0.09^{a}	2.03 ± 0.19^{ac}	2.41 ± 0.08^{def}	1.78 ± 0.14^{a}	2.26 ± 0.15^{bce}	2.55 ± 0.07^{ef}
101.0953	101.096	$C_6H_{12}OH^+$	Hexenol	0.95 ± 0.15^{ab}	1.21 ± 0.13^{deg}	1.28 ± 0.02^{fg}	1.05 ± 0.08^{bd}	1.18 ± 0.06^{cdef}	1.39 ± 0.07^{g}	0.75 ± 0.05^{a}	1.00 ± 0.07^{bc}	1.24 ± 0.03^{deg}	0.88 ± 0.09^{ab}	1.07 ± 0.06^{be}	1.27 ± 0.15^{eg}
107.0481	107.049	$C_7H_6OH^+$	Benzaldehyde	1.11 ± 0.02^{ac}	1.16 ± 0.05^{ac}	1.19 ± 0.14^{ac}	1.23 ± 0.11^{ac}	1.31 ± 0.19^{c}	1.28 ± 0.06^{bc}	0.99 ± 0.18^{ab}	1.01 ± 0.05^{ac}	1.09 ± 0.06^{a}	1.03 ± 0.24^{ac}	0.96 ± 0.03^{ac}	1.19 ± 0.25^{ac}
109.1005	109.101	$C_8H_{12}H^+$	Cyclooctadiene	1.36 ± 0.05^{ac}	1.48 ± 0.11^{ac}	1.48 ± 0.19^{ac}	1.38 ± 0.08^{ac}	1.52 ± 0.22^{acd}	1.62 ± 0.14^{c}	1.23 ± 0.11^{a}	1.36 ± 0.03^{ac}	1.45 ± 0.12^{ac}	1.25 ± 0.15^{ab}	1.49 ± 0.14^{ac}	1.57 ± 0.28^{bc}
111.0798	111.080	$C_7H_{10}OH^+$	Heptadienal	0.97 ± 0.08^{ab}	1.35 ± 0.18^{bcd}	1.48 ± 0.18^{cd}	0.98 ± 0.14^{ab}	1.44 ± 0.35^{cd}	1.77 ± 0.22^{d}	0.75 ± 0.14^{a}	1.05 ± 0.10^{ac}	1.41 ± 0.19^{bcd}	0.77 ± 0.06^{a}	1.15 ± 0.08^{ac}	1.63 ± 0.35^{d}
113.0955	113.096	$C_7H_{12}OH^+$	Heptenal	0.25 ± 0.04^{ab}	0.31 ± 0.03^{bd}	0.36 ± 0.03^{d}	0.25 ± 0.04^{ab}	0.32 ± 0.04^{bd}	0.38 ± 0.06^{d}	0.22 ± 0.01^{a}	0.26 ± 0.01^{ab}	0.34 ± 0.03^{cd}	0.22 ± 0.02^{a}	0.27 ± 0.03^{abc}	0.36 ± 0.02^{d}
115.1113	115.112	$C_7H_{14}OH^+$	Heptanone	0.60 ± 0.05^{ab}	0.71 ± 0.06^{bcdf}	0.73 ± 0.04^{cdf}	0.65 ± 0.06^{ad}	0.67 ± 0.02^{bcde}	0.81 ± 0.05^{f}	0.54 ± 0.03^{a}	0.61 ± 0.05^{ac}	0.75 ± 0.03^{df}	0.55 ± 0.05^{a}	0.64 ± 0.04^{ad}	0.78 ± 0.10^{ef}
121.1006	121.101	$C_9H_{12}H^+$	Methylethylbenzene	0.33 ± 0.06^{ab}	0.38 ± 0.08^{ac}	0.4 ± 0.02^{bc}	0.36 ± 0.06^{ac}	0.43 ± 0.05^{bc}	0.46 ± 0.06^{c}	0.34 ± 0.01^{ab}	0.36 ± 0.03^{ac}	0.42 ± 0.02^{bcd}	0.29 ± 0.01^{a}	0.36 ± 0.05^{ac}	0.43 ± 0.03^{bc}
123.1163	123.117	$C_9H_{14}H^+$	Santene	0.74 ± 0.08^{ac}	0.91 ± 0.13^{ef}	0.91 ± 0.06^{ef}	0.74 ± 0.05^{ac}	0.89 ± 0.03^{def}	1.02 ± 0.07^{f}	0.65 ± 0.02^{ab}	0.75 ± 0.03^{bcd}	0.94 ± 0.04^{ef}	0.61 ± 0.05^{a}	0.81 ± 0.04^{ce}	0.97 ± 0.05^{f}
127.1112	127.112	$C_8H_{14}OH^+$	Octenone Methylheptenone	0.48 ± 0.01^{ac}	0.52 ± 0.08^{bc}	0.54 ± 0.06^{bc}	0.46 ± 0.03^{ac}	0.55 ± 0.10^{bc}	0.57 ± 0.07^{c}	0.37 ± 0.03^{a}	0.43 ± 0.03^{ab}	0.51 ± 0.06^{bc}	0.43 ± 0.08^{ab}	0.50 ± 0.02^{ac}	0.56 ± 0.07^{bc}
131.1059	131.107	$C_7H_{14}O_2H^+$	Heptanoic acid hexyl formate	0.15 ± 0.03^{ab}	0.16 ± 0.04^{ab}	0.14 ± 0.01^{ab}	0.16 ± 0.02^{ab}	0.14 ± 0.01^{a}	0.17 ± 0.01^{ab}	0.16 ± 0.01^{ab}	0.18 ± 0.04^{ab}	0.18 ± 0.01^{ab}	0.15 ± 0.02^{ab}	0.18 ± 0.02^{ab}	0.19 ± 0.00^{b}
135.1158	135.117	$C_{10}H_{14}H^+$	Methylpropylbenzene	0.17 ± 0.02^{bcde}	0.18 ± 0.03^{cde}	0.19 ± 0.00^{ef}	0.17 ± 0.02^{bde}	0.18 ± 0.01^{cdf}	0.22 ± 0.00^{f}	0.14 ± 0.02^{ab}	0.15 ± 0.01^{ac}	0.17 ± 0.01^{acfe}	0.13 ± 0.01^{a}	0.15 ± 0.02^{ad}	0.18 ± 0.01^{ef}
137.1317	137.133	$C_{10}H_{16}H^+$	Various monoterpenes	0.31 ± 0.04^{ac}	0.36 ± 0.07^{bce}	0.36 ± 0.04^{ce}	0.31 ± 0.01^{ac}	0.36 ± 0.05^{ce}	0.44 ± 0.04^{e}	0.27 ± 0.05^{ab}	0.33 ± 0.03^{acd}	0.34 ± 0.02^{acd}	0.26 ± 0.00^{a}	0.34 ± 0.02^{acd}	0.41 ± 0.03^{de}
139.1109	139.112	$C_9H_{14}OH^+$	Nonadienal	1.09 ± 0.09^{ac}	1.30 ± 0.21^{ce}	1.35 ± 0.10^{def}	1.07 ± 0.08^{ac}	1.29 ± 0.08^{ce}	1.56 ± 0.13^{f}	0.97 ± 0.06^{ab}	1.13 ± 0.04^{acd}	1.34 ± 0.02^{def}	0.89 ± 0.06^{a}	1.20 ± 0.09^{bce}	1.42 ± 0.16^{ef}
141.1263	141.127	$C_9H_{16}OH^+$	Nonenal	0.28 ± 0.02^{ac}	0.31 ± 0.03^{ce}	0.31 ± 0.01^{cd}	0.28 ± 0.02^{ac}	0.28 ± 0.02^{acd}	0.36 ± 0.04^{e}	0.25 ± 0.02^{a}	0.29 ± 0.01^{acd}	0.32 ± 0.01^{ce}	0.26 ± 0.00^{ab}	0.30 ± 0.02^{bcd}	0.34 ± 0.03^{de}

Table 2. Results from Hierarchical Cluster Analysis on the mean value of the three replicates analyzed for each of the different infusions (3 temperatures x 2 leaf sizes x 2 waters x 10 time points)

Size	Water	Temp. °C	Cluster				
			1	2	3	4	5
Full	AP	60	0.5 / 1 / 1.5	2 / 2.5 / 3 / 3.5	4 / 4.5 / 5		
		70	0.5 / 1	1.5 / 2	2.5 / 3	3.5 / 4 / 4.5 / 5	
		80	0.5	1 / 1.5	2	2.5 / 3	3.5 / 4 / 4.5 / 5
	SB	60	0.5 / 1 / 1.5	2 / 2.5 / 3 / 3.5	4 / 4.5	5	
		70	0.5 / 1	1.5 / 2	2.5	3 / 3.5 / 4 / 4.5 / 5	
		80	0.5	1 / 1.5		2 / 2.5 / 3 / 3.5	4.5 / 5
Broken	AP	60		0.5 / 1 / 1.5 / 2	2.5 / 3 / 3.5 / 4	4.5 / 5	
		70		0.5 / 1	1.5 / 2 / 2.5	3 / 3.5 / 4 / 4.5	5
		80		0.5	1 / 1.5	2 / 2.5 / 3	3.5 / 4 / 4.5 / 5
	SB	60	0.5	1 / 1.5 / 2	2.5 / 3	3.5 / 4 / 4.5 / 5	
		70		0.5 / 1	1.5	2 / 2.5 / 3	3.5 / 4 / 4.5 / 5
		80		0.5	1	1.5 / 2	3 / 3.5 / 4 / 4.5 / 5

The second approach performed for grouping the samples was to use the whole time-intensity profile for each of the infusion conditions. Thereby, the matrix used for HCA consisted of 36 observations (3 temperatures x 2 leaf sizes x 2 waters x 3 replicates) and 880 variables (88 m/z x 10 time points). This clustering gave information about which infusion conditions resulted in similar time-intensity profiles. As it is shown in Figure 2A, HCA resulted in clustering of the samples according to the leaf size and infusion temperature, with minor exceptions. One of the replicates of full leaves infused at 80°C was grouped with samples infused at 70°C; broken leaves infused at 70 and 80 °C were grouped together. No

difference between the two waters was observed within the clusters. To eliminate the differences in total volatile intensity when extracting with different infusion conditions, and hence to compare the way volatiles were extracted, the time intensity profiles were normalized to the intensity at the end of the infusion for each m/z and a HCA was performed again (Figure 2B). In this case, only separation between broken and full leaves was achieved. The fact that samples infused at different temperatures within one leaf size were clustered in different groups when the raw intensity was used but they all belonged to the same group when normalized intensity was considered, indicates that a change in temperature resulted in different overall amounts of volatile compounds being extracted –higher extraction with higher temperatures – however the VOCs had similar extraction profiles. Only the change in the leaf size produced different extraction profiles and, therefore, different dynamics of the extraction. In other words, breaking the leaves not only increased the extraction rate, it also changed the extraction dynamics of compounds.

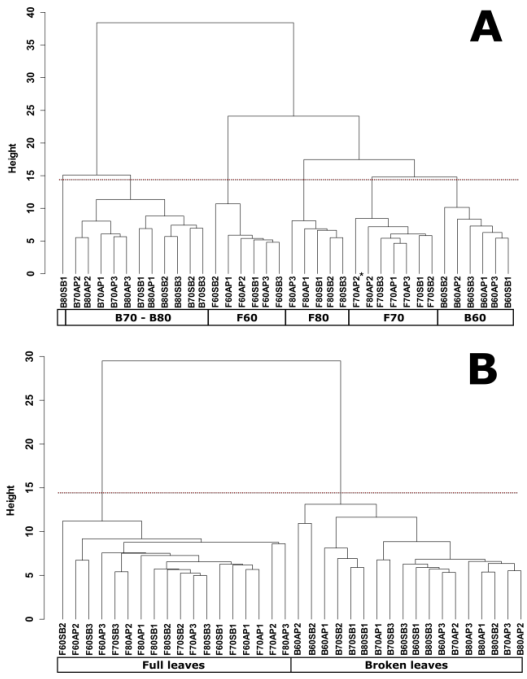

Figure 2. Hierarchical Cluster Analysis of the time intensity profile for all samples (3 temperatures x 2 leaf sizes x 2 waters x 3 replicates) using (A) absolute intensity or (B) intensity normalized to the intensity at the last time point (5 minutes). The coding for samples is: leaf size (F – full leaves; B – broken) – Temperature (60,70 or 80 °C) – Water (SB – San Benedetto; AP – Aqua panna) – Replicate (1, 2 or 3).

3.3. Effect of leaf size on volatile extraction.

Leaf size was responsible for the first observed differences in volatile extraction – already after 30 second of extraction, the VOC profiles of full vs broken leaves could be separated (Figure 1). It mainly affected aroma compounds extraction during the first part of the brewing process. This early separation is mainly

based on the overall intensity of extracted VOC with faster extraction of VOCs from broken leaves. Over longer extraction time, the difference between full and broken leaves tend to fade.

Tea leaf size produced a minimum effect on the equilibrium concentration of some soluble tea compounds (i.e. caffeine and theaflavin)[89] but it significantly affected their extraction kinetics[78]. The kinetics of tea infusion have traditionally been modeled by considering the tea leaf as a lamina where compounds are extracted from the two large surfaces[77,80], but the effect of the edges becomes important when the leaf size decreases and the leaf particles start resembling spheres [78]. This implies that in an agitated system, the extraction of soluble compounds from the tea leaves will be faster for smaller leaf sizes as has also been observed in the case of polyphenols[91,212], caffeine[212] or minerals like calcium or aluminum[93]. When we take a closer look into the individual volatile compounds it is also possible to observe the differences revealed in the PCA between both leaf sizes, especially at the beginning of the extraction. An example is given in Figure 3 for the mass peak at m/z 63.026 which can be tentatively identified as dimethyl sulfide, a key green tea aroma compound[207,220,221]. It can be clearly seen from the figure that the differences between both leaf sizes were significant at all brewing temperatures but only at the beginning of extraction. After 1.5 minutes of infusion those differences became no long significant.

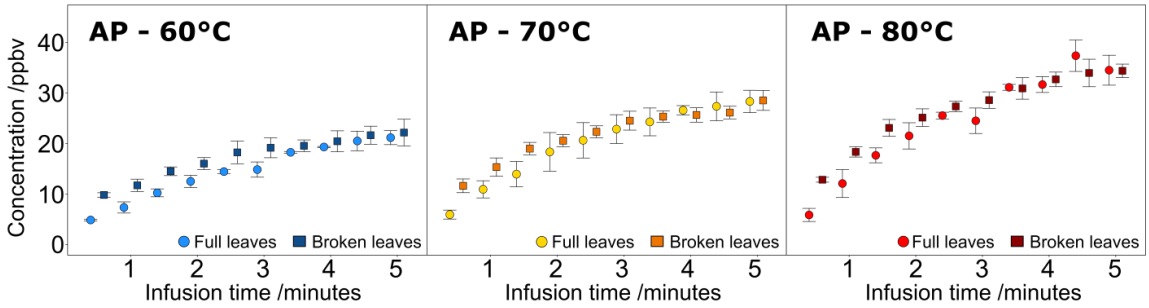

Figure 3. Effect of leaf size on dimethyl sulfide extraction at different temperatures. In each plot, the mean and the standard deviation of the three replicates is shown. Samples marked with an asterisk (*) are significantly different at that time point ($p<0.01$). Points have been slightly moved along the x axis to ease differentiation of samples and correspond to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 minutes.

3.4. Effect of water temperature on volatile extraction.

Temperature had a greater effect on the extraction of volatiles from tea leaves than leaf size, but its effect was evident only after the first minutes of extraction as shown in the PCA. At short extraction times of below 1 minute, the temperature (over the range from 60 °C to 80 °C investigated here) did not reveal any differences, neither for overall intensity nor profile. The same interpretation holds for the time evolution of dimethyl sulfide (Figure 4). Differences amongst the three temperatures studied are significant from 1.5 minutes of brewing until the end. The two extreme temperatures (60°C and 80°C) resulted in significantly different amounts of dimethyl sulfide for all time points in the case of broken leaves and for all but 0.5 minutes when infusions were prepared with full leaves. Increased extraction with temperature has been reported for soluble, non-volatile constituents of tea[82], such as polyphenols or caffeine[84,91,222], and for formation of tea foam in the case of black tea[223]. Wright and co-

workers[116] also studied the effect of temperature on black tea volatiles by analyzing the headspace of the final infusions by Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS). They also found that the amount of volatiles in the final infusions increased as a function of the temperature. In our case, as the volatile content was monitored over time, we were able to observe how those differences in extraction with temperature developed with time and became greater as the brewing time increased.

3.4. Effect of water composition on volatile extraction.

The last brewing parameter studied was water hardness. According to the PCAs, water composition had the smallest impact of all the parameters studied here with respect to volatile extraction. In fact, at an individual compound level, significant differences between the two types of water were found only for four compounds tentatively identified as methanol, acetaldehyde, dimethyl sulfide and pentenal, and only in the case of broken leaves (Figure 4). For these compounds, infusion of broken leaves resulted in higher headspace intensity when hard water was used. Hard water infusions have shown lower extractability of caffeine, theaflavins and other organic compounds from tea leaves than soft waters[80,81,93]. This effect has been attributed to the uptake of calcium by the leaves which can be complexed with pectin on the cell walls producing their gelification and modifying the diffusion of organic compounds through the cell wall. That would have implied that infusions in soft water would have resulted in higher volatile intensities. In our case, the differences in calcium content were small (32.0 mgL^{-1} in soft water and 50.30 mgL^{-1} in hard water) which might have had low impact on the extractability of the compounds but still cannot explain the higher concentration of some volatiles when teas were prepared in hard water. Another possible explanation would be that the higher salt content in the hard water produced the salting-out of volatiles to the headspace, but in this case, the same effect should have been observed in samples prepared from full leaves. Further research would be needed to explain the effect of water composition on the extraction of volatile compounds from tea leaves.

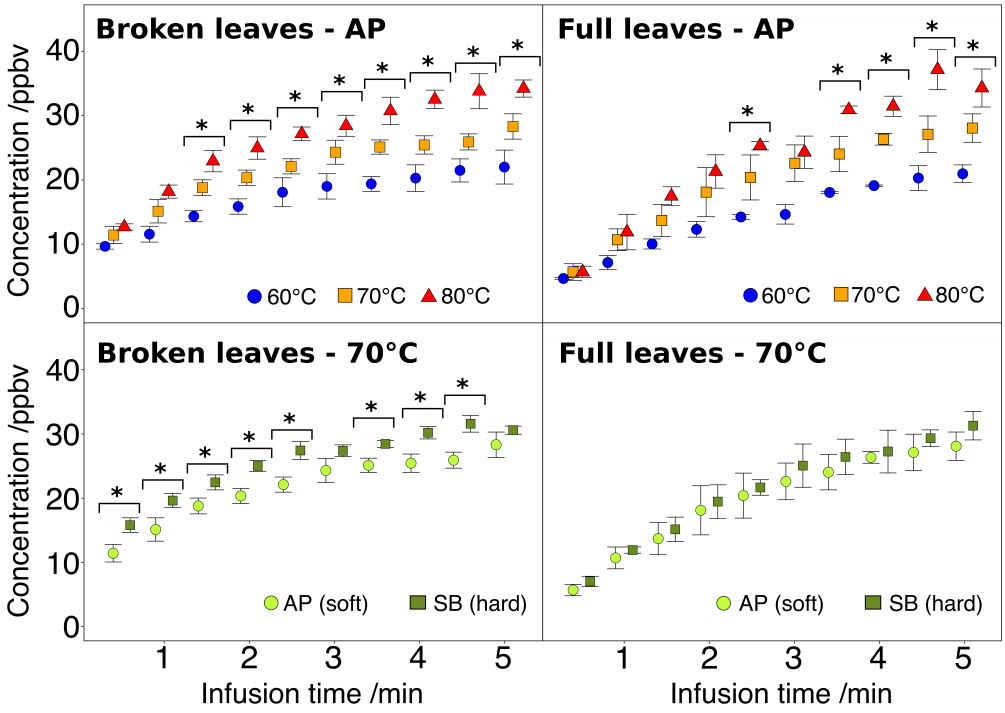

Figure 4. Effect of different infusion conditions on dimethyl sulfide extraction. In each plot, the mean and the standard deviation of the three replicates is shown. Samples marked with an asterisk (*) are significantly different at that time point ($p<0.01$).Points have been slightly moved along the x axis to ease differentiation of samples and correspond to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 minutes.

4 CONCLUSIONS

Changes in the volatile profile of tea infusions during brewing have been analyzed for the first time with PTR-ToF-MS. The combination of a direct injection mass spectrometry technique with multivariate analysis has proven to be a useful tool to follow the tea brewing process and how it is affected by different brewing parameters. At the beginning of extraction, the leaf shape is responsible of most of the observed differences in the volatile profile, but at longer extraction times, those differences become smaller. The opposite is found for temperature. At the beginning of the brewing no differences could be observed between the three extraction temperatures 60 °, 70 °C and 80 °C, but differences appeared, and increased, with time. The mineral content of the extraction water was the parameter that had least impact on the volatile profile of tea infusions (within the parameter range investigated here) – differentiation between low and high mineral content was only observed at two minutes extraction time. Furthermore, we were able to classify samples according to their volatile profile and therefore determine which combinations of parameters resulted in similar aroma. From an academic perspective, this approach will help in obtaining a more detailed insight into the extraction process of tea flavor compounds. From an economic perspective, it can assist in new product developments (i.e. tea bag, capsules or instant tea) to optimize and recommend extraction parameters, achieve a similar profile to another product format or benchmark (e.g. market leader) or approach the profile of a gold standard (i.e. loose-leaf tea). In summary, the approach outlined here opens new perspectives towards a deeper understanding of the tea

extraction process and can be used in new product developments and the improvement of existing products.

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