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Vacuum-Assisted Headspace Solid-Phase Microextraction for Enhanced Aroma Profiling of Fish Samples

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VACUUM-ASSISTED HEADSPACE SOLID-PHASE MICROEXTRACTION FOR ENHANCED AROMA PROFILING OF FISH SAMPLES

NATHALIE DELBECQUE

MASTER THESIS PRESENTED IN ORDER TO OBTAIN THE BIOENGINEER MASTER DIPLOMA
ORIENTATION CHEMISTRY AND BIO-INDUSTRIES

ACADEMIC YEAR 2018-2019

SUPERVISOR: GIORGIA PURCARO

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Symbols and abbreviations

Instruments

SPME: Solid Phase Microextraction

DI-: direct

HS-: headspace

VAC-: vacuum

Norm: normal

HPLC: High pressure liquid chromatography

GC: Gas chromatograph

CAR: carboxen

PDMS: polydimethylsiloxane

DVB: divinylbenzene

PA: polyacrylate

CW: carbowax

Other

SSOs: specific spoilage organisms

TVC: total viable count

HACCP: hazard analysis critical control point

GMP: good manufacturing practices

FAO: food and agriculture organization

QIM: quality index method

TMA(O): trimethylamine (oxide)

TVB-N: total volatile base nitrogen

VOCs: volatile organic compounds

ω: omega

BHT: Butylated hydroxytoluene

Résumé

La SPME est une méthode introduite par Belradi et Pawliszyn en 1989 et qui ne nécessite pas de solvant. Elle est basée sur le transfert de masse entre un échantillon, l'espace de tête et une fibre. Cette dernière est ensuite injectée dans un instrument de chromatographie gazeuse pour séparer et identifier les composés extraits. C'est une méthode utilisée régulièrement puisqu'elle rapide et ne nécessite pas beaucoup de manipulation (Chang-hua &al, 2016; E. Psillakis, 2017). Elle a été utilisée pour beaucoup de produits alimentaires dont les poissons qui sont utilisés dans le cadre de ce travail de fin d'étude.

Les fruits de mer (poissons, algues, crustacés,…) sont fortement consommés dans le monde. Cependant, ces produits ont une durée de vie limitée et leur fraicheur est un paramètre important afin de s'assurer de la satisfaction et de la sécurité du consommateur (Reilly A., 2018). Or la SPME a déjà été utilisée sur plusieurs espèces de poisson pour mettre en avant des marqueurs de fraicheur (alcools, aldehydes, cétones ou amines) (Miyasaki &al, 2011; Triqui &al, 2003; Wierda &al, 2006; Iglesias &al, 2007; Iglesias and Medina, 2008; Iglesias &al, 2009; Zhang &al, 2010). Ils ont utilisé des températures supérieures à 40°C et de longs temps d'extraction, il n'est dès lors plus possible de considérer le poisson comme étant frais et cru. La méthode présentée dans ce travail se base sur le tutoriel de Psillakis (2017). Celle-ci introduit le vide comme un nouveau paramètre à considérer pour l'optimisation de la SPME. Sous-vide, la cinétique de transfert de masse s'accélère et l'équilibre est atteint plus rapidement.

18 composés ont été sélectionnés selon leur volatilité et ils ont permis d'optimiser la méthode (temps et température d'extraction) avec des conditions sous-vide et normales. Une fois optimisée, la méthode a été appliquée pour étudier la dégradation de poissons. La dégradation de quatre espèces a été suivie sur une période de cinq jours consécutifs.

Les résultats conduisent à des perspectives intéressantes. L'extraction réalisée à 5°C et sous-vide permet une extraction similaire ou supérieure à celle réalisée à 40°C avec des conditions normales. Le BHT, présent à l'état de trace dans certaines espèces, n'a pu être détecté seulement lorsqu'il avait été extrait sous-vide. Ce travail a donc permis de justifier l'emploi du vide pour permettre de travailler à de plus basses températures, évitant de ce fait la production d'artéfacts par la chaleur.

Abstract

SPME is a solvent less method, first described by Belradi and Pawliszyn in 1989, that relies on the mass transfer between the sample and the headspace then the headspace and the fiber. The latter is then injected in a gas chromatography instrument to separate and identify the compounds extracted. SPME is commonly used as it fast and it does not require a lot of particular sample manipulation (Chang-hua &al, 2016; E. Psillakis, 2017). It has been widely used on food products and it was used on fish for this project.

Seafood (fish, plants, shellfish,…) is widely consumed all over the world. Such products however have a limited shelf-life and freshness is an important parameter to ensure the consumer's safety and satisfaction (Reilly A., 2018). SPME has already been used on many fish species in order to highlight some freshness markers such as alcohols, aldehydes, ketones or amines (Miyasaki &al, 2011; Triqui &al, 2003; Wierda &al, 2006; Iglesias &al, 2007; Iglesias and Medina, 2008; Iglesias &al, 2009; Zhang &al, 2010). They used temperature over 40°C and considering the long extraction time, the fish can no longer be considered fresh and raw. The method presented in this thesis is based on E. Psillakis' tutorial review (2017). It introduces vacuum as a new parameter to consider in HSSPME. Under vacuum the kinetic increases as the equilibrium state is reached faster.

18 selected compounds with different volatility were followed to optimize the method (time and temperature of extraction) under vacuum and normal conditions. Once optimized, the method was applied to fish spoilage. The degradation of four fish species was assessed during five consecutive days.

Results lead to promising perspectives for the method (VAC-HS-SPME). At 5°C, under vacuum the extraction was similar or even better than the extraction at 40°C with normal conditions. BHT, present as trace in some species, could only be detected when it was extracted under vacuum. In conclusion, this work could justify the use of low temperature and vacuum in order to avoid artifacts production due to heat.

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I.Introduction

Chapter 1: Solid-phase microextraction (SPME)

In 1989 Belradi and Pawliszyn introduced a new solventless method to extract organic compounds from water matrix samples. They used silica microfibers (diameter: 0,05 to 1mm) modified by polymerizing an organic coating on their tip. The modified fiber was directly immersed in the water sample to absorb the analytes of interest. Then the analytes were desorbed and injected into a separating instrument such as HPLC or GC. The extraction is not complete but rather an equilibrium between the compounds in the matrix and those absorbed on the fiber. In fact, the extraction yield increased with the extraction time up to a point where the equilibrium was reached and the extracted compounds stayed at the same level. They noticed that the time needed to reach equilibrium was dependent on the kind of coating they chose. Some problems rose from these first experiments: some coating leaks happened causing the fiber to absorb less analytes in following extractions (Belardi & Pawliszyn, 1989). Due to the easiness and rapidity of application, this technique has gained esponential interest and has been improved continuously since its first publication.

Improvements include the optimization of both the method and the instrument for on-site analysis and monitoring (Zhang &al, 1994; Ouyang & Pawliszyn, 2006). Different extraction modes were investigated: coated fibers used in direct, headspace or membrane-protected conditions and in-tube SPME setup was introduced (Lord & Pawliszyn, 2000). It has been used in many fields including environmental, food, pharmaceuticals and more recently in in-vivo applications (Wang &al, 2011; Merib &al., 2014; Souza-Silva &al., 2015; Filipiak & Bojko, 2019). It has been used coupled to different analytical instruments, such as GC, LC, comprehensive GC (GC×GC) (Tranchida &al, 2015), but also directly desorbed in the MS (Ziółkowska &al, 2016).

1. Device

The device used for SPME is made of two distinct parts: a needle-like stainless steel that contains the fiber and a holder with a plunger to push the fiber out of the needle. The whole setup looks like a syringe and thus is very easy to use even for untrained people (Zhang &al, 1994; Kataoka &al., 2000). Figure 1 shows a more detailed representation of the different parts. The stainless steel tube is mainly used to increase the mechanical strength as it

will allow multiple extractions without bending the fiber as well as piercing septum to reach the headspace. On the other hand, the holder is made of a barrel that can contain replaceable needles (Kataoka &al., 2000). The plunger can be pushed to the retaining screw thus exposing the fiber then back up when the extraction is finished. The device is so simple and compact that it can be used directly on the field. In this case, the needle should be closed with a septum piece and cooled during transportation back to the laboratory (Zhang &al, 1994).

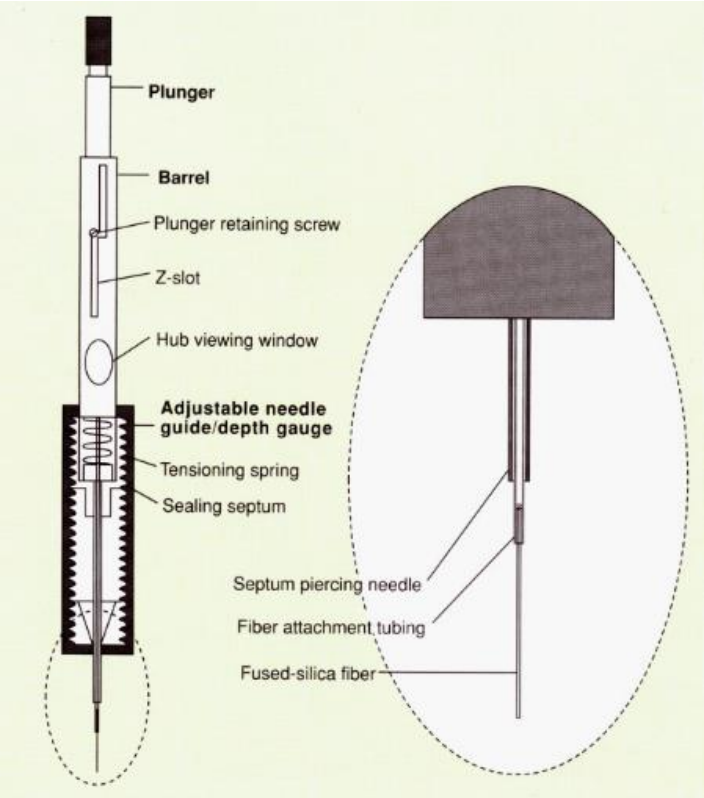

Figure 1: SPME device: needle and holder (Zhang &al, 1994).

2. Theory

Solid-phase microextraction (SPME) consists basically in two steps.

I. Extraction: absorption or adsorption of analytes on the fiber based on a two or three phases equilibrium;

II. Desorption: of the analytes into the analytical instrument for their determination

2.1 Extraction

2.1.1 Equilibrium

The first step, which consists in the extraction of the analytes from the sample matrix, can be carried out in two different mode: by immersing the fiber in the liquid sample (direct immersion: DI- SPME) or by exposing the fiber to the vapor phase above the sample (headspace: HS-SPME) (Balasubramanian & Panigrahi, 2011; Zhang &al., 1994; Pawliszyn J., 1997). The two modes are regulated by a slight different equilibrium theory, in fact the former is a two-system equilibrium (sample-fiber), while the latter is a three-system equilibrium (sample-headspace and headspace-fiber). In both cases the equilibrium between the sample matrix and the coating is reached when the amount extracted remains constant.

Equilibrium conditions are described as (Pawliszyn J., 1997):

$$n = \frac{KV_f V_S C_0}{KV_f + V_S}$$

Where,

n: amount extracted by the coating

K: fiber coating/sample matrix distribution constant

V_f: fiber coating volume

V_s: sample volume

C_0: initial concentration in the sample (given analyte)

Moreover when the sample volume is big enough ($V_s >> KV_f$), the equation can be simplified to $n = KV_fC_0$. This shows how practical SPME is even in field applications as the amount of extracted analyte is completely independent of the sample volume. This has opened the way to many *in-vivo* and *on-field* applications (Zhang &al., 1994; Pawliszyn J., 1997; Balasubramanian & Panigrahi, 2011).

When headspace SPME is used the mass transfer involves three phases: sample matrix, headspace and the fiber's coating (Pawliszyn J., 1997; Psillakis E., 2017). Therefore, the equilibrium is expressed as (Pawliszyn J., 1997):

$$n = \frac{K_{fh}K_{hs}V_f C_0 V_s}{K_{fh}K_{hs}V_f + K_{hs}V_h + V_s}$$

Where,

V_h: headspace volume

K_{fh}: coating/headspace distribution constant, equals the ratio of the concentration of a given analyte extracted in the coating and in the headspace

K_{hs}: headspace/sample matrix distribution constant, equals the ratio of the concentration of a given analyte in the headspace and in the sample

The distribution constant is an indication of how sensitive the SPME extraction will be. Some compounds may have a small constant but it is possible to improve them by changing some extraction parameters (e.g. temperature, pH or salt addition).

This theory works for coatings that extract molecules via absorption (liquid coatings). However some coatings work with adsorption (solid coatings). In this case, the equation to express equilibrium changes. Either with DI or HS- SPME, adsorption is a competitive process thus when giving the concentration equation of analyte A, one must take into account at least one other competitive compound B (Górecki &al, 1999). The resulting theory is much more complicated.

For DI-SPME, the equilibrium is expressed as (Górecki &al, 1999):

$$n_A = C_{fA}^{\infty} V_f = \frac{K_A C_{0A} V_s V_f (C_{fmax} - C_{fA}^{\infty})}{(1 + K_B C_{sB}^{\infty}) V_s + K_A V_f (C_{fmax} - C_{fA}^{\infty})}$$

And for HS- SPME (Górecki &al, 1999):

$$n_A = C_{fA}^{\infty} V_f = \frac{K'_A C_{0A} V_s V_f (C_{fmax} - C_{fA}^{\infty})}{(1 + K'_B C_{sB}^{\infty}) V_s + K'_A V_f \left(C_{fmax} - C_{fA}^{\infty}\right) + a V_s K_{H_A} (1 + K'_B C_{sB}^{\infty})}$$

These equations are complicated and it is difficult to predict how each term will affect the amount of A extracted beforehand. However it can be used in theoretical models (Górecki &al, 1999).

2.1.2 Mass transfer

In HS-SPME sampling, one of the main limiting factors is the transfer of analytes in the headspace. The mass transfer of molecules in headspace from a liquid sample can be explained with a two-resistance concept (Psillakis E., 2017; Zhakupbekova &al., 2019). It is the same process as the one describing the evaporation rate from water bodies to the atmosphere (Mackay & Leinonen, 1975). Figure 2 represents a diagram of the model. There are some assumptions followed for the model:

- Air and water phases are well mixed and separated by an inter-phase
- There are two near stagnant films (gas and liquid) on each side of the inter-phase

- Most of the resistance to diffusion lies in these films
- Most of the concentration gradient lies in these films

During extraction, several processes take place in this model. First, molecules from the water body migrate to the liquid-film. Then they are transferred to the gas-film through molecular diffusion. From the gas-film, molecules are transported closer to the fiber's coating. They get adsorbed on the coating surface and depending on the coating's nature they migrate into the bulk or stay on the surface (Górecki &al, 1999; Zhakupbekova &al., 2019). When the extraction is conducted before the equilibration state is reached, the chemical mass balanced is expressed as (Psillakis &al., 2012a):

$$V_s \frac{dC_s}{dt} = -K_L A(C_s - C_i)$$

Where,

C_i: analyte concentration in the inter-phase between liquid and gas

A: interfacial contact area between liquid and gas

K_L: overall mass transfer coefficient

K_L can be described using the 2-resistance theory mentioned above as (Mackay & Leinonen, 1975; Psillakis &al., 2012a):

$$K_L = \left[\frac{1}{k_l} + \frac{RT}{K_H k_g}\right]$$

k_l and k_g: mass transfer coefficient of the liquid-film and gas-film, respectively

K_H: Henry's law constant (ratio of the partial pressure to aqueous concentration)

R: gas constant ($8{,}2057.10^{-5}$ $m^3.atm.mol^{-1}.K^{-1}$)

T: temperature

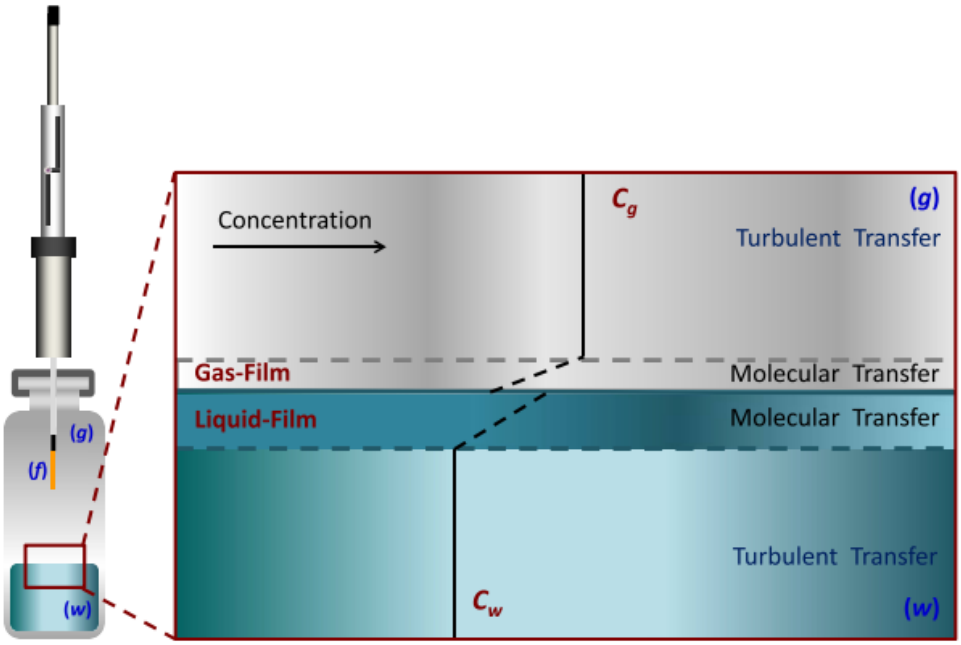

Figure 2: Headspace SPME experimental setup and diagram of the 2-resistance model (gas-liquid) (Psillakis E., 2017)

2.2 Desorption

Thermal desorption is used to remove all analytes from the fiber into the analytical instrument. Parameters (e.g. temperature, exposition time or injection depth) are optimized in order to desorb everything in a short period of time in order to avoid carry-over. Most modern GC have an injector suitable to directly inject the fiber and a dedicated liner has been manufactured thus simplifying the whole process (Kataoka &al., 2000).

3. Optimisation

Although a well-defined theory, in the practical application of SPME, a serie of parameters needs to be experimentally optimized. In this section, the main parameters are summarized.

3.1 Extraction mode

As mentioned above, extraction can be performed in two different ways. Direct-SPME is usually more sensitive than HS-SPME and is more suitable for less-volatile compounds. But the fiber's lifetime is shorter as it is directly exposed in the sample. On the other hand, HS-SPME shows less background noise and is more suitable for volatile compounds (Kataoka &al., 2000).

3.2 Coatings

The affinity between a molecule and the coating is based on the "like dissolve like" principle and different types of coating as well as different thickness can be selected according to the different applications.

Thickness: Usually thicker coatings are used for volatile compounds. They extract more molecules as a larger volume can retain more. On the other hand, larger compounds are slower to diffuse into the coating so thinner ones are preferred. Thinner coatings are also used to reduce time extraction and to improve the precision as well as reduce carry-over (Scheppers W., 1999; Wardencki &al, 2004).

Types of coatings: it determines the selectivity of the extraction and the optimization needs to be adapted accordingly. The silica fiber only serves as support as it is chemically inert (Zhang &al, 1994). It is then modified by adding a layer of coating: either only one compound such as polydimethylsiloxane (PDMS), or 2 compounds as PDMS and divinylbenzene (DVB/PDMS) or even 3 different compounds as carboxen (CAR)/PDMS/DVB (Sigma Aldrich brochure, 2018). The coatings can be immobilized with different methods that will results in different stability towards solvents (Kataoka &al., 2000; Scheppers W, 1999):

- Non-bonding: stable with water-miscible solvents but bleed with non-polar solvents. Less thermal stability than the others.
- Bonding: stable with most organic solvent except some non-polar one
- Partial crosslinking: contain crosslinking agents (vinyl groups) however it does not bond to the silica core. Stable with water-miscible organic solvents and some non-polar ones
- High cross-linking: similar to partial crosslinking (stable with water-miscible organic solvents and some non-polar ones) but there is some bonding to the fiber's core.

Table 1 shows a list of some commercially-available coatings and their main applications (Kataoka &al., 2000; Wardencki &al, 2004; Supelco Fiber selection guide 2018).

Table 1: commercially available SPME fibers.

Stationary phase	Polarity	Extraction	Thickness (µm)	Max. Temperature (°C)	Analyte type recommendation
PDMS	Non-polar	Absorption	7 to 100	280 (320 for 7µm thickness)	Non polar high, semi and low volatiles
PDMS/DVB	Semi-polar	Adsorption	65	270	Volatiles, Amines, and Nitro-aromatic Compounds
PA	Polar	Absorption	85	280	Polar semi-volatiles
CAR/PDMS	Semi-polar	Adsorption	all	310	Gases and low molecular weight compounds
CW/DVB	Polar	Adsorption	65	250	Alcohols and polar compounds
CAR/PDMS/DVB	Semi-polar	Adsorption	50/30	270	Flavor compounds: volatiles and semi-volatiles (C3-C20) + Trace compounds

An important distinction that needs to be done is between coatings extracting through adsorption and the ones through absorption. Figure 3 represents a schematic representation of both mechanisms. On the bottom, diagrams show the initial state when the fiber is just exposed to the sample matrix or headspace. On top, they represent the equilibrium state. This is where adsorption and absorption differ significantly. In coatings such as PDMS, analytes migrate into the bulk of the coating as the diffusion coefficient is similar as the one in organic solvents. Concerning mixed coatings the diffusion coefficients of analytes are much smaller and, for the time frame of the SPME extraction, they stay on the surface of the coating, since they do not have the time to diffuse. However, if those molecules stayed for a longer period of time (days or weeks) they would eventually diffuse in the coating and it would result in persistent carry-over (Górecki &al, 1999). From a practical view point, the different extraction modes affect extraction time, linearity of the response and the occurrence of the displacement effect.

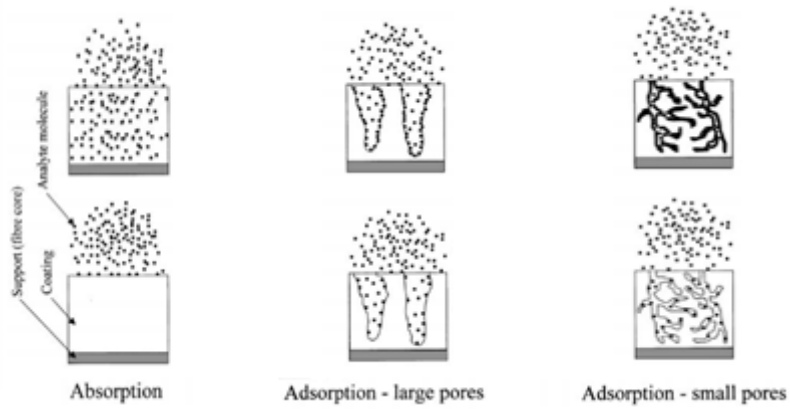

Figure 3: Comparison of absorption and adsorption mechanisms that can occur depending on the SPME coating. The step on the left is the initial state and on the right is the equilibrium state (Górecki &al, 1999).

Apart from the commercial coatings, listed in table 1, several lab-made coatings have been proposed, particularly suitable for specific applications. Some examples are conducting polymers (polyaniline or polystyrene), ionic liquids or carbonaceous materials (graphene and carbon nanotubes) (Spietelun &al., 2010; Xu &al, 2013; Zheng &al., 2018; Lashgari & Yamini, 2019).

3.3 Extraction parameters

The extraction can be optimized with several parameters: temperature, salt addition, pH, the matrix polarity, water addition and magnetic agitation. Annex 1 shows how the first ones will affect the distribution constant. This will in turn change the equilibrium according to the different equations introduced above.

3.3.1 Temperature

It is a very important parameter to consider when optimizing SPME as it will affect greatly the kinetic of the process kinetic (Pawliszyn Janusz, 1997). This parameter is even more important in HS-SPME since heating the sample will allow more analytes in the gaseous phase (Kataoka &al., 2000). However, too high temperatures will increase the extraction speed but decrease at the same time the distribution constant thus impacting the sensitivity and, when excessive, might cause premature desorption (Wardencki &al, 2004). Moreover, the use of high temperature may cause the formation of undesired artifacts. A compromise must be made between the extraction rate and the sensitivity (Kataoka &al., 2000; Balasubramanian & Panigrahi, 2011; Pawliszyn, J., 2012). In 2001 Brunton &al. showed that the total volatile compounds extracted with higher temperature showed increased peak areas. However only low-volatile compounds showed an increase. For medium and highly volatile

molecules, peak areas tended to decrease over very high temperatures (almost a 5-fold decrease between 60 and 100°C for medium volatile compounds).

3.3.2 Salts and pH

NaCl or K_2CO_3, for example, can be added for the salting-out effect (the analytes solubility decreases). And pH can be increased or lowered to target alkaline or acidic analytes respectively. However those two parameters must be changed with caution with direct SPME as extreme conditions can damage the fiber (Kataoka &al., 2000).

3.3.3 Agitation

Agitation plays an important role in the kinetics process, facilitating the transfer of molecules into the liquid sample and their release into the headspace. Obviously it will not affect the equilibrium state (Psillakis, E., 2017).

3.3.4 Extraction time

The length of the fiber exposition in the headspace or the sample is a very significant parameter to assess. Longer exposure will allow for more fiber sites to be occupied. It is also closely related to the extraction temperature as higher temperatures shorten the time needed to reach equilibrium. However, once the equilibrium state has been reached, longer exposure will not improve the extraction efficiency, on the opposite, displacement of the molecules adsorbed in the phase may occur (Wardencki &al, 2004).

3.4 Vacuum

Recently, the use of reduced pressure conditions was studied to improve the SPME kinetics. In 2017, Psillakis published a complete tutorial on the vacuum-assisted HS-SPME and presented both theoretical aspects as well as practical tips. The first consideration to take into account is that, according to thermodynamics, reduced pressure will not improve the amount of analyte extracted. Indeed the partial pressure and concentrations at equilibrium do not depend on the system pressure except for very high pressure (Moran &al., 2010). However this reduced pressure was found to increase the extraction rate and thus to decrease the overall extraction time (Brunton &al., 2001 ; Psillakis &al., 2012a). Figure 4 shows how the vacuum speeds up the equilibrium. It is also possible that, if the graph was extended to longer extraction times, the trend would go down for some compounds. This would be due to a displacement effect: compounds that did not reach easily the headspace are helped with the vacuum, if they have a good affinity with the fiber, they could take the place of other volatiles

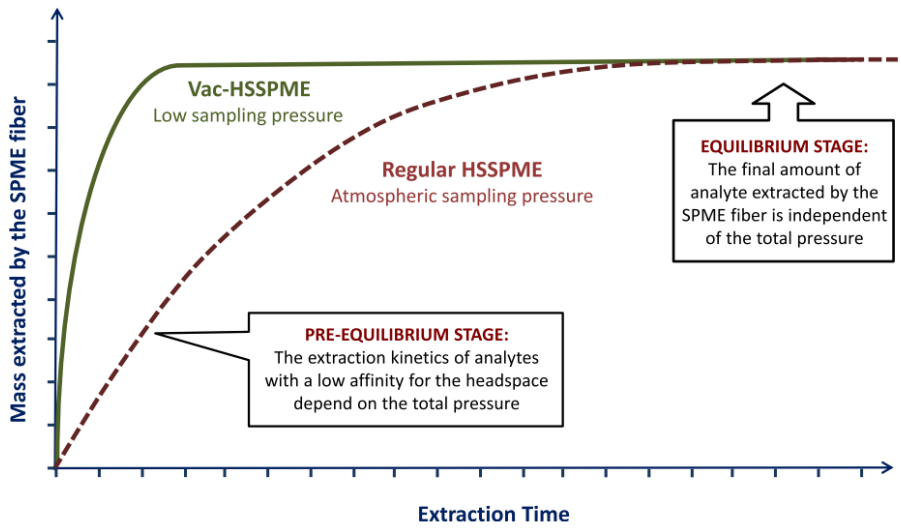

Figure 4: equilibrium curve under vacuum and regular HS-SPME (Psillakis E., 2017).

In regular HSSPME, when the fiber extracts molecules from the gas phase, their concentration falls under the equilibrium concentration. As a response, molecules from the liquid phase start to diffuse to headspace. However semi- and low-volatile molecules are slower to diffuse thus slowing the equilibration process. Reducing the pressure in the headspace would enhance the evaporation rate of molecules thus improving the equilibration rate (Psillakis &al., 2012a). This improvement is explained by the two-resistance model presented above (figure 2). Reduced pressure increases the mass transfer coefficient in the gas-film (k_g) hence increases the overall mass transfer coefficient (K_L). In other words, compounds with low K_H will react faster to the concentration drop in the headspace thus improving the equilibration rate (Psillakis E., 2017). An empirical K_H value was found to predict how vacuum will affect the analyte extraction (Psillakis E., 2017; Glykioti &al., 2016; Trujillo-Rodríguez &al., 2017). Above $1{,}6 \times 10^{-4}$ (atm m^3 mol^{-1}) it is considered that the liquid-phase resistance controls most of the evaporation rate and vacuum and regular SPME are expected to have similar performance. Below this value, the gas-phase resistance is limiting the evaporation and vacuum should significantly improve the extraction rate (Psillakis E., 2017).

The use of reduced pressure conditions requires only a modification of the sample container to guarantee the maintenance of the pressure conditions over a rather long time. The first experiments were conducted using a rather large vessel (500 to 1000mL) but to simplify the process and have the possibility to automate the extraction a smaller vial (22mL) as well as a specific vial cap were designed. (Psillakis &al., 2013). Figure 5 shows the design of the

container optimized by Psillakis &al. in 2017. The O-ring guarantees that low pressure is maintained inside the vial throughout the incubation and extraction phases. The septum allows needle-like injections but also keeps the low pressure during the incubation.

Figure 5: modified cap in order to keep vacuum conditions in the vial throughout the equilibration phase and extraction (Psillakis E., 2017).

A vacuum-pump should be used to generate low-pressure condition. It is possible to remove air before introducing the liquid sample in the vial using a syringe. The final pressure will increase a little as the gas volume will decrease but it is not significant (Psillakis E., 2017). The air removal can also take place after the sample is introduced in the vial (as for solid samples). In this case, the total pressure will be the sum of analytes partial pressure, water pressure (if water is added) and the final pressure in the chamber after being vacuumed (Psillakis E., 2017).

Finally, the analytical performance of the method was assessed and showed promising results (Psillakis &al., 2012b). Afterwards method was applied in several fields including food (Alberdi-Cedeño &al., 2017; Vakinti &al., 2019) and environmental (Lee &al., 2015; Yiantzi &al., 2015; Glykioti &al., 2016; Beiranvand & Ghiasvand, 2017; Orazbayeva &al., 2018; Zhakupbekova &al., 2019). And it is likely to see more applications rise in the future.

4. Applications

SPME is a well-established method that is used in many different fields including environmental, toxicological, pharmaceuticals and food studies (Aulakh &al; 2005; Filipiak & Bojko, 2019). Moreover SPME has not reached its limits yet as research continues to improve the method (Reyes-Garcés &al, 2018). New coatings, especially lab-made can now achieve better efficiency, less swelling as well as longer lifetime because of the improvement in thermal and mechanical stability (Kabir &al., 2013; Souza-silva &al., 2015; Godage &

Gionfriddo, 2019; Lashgari & Yamini, 2019). Applications are also broadening to endogenous non-volatile compounds (Zhang &al., 2014) and even real-time monitoring of complex matrix (Gómez-Ríos & Pawliszyn, 2014). Moreover SPME has many advantages such as low cost, ease of operation and automation and it combines sampling, isolation and enrichment in one step (Souza-silva &al., 2015).

4.1 Food analysis

Food analysis using SPME were conducted since the early beginning of the method (Hawthorne &al., 1992; Page & Lacroix, 1993; Kataoka &al., 2000; Béné &al., 2001; Rouseff & Cadwallader, 2001; Balasubramanian & Panigrahi, 2011; Chan &al., 2006; Lee &al., 2015; Souza-silva &al., 2015; Chang-hua &al., 2016 ; Reyes-Garcés &al., 2018). They include application in volatiles: wine (Pozo-Bayón &al., 2001; Slaghenaufi &al., 2014) and other alcoholic beverages (Demyttenaere &al., 2003; Vallejo-Cordoba &al., 2004; Cheng &al., 2014; Riu-Aumatell &al., 2014), fruits (Beaulieu & Lea, 2006; Pereira &al., 2011; Steingass &al., 2015), juices and other soft drinks (Hawthorne &al., 1992; Mahattanatawee &al., 2013; Schmutzer &al., 2014), meat (Moon & Li-Chan, 2004; Liu &al., 2007; Bhattacharjee &al., 2011; Flores &al., 2013; Benet &al., 2015), cereals products (Sides &al., 2001; Poinot &al, 2007; Plessas &al., 2008) and off-flavors (Wu &al., 2012). But SPME can also be used for non-volatile compounds such as pesticides or other contaminants (Robles-Molina &al., 2011; Abdulra'UF &al., 2012; Merib &al., 2014). SPME is a very powerful tool to evaluate the food quality, of either fresh or processed food as it can assess the nutritional value, flavor, aroma profile or contamination (Souza-Silva &al., 2015; Chang-hua &al., 2016).

Chapter 2: Fish

Seafood (fish, plants, shellfish,…) is widely consumed all over the world and worldwide production reached 170,9 millions of tons in 2016 (FAO, 2018). The average consumption per person is estimated at around 20,3 kg per year and has followed a continuous growth in the last years (FAO, 2018). More specifically, Mediterranean countries such as Spain, Greece, Croatia and Italy are among the world's biggest consumers and they spent over 34 millions of Euros on fishery products. Their average consumption reaches over 34kg per person and per year (WWF, 2017).

In 2014, the FAO estimated that about 80% of the world production was used for direct human consumption as food products, of which 40% as live, fresh or chilled, 60% underwent some processing: freezing, canning or extraction of oil to name a few. Over 30% of this processed fish production was not meant for human consumption, making fresh or chilled fish the most common way to consume fish.

1. Composition

Fish are an important part of a healthy human diet because of their composition (Khalili & Sampels, 2018). Figure 6 shows a summary of the average fish composition. The main component in fish is water which represents 70-80% of its mass. The content may vary depending on several parameters such as the age, the sexual maturity, the part of the fish considered, if they were farmed or wild or the lipid content as the latter has an inverse relationship with water (Yeannes & Almando, 2003).

The second major compound is proteins as they represent 17 to 22%. They play a major role in the diet as they bring essential amino acids (including methionine and lysine) that human bodies cannot synthesize. Fish proteins are easily digested because they do not have a lot of connective tissues and the fibers are short (Gokoglu & Yerlikaya, 2015; Khalili & Sampels, 2018).

Lipids are the third components for abundance (1-10%). They are used as a parameter to classify fishes: lean (<1%), low fat (1-5%), medium fatty fish (5-10%) and fatty fish (>10%) (Oehlenschläger J., 2011). However their content may vary a lot depending on different parameters such as age, sex and sexual maturation, season, species and organs. Fishes contain long-chain polyunsaturated ω-3 fatty acids and with a good ratio to ω-6 fatty acids that are essential to human health (Williams C. M., 2000; Khalili & Sampels, 2018). For this reason it is recommended to eat fish twice a week for a balanced diet. (Gokoglu & Yerlikaya, 2015; Khalili & Sampels, 2018). Lipids play a big role in fish spoilage since they may undergo different oxidation processes which are of both quality and health concern. For instance, free fatty acids bring unpleasant taste and odor (Franckel, 1980; Ghaly &al, 2010; Ryder &al, 2014).

Carbohydrates are not abundant in fishes (<1%) and their content is usually close in trace. If the capture conditions are stressful for the fish, glycogen which is made of carbohydrates enters glycolysis and under anaerobic conditions lactic acid is produced thus changing the pH. (Gokoglu & Yerlikaya, 2015).

Finally, fishes contain a lot of minerals such as calcium, phosphorus, potassium or sodium as well as vitamin D. This is also a reason fishes are good for the human health however they do not play any role in the assessment of fish spoilage. (Gokoglu & Yerlikaya, 2015; Khalili & Sampels, 2018).

2. Fish storage and spoilage

Once caught, the processing of the fish needs to start directly on the fishing boat: fishes are usually cleaned, gutted and separated in different storage boxes. They are chilled on ice and identified for traceability. Once on land, fish can be processed through primary and secondary production. The first step is to weigh and measure them. Then they can be directly sold to the retailer or final consumer or further processed. The first production consists in heading, filletting, skinning the fish and keeping it in ice water before freezing it. The secondary production can take place directly after the first one or later, after distribution and consists in cooking, grilling, marinating or other processing techniques to stabilize the fish and prepare the final product for customers (Hameri & Pálsson, 2003; Karlsen &al., 2011). Most post-harvest losses occur because of inadequate use of ice, poor access to roads and electricity, and inadequate infrastructure and services in physical markets (Ryder &al, 2014).

Apart from hazard fish consumption may pose due to the presence of pathogenic microorganisms (e.g. *Vibrio cholerae*) or bacteria producing chemical contaminants (e.g. *Gambierdiscus toxicus* causing ciguatera, *Listeria monocytogenes*) (Sumner & Ross, 2002; Sharp & Lopata, 2014), fish can be of concern due to allergic or allergic-like reactions caused by degradation products of the different fish component, such as amino acids (histamine consumption leading to scombroid poisoning) (Sumner & Ross, 2002; Hungerford J. M., 2010). It is in fact important to stress that before the secondary processing. Fishes are highly perishable goods, which may undergo many mechanisms that lead to spoilage and make them improper for consumption. Those mechanisms take place as soon as the fish is caught. They

are all related as some reactions depend on each other but they can be grouped in 3 main types of activities: i) enzymatic, ii) chemical and iii) microbial and they all lead to sensory (odor, taste and appearance) and quality (composition, toxic compounds) changes.

2.1 Enzymatic reactions

The first process is the loss of fish muscles flexibility. It is called Rigor Mortis and occurs just a few hours after the fish death. Just after death, muscles are still soft but because ATP level decrease they will stiffen and will never contract again even under stimulation. The timing for the occurrence of this process depends on different factors such as species, size, physical condition and exhaustion of the fish before its death and the storage temperature. The muscles are then softened because of enzymatic autolysis (Gokoglu & Yerlikaya, 2015).

Digestive enzymes cause autolysis of proteins and lipids which will mostly impact the fish texture. Table 2 reports the enzymes involved in this process as well as their substrate and the changes that occur when the fish is chilled. Proteolysis degrades proteins into peptides and amino acids. Enzymes related can also cause "belly bursting" when they reach the ventral muscle. When they use glycogen as substrate (glycolysis), there will be a production of lactic acid resulting in a pH drop. Some enzymes, either endogenous or bacterial, use trimethylamine oxide producing formaldehyde, a highly volatile compound (Ryder &al, 2014; Gokoglu & Yerlikaya, 2015).

Table 2: Summary of enzymes and the change they induce in chilled fish. Source: Ryder &al, 2014

Summary of autolytic changes in chilled fish

Enzyme	Substrate	Changes encountered	Prevention/inhibition
Glycolytic enzymes	Glycogen	Production of lactic acid, pH of tissue drops, loss of waterholding capacity in muscle High-temperature rigor may result in gaping	Fish should be allowed to pass through rigor at temperatures as close to 0 °C as practically possible Pre-rigor stress must be avoided
Autolytic enzymes, involved in nucleotide breakdown	ATP, ADP, AMP, IMP	Loss of fresh fish flavour, gradual production of bitterness with Hx (later stages)	Same as above Rough handling or crushing accelerates breakdown
Cathepsins	Proteins, peptides	Softening of tissue, making processing difficult or impossible	Rough handling during storage and discharge
Chymotrypsin, trypsin, carboxy-peptidases	Proteins, peptides	Autolysis of visceral cavity in pelagics (belly-bursting)	Problem increased with freezing/thawing or long-term chill storage
Calpain	Myofibrillar proteins	Softening, moult-induced softening in crustaceans	Removal of calcium thus preventing activation?
Collagenases	Connective tissue	"Gaping" of fillets, softening	Connective tissue degradation related to time and temperature of chilled storage
TMAO demethylase	TMAO	Formaldehyde-induced toughening of frozen gadoid fish	Store fish at temperature ≤ –30 °C Physical abuse and freezing/thawing accelerate formaldehyde-induced toughening

ATP is also degraded in several catabolic compounds (Figure 6) and the ratio of their concentrations is used to assess fish freshness (Gokoglu & Yerlikaya, 2015).

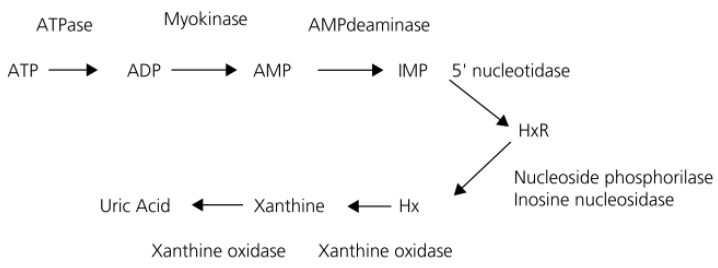

Figure 6: ATP breakdown. (Gokoglu & Yerlikaya, 2015)

2.2 Chemical reactions

One of the major mechanisms causing fish spoilage is lipid rancidity, both hydrolytic (or lipolytic) and oxidative.

Hydrolytic rancidity can follow two pathways. The first occurs in the presence of water and heat. Water reacts with triglycerides to form keto acids, which lose carbon dioxide to form methyl ketones and hydroxyl fatty acids. The second way is mediated by the presence of water and enzymes (lipases) and cause the hydrolysis of triglycerides forming free fatty acids and glycerol (and/or mono- and diglycerides as intermediates). According to the fatty acid chain length off-flavour may be produced (short fatty acid have a very low odor threshold). The free fatty acids can then undergo oxidative rancidity, which leads to quality degradation and product rejection due to the formation of undesirable aroma and off-flavour, forming low molecular weight compounds such as ketones or aldehydes (Ryder &al, 2014; Gokoglu & Yerlikaya, 2015). Moreover, the oxidation products are suspected to be toxic (Esterbauer H., 1993).

The high content of polyunsaturated fatty acids in fish leads to very high susceptibility to oxidative rancidity. This process takes place in three stages. Figure 7 shows a synthesis of those stages.

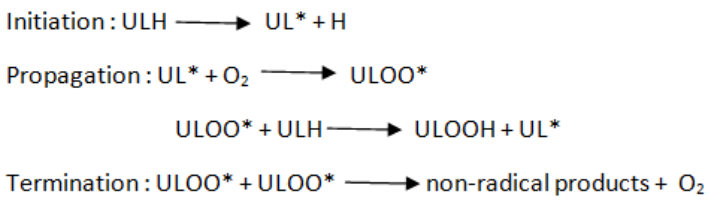

Figure 7: 3 steps of lipid oxidation. UL = unsaturated lipids. (Source: Franckel, 1980)

During the initiation, in the presence of initiators such as metals or light, unsaturated lipids lose hydrogen to form a lipid-free radical. During the second step (propagation), this highly reactive free radical reacts with dioxygen to form a peroxyl radical. There is then a hydrogen transfer from another unsaturated lipid to the peroxyl radical to form hydroperoxide

and a new lipid free radical. The latter reaction occurs at a slower rate than the formation of peroxyl radicals determining the accumulation of those compounds. When the level of these compounds is high enough, they start to interact together to form non-radical products terminating the reaction (Franckel, 1980; Ghaly &al, 2010; Ryder &al, 2014).

2.3 Microbiological reactions

Microbial spoilage occurs in two different ways. First, microorganisms can live and multiply on the fish, even at low temperature and this accumulation leads to a sensory rejection of the fish because of the colonisation aspect and taste (Gokoglu & Yerlikaya, 2015). Some bacteria are pathogenic, for example *Shewanella putrefaciens* or *Vibrio Cholerae* and their presence leads to a direct safety rejection (Austin & Austin, 2016). Second of all, they can produce metabolites that can give off-odors or flavors or can even be toxic (e.g. ciguatoxins) (Brett M. M., 2003; Dickey &al, 2008; Ghaly &al, 2010). When the fish is alive microbiota can be found on the skin, gills and in the intestines. When the fish dies microbiota can attack the muscles due to the lack of the normal immunological defence (Gokoglu & Yerlikaya, 2015).

The kind of microorganisms that live on the fish depends on the environment where it lives. Freshwater or seawater will have different microflora as well as tropical or cold water. Only some microorganisms called specific spoilage organisms (SSOs) account for fish spoilage (Ryder &al, 2014). Depending on the storage temperature after death, different SSOs will be able to grow: psychrotrophs bacteria such as *Pseudomonas spp.* or *Shewanella spp.* will grow on chilled fish whereas mesophile organisms will grow on fish kept at room temperature. They will produce spoilage compounds such as ketones, aldehydes, alcohols, organic acids or amines that result in inappropriate smell and flavors (Ghaly &al, 2010; Ryder &al, 2014; Gokoglu & Yerlikaya, 2015; Odeyemi &al, 2018).

Some bacteria, such as *Clostridium perfringens* or *Morganella morganii* have enzymes that can degrade fish components. The most famous example is the histamine decarboxylase that produces histamine from free histidine (amino acid) at temperatures of 4°C or above (Brett M. M., 2003; Hungerford, 2010). It occurs after mishandling practices especially regarding temperature. When ingested the consumer risks scombroid poisoning that results in an allergic-like reaction. On the bright side, careful establishment of standards and the use of HACCP principles reduce greatly the occurrence of such disease (Hungerford, 2010).

3. Freshness assessment

As aforementioned, fish is a perishable food, which, if not properly handle can pose severe risk to human health. Many guidelines exist to assure good manufacturing practices (GMP) as well as the application of HACCP (Hazard Analysis Critical Control Point):

- World Trade Organization, (https://www.wto.org/english/docs_e/legal_e/legal_e.htm),
- Codex Alimentarius (http://www.fao.org/fao-who-codexalimentarius/codex-texts/en/),
- FAO (FAO, 1995)

But there is still a need for science-based methods to test their freshness and quality (Reilly A., 2018).

The first problem is the lack of a clear definition of fish freshness. In 2011 Oehlenschläger defined fresh fish as: "[…] a fish in its entire properties [that] is not far away from those properties it had in the living state, or that only a short period of time has passed since the fish has been caught or harvested." And the Cambridge Dictionary gives the shelf life definition: "the length of time that a product, especially food, can be kept in a shop before it becomes too old to be sold or used." This part will present the methods used to assess fish freshness and/or predict the remaining shelf life.

3.1 Traditionnal methods

Sensory methods: are widely used and there are three kinds of tests that can be performed: descriptive and discriminative tests that need a trained panel or hedonic test which is based on the consumer liking (Oehlenschläger J., 2011). The EU quality grading scheme, the Torry scheme and the quality index method (QIM) are tests specially designed for fish evaluation (Olafsdottir &Al, 1997; Oehlenschläger J., 2011). However, QIM is the most applied method in research and industries applications. Annex 2 shows an example of this method applied to bogue (*Boops boops*). The panel takes into consideration different parameter and depending on the assessment, they give a grade between 3 and 0. The highest score is given to spoiled characteristics and the overall score gives an idea of the fish freshness (Lougovois &al, 2003; Oehlenschläger J., 2011; Bogdanovic &al., 2012). The parameters considered can change between studies and many different schemes can be found on the QIM eurofish website (http://www.qim-eurofish.com/).

Physical measurements: rely on the fish structure. However fish muscles lack uniform structure thus making the evaluation difficult. Some methods use changes in the electrical properties (when enzymes reduce TMAO to TMA, this reduction can be used for conductance assays) but these methods cannot be used when the fish is stored in chilled sea water or when the fish is mechanically damaged (Olafsdottir &Al, 1997). Color can also be used to assess freshness. This method main advantage is that the instrumentation is not expensive however more works needs to be done regarding its standardization (Olafsdottir &Al, 1997; Ocaño-Higuera &al, 2011; Oehlenschläger J., 2011).

Biological tests: rely on the estimation of total viable counts (TVC) or the presence of specific spoilage organisms (SSO) which gives useful information on fish freshness but needs long incubation step that takes place at least overnight (Olafsdottir &Al, 1997, http://www.fao.org/3/v7180e/v7180e09.htm).

Chemical methods: many changes that occur after fish death can be measured with chemistry methods. Some authors have studied amines (e.g cadaverine, putrescine) as potential markers of freshness (Pacquit &al., 2006; Rodríguez-Méndez &al., 2009; Henao-Escobar &al., 2013). They are originated from the degradation of amino acids and are expressed as TVB-N (total volatile base nitrogen). However, their concentration rises significantly after 10 to 20 days from death and can be present also on the live fish; therefore the use of volatile amine is not considered as valuable as freshness indicators (Caballero &al., 2006; Castro &al, 2006; Oehlenschläger J., 2011).

The breakdown of ATP into inosin (Ino) and hypoxanthine (Hx) is used to define a K-value, based on the ratio between the concentration of Hx, Ino and the intermediates compounds (ATP metabolites). A low K-value means that the concentration of the final products is not high compared to the other compounds thus the fish is fresh (Olafsdottir &Al, 1997; Oehlenschläger J., 2011; Gokoglu & Yerlikaya, 2015).

$$K - value\ (\%) = \frac{[Ino] + [Hx]}{[ATP] + [ADP] + [AMP] + [Ino] + [Hx]}\ x100$$

The pH could also be an indicator as it has a value between 5,8 and 6,5 at the fish death and it increases up to 8 when the fish is spoiled. However Oehlenschläger reports that

the value varies a lot between specimens and that single values do not show good correlation with the freshness (Oehlenschläger J., 2011).

3.2 New methods

Recent publications present new techniques used to assess fish freshness. Table 3 shows their principle as well as some of their advantages and disadvantages. In general, they have common advantages: they are fast, simple to use, non-destructive as well as environmental-friendly and highly effective (Wu &al., 2019). However none of them is recommended to be used as a single method for the assessment. Moreover "traditional" methods such as QIM or K-value are still dominant in the fish industry and market.

It is worthy to stress that studies on fish are really challenging because there are a lot of different species with their own specific composition. Moreover, there is also a large variability between fishes of the same species. As mentioned before, fat or protein content may vary depending on several parameters including the season and the sexual maturity. That means that during transition periods, it is possible to find specimens with very different composition (Oehlenschläger J., 2011). That said, it is difficult to develop a method that can be successfully applied in all the cases. Moreover, any new method proposed needs a long testing period to be prove robust and efficient.

Some of these new techniques are based on the evaluation of volatile compounds as many spoilage mechanisms lead to their formation. Some of these compounds could be considered as fresh or spoilage markers. To monitor them, headspace techniques are usually used. They can be used either as static or dynamic extractions but the aim is always the same: volatile compounds need to be collected then separated to be identified and quantified. This last step is usually done with chromatographic instruments such as gas chromatography (GC). On the other hand, there are a lot of methods to collect the VOCs: SPME with or without sample preparation before exposition to the fiber, solvent extraction or even distillation (Bowadt &al., 1994; Koutsoumanis & Nycha, 1999; Morita &al., 2003; Triqui & Bouchriti, 2003; Wierda &al, 2006; Iglesias &al., 2007; Iglesias & Medina, 2008; Iglesias &al., 2009; Selli & Cayhan, 2009; Giri &al., 2010; Zhang &al., 2010; Miyasaki &al., 2011; Wichaphon &al., 2012; Giogios &al., 2013; Lee &al., 2015; Parlapani &al., 2015; Parlapani &al., 2017). The use of volatiles for quality assessment of fish will be discussed in more details in chapter 3.

Table 3: New methods to assess fish freshness. Vis/NIR= visible/near-infrared. HIS= hyperspectral imaging. TVB-N= total volatile basic nitrogen

Type	Techniques		Principle	Advantages	Disavantages	Reference
Bio sensor	Electro chemical biosensor		The electrode will react to different fish deterioration reactions (e.g. redox) or products	Simple and fast	Not as sensitive as enzymes biosensors. Not selective.	Chang &al, 2017 ; Heising &al, 2012 ; 2014
	Enzyme biosensor		Enzymes are immobilized on electrodes and react with target compounds. Mostly used on ATP degradation products, especially Xanthine.	Highly selective. Simple and fast	Expensive and difficult for quantification. Enzymes can be desactivacted by external conditions.	Borisova &al, 2016 ; Gumpu &al, 2016 ; Thakur & Ragavan, 2013
Sensory bionics technology	Electric tongue		The sensors are made to imitate the gustative process.		More appropriate for liquid samples.	Jiang &al, 2018; Ruiz-Rico &al, 2013
	Colorimetric sensor array		Based on the fish color change.	Accurate and sensitive. Portable	Cannot be re-used	Kuswandi &al, 2012; Morsy &al, 2016
	Computer vision technique		Follow fish color change by using computer to analyze an image.	Simple and fast	Only witnesses surface changes.	Balaban & Alçiçek, 2015; Shi &al, 2018
Spectroscopic techniques	Vis/NIR spectroscopy (400-2500 nm)		It reacts specifically to groups such as C-H, O-H, N-H, C-O and S-H that are prone to change during fish degradation.	Detects changes from initial degradation phases.	Requires different models for each scenario	Cheng &al, 2013;
	HSI techniques	Sensory indicator. Chemical compounds. Microbiological inspection. Texture.	Combines traditional computer vision and Vis/NIR spectroscopy to obtain spectral and spatial data that translate the chemical and physical characteristics of the sample.			Cheng & Sun, 2015; Cheng, Nicolai & Sun, 2017 Cheng, Sun & Wei, 2016 Wu & Sun, 2013 Wu, Sun & He, 2014
	Fluorescence spectroscopy		Based on fluorophores in early post-mortem changes	no pre-treatment	Needs more studies	Elmasry &al, 2016
Volatile based techniques	HS-SPME		Volatiles, secondary metabolites	Simple setup, solvent-less	Needs more studies	Triqui & Bouchriti, 2003
	Electric nose (gas sensor array)		Create untargeted odour patterns associated to freshness	Fast, good repeatability	Needs extensive database creation	Alimelli &al, 2007; Hui &al, 2012

Chapter 3: fish volatiles

Over 30 different papers were found to work on seafood volatiles, in annex 3 there is the complete list. Different methods were used including a vast majority of HS-SPME. The rest includes gas sensor array, simultaneous distillation-extraction and the total volatile base nitrogen. In the work on HS-SPME different goals were set as well as different sample prep. In the 22 papers using HS-SPME, many used complicated sample preparation including the reduction of the sample into powder with N_2, the help of microwave or cooking the sample. Some works were based on seafood related products (fish sauce, fish oil emulsion,…). And some papers were focused on specific volatile molecules (volatiles amines, PAHs). In the end, 7 articles worked on the aroma profile of raw fish. The list as well as some information on each work can be found in table 4. Three different fibers were used: CAR/PDMS, PDMS/DVB and CAR/PDMS/DVB. Concerning the sample size, they all work with different weights and different vial sizes. For the incubation, the information is variable too: from 10 to 30 minutes, at the same extraction temperature or at room temperature. On the other hand the temperature used was either 40 or 50°C and the extraction time was usually between 15 and 45 minutes. Overall there is no clear trend or method to follow for raw fish aroma profiling.

In those 7 articles, over 160 (166) different compounds were identified, the complete list can be found in annex 4. They are ordered according to their occurrence in those papers and 35 of them appeared in 3 or more different works. They include 11 aldehydes, 9 alcohols, 6 alkanes, 4 ketones, 2 hydrocabons and 3 other chemicals. This list will be used to choose several compounds to follow throughout the project as fishes give complex aroma profiles.

Tableau 4: Literature on the aroma profiling of raw finfish.

N°	Ref	Method	Fiber	Fish Species	Sample size	Sample prep	Incubation	Temperature (°C)	Extraction time (min)	Analytical instrument
A	Duflos &al, 2010	HS-SPME	CAR/PDMS (65µm)	Whiting (Merlangius merlangus)	11mL of supernatant	50g of sample + 100mL ultrapure water saturated with NaCl. Then centrifugation (12000g, 10', 4°C)	10 min at 50°C and 500rpm	50	40	GC-MS
B	Pratama &al, 2018	HS-SPME	CAR/PDMS/DVB	Patin catfish + Nile Tilapia	no mention	Fillets	no mention	40	45	GC-MS
C	Triqui & Bouchriti, 2003	HS-SPME	CAR/PDMS/DVB (50/30m)	Sardines (Sardina pilchardus)	Whole fish (45± 3 g)	-	no mention	no mention	15	GC-FID + olfactometry
D	Iglesias &al, 2009	HS-SPME	CAR/DPMS/DVB (50/30m)	Gilthead sea bream	20g	Miced fish muscle	15 min at 40°C	40	30	GC-MS
E	Miyasaki &al, 2011	HS-SPME + electric nose	PDMS/DVB	Sardine, Jack mackerel, chub mackerel, yellowtail, skipjack, bluefin tuna, red seabream, Japanese seabass, flatfish, puffer, bartail flathead	1g	2-3mm cubes of fish muscle	no mention	40	30	GC-MS
F	Duflos &al, 2006	HS-SPME + HS-MS	CAR/PDMS (75µm)	Whiting, cod, mackerel	24g	1 cm cubes	30 min at RT	50	2h	GC-MS
G	Li &al, 2018	HS-SPME + TVBN	PDMS/DVB (65µm)	Silver carp (Hypophthalmichthys molitrix)	2g	Fillets	20 min at 50°C	50	30	GC-MS

Chapter 4: aim of the project

The first part of the project was devoted to HS-SPME method optimization. Several parameters (fiber coatings, temperature, extraction time) were investigated. Since the experiments were carried out partially through manual sampling (<30°C) and partially using an autosampler (experiments ≥30°C), agitation was not used in order to avoid variability due to the different kind of agitation in the two cases. Salt addition was not tested, since the salting-out effect is limited in case of solid sample. Three types of fiber were chosen for comparison as they are the most used in the literature (CAR/DPMS, PDMS/DVB, CAR/PDMS/DVB) (see table 4). The extraction time and temperature profile was investigated to study the possibility to use refrigerated temperature for sampling (*i.e* 5°C).

The second part focuses on different fish species (Salmon, Cod, Redfish and Pollock) and their degradation. The fish were kept in a fridge, similar to a domestic one, up to five days. The aromatic profile was investigated every day and 18 chosen compounds were followed. The results obtained at 5°C under vacuum were compared to those obtained at 40°C with normal conditions. The general trend followed by each compound was also studied.

Throughout the entire project, reduced and normal pressures are compared in order to highlight the improvement brought by vacuum compared to the method first described. Indeed, the main interest is to investigate the performance and potentiality of VAC-HS-SPME towards different kind of matrix. Work has already been done on aqueous samples but this project takes interest in an organic, solid sample. This project may confirm what has already been discussed before or bring new leads to follow in order to understand better HS-SPME and the effect of vacuum on the volatile extraction.

II. Material and method

Standards

Saturated C_7-C_{30} alkanes standard (certified reference material, SIGMA-ALDRICH), Hexane (HPLC grade, Biosolve Chimie).

Fish preparation

All fishes (Cod (*Gadus sp),* Salmon *(Oncorhynchus sp),* Pollock or Saithe *(Pollachius virens),* Redfish *(Sebastes norvegicus)*) were purchased in a local supermarket (Carrefour market) in Gembloux, Belgium. For the optimization of time and temperature, *Oncorhynchus sp* was left for 24h at room temperature in order to intensify the presence of volatiles. For the spoilage assessment the fish was purchased in the morning and processed directly. In all cases, the fish was homogenized in a blender (VEO Home, KWG-130B) then 5,0±0,5g were put in closed 22-mL vials. For the optimization, those vials were kept in a freezer (ZANKER) at -17°C and defrosted for 10 minutes at room temperature before analysis. For the spoilage assessment, vials were kept in a fridge (Bauknecht) at 5-7°C up to 5 days.

HSSPME

CAR/PDMS (carboxen/polydimethylsiloxane), PDMS/DVB (divinylbenzene) and CAR/PDMS/DVB fibers, kindly provided by SupelCo (Bellefonte, PA) were used. Some parameters stayed the same throughout the experiments: 10 minutes of incubation, 2 minutes of desorption and 10 minutes at 250°C to clean the fiber after each run.

For the optimization, different extraction times were tested (10, 20, 30, 40, 60 minutes) at 5, 30 and 40°C to establish a time profile. A temperature profile was also achieved for 30 minutes of extraction at 5, 10, 20, 30, 40 and 60°C. Thirty minutes of extraction at 5°C and 40°C were kept as conditions to be compared for the spoilage assessment.

Regular HSSPME consists in closing the vials with the adequate screw-cap, incubation in order to reach equilibrium, extraction with the fiber then injection in the GC-MS.

For the vacuum HSSPME, there is one extra step before incubation which removes the air from the vial. The cap was slightly changed as it needs an O ring to be gas-tight and a septum in order to use a syringe to remove air from the vial. The set up was the same as the one presented by Yiantzi &al., 2016. A vacuum pump (Diaphragm vacuum pump, VacuumBrand GmbH &Co. KG, model MZ 2C NT, Wertheim, Germany) was used and it

was tightly connected to a 5mL plastic syringe equipped with a stainless steel needle to remove the air.

Incubation and extraction for both regular and vacuum HSSPME were conducted either in a water bath cooled with ice for temperatures under 30°C or in the auto sampler for 30, 40 and 50°C.

GC-MS

An Agilent 7890B GC coupled to a 5977 MSDquadrupole mass spectrometer was used for all analyses. Separation was performed on a 30 m × 0,25 mm i.d × 0,25 µm OPTIMA 5MS Accent column (Macherey-Nagel). The coating is a 5 % diphenyl – 95 % dimethylpolysiloxane (non polar column). It was also equipped with a Gerstel Multipurpose Sampler. The injector and interface temperature was 250 and 280°C respectively and the over program was as follow: 35°C held for 2 minutes, then increased to 250°C at 12°C/min then to 300°C at 25°C/min, hold for 2 minutes. There was a 2 minutes desorption time with a split injection at a split ratio of 5. Carrier gas: helium at a flow rate of 1mL/min. For the detector, it was operating in EI at 70eV. The quadrupole was set at 150°C and the mass range was set from 35 to 350 *m/z* with a scan interval every 0,5s at 1,5kV. Data were acquired by MassHunter GC/MS Acquisition software B.07.06.2704 (Agilent, USA), converted into AIA by MSD ChemStation F.001.03.2357 (Agilent) and processed using Shimadzu GCMSolution ver 4.45 (Shimadzu, Japan).

III. Results and discussion

Chapter 1: Optimization

1. Fibers selection

The first part of the optimization was the selection of the fiber. Three different fibers were tested: PDMS/DVB, CAR/PDMS and CAR/PDMS/DVB. 18 compounds were selected for comparing the different conditions tested. They were selected to cover the entire volatility range and different chemical classes (alkanes, aldehydes, alcohols) and based on the compounds reported as important markers for fish in the literature (see annex 4). Their identification was based on the similarity match with commercial database (NIST and FFNSC) and an experimental LRI (linear retention index) within a ±10units compared to the literature. Table 5 presents the list of those compounds as well as some of their physico-chemical characteristics.

Table 5: Selected peaks with their molecular weight, boiling point and Henry's constant.

n°	NAME	CAS n°	MW (g/mol)	BP (°C)	K_H (atm.m3/mol)	LRI exp	LRI Lib	MS %	Ion Ref.
1	Butanal, 2-methyl-	96-17-3	86,132	94	1,05E-04	658	662	98	44
2	Butanal, 3-methyl-	590-86-3	86,132	92	5,21E-04	665	676	96	57
3	Heptane, 2,4-dimethyl-	2213-23-2	128,255	115	5,86E-01	792	788	92	43
4	Styrene	100-42-5	104,149	145	2,01E-03	891	891	90	104
5	Heptanal	111-71-7	114,185	153	4,53E-04	901	906	87	70
6	2,6-Dimethyl-4-heptanol	108-82-7	144,254	178	1,03E-04	949	950	85	43
7	Octanal	124-13-0	128,212	171	6,38E-04	1003	1006	89	43
8	Benzeneacetaldehyde	122-78-1	120,148	195	1,85E-05	1048	1045	97	91
9	Nonanal	124-19-6	142,239	214	8,02E-04	1104	1107	93	57
10	Dodecane	112-40-3	170,335	216	4,81E-01	1199	1200	93	57
11	Decanal	112-31-2	156,265	208	1,11E-03	1206	1208	88	57
12	Tridecane	629-50-5	184,361	235	8,14E-01	1299	1300	88	57
13	Tetradecane	629-59-4	198,388	253	1,05E+00	1399	1400	94	57
14	Pentadecane	629-62-9	212,415	269	1,49E+00	1499	1500	95	57
15	BHT	128-37-0	220,35	265	8,93E-05	1507	1503		205
16	Hexadecane	544-76-3	220,35	287	2,26E+00	1598	1600	90	57
17	Heptadecane	629-78-7	240,468	302	3,49E+00	1699	1700	95	57
18	Octadecane	593-45-3	254,494	317	5,22E+00	1798	1800	90	57

The uptake of those compounds by the different fibers was compared both under conventional and reduced pressure sampling. Figure 8 shows a comparison of the chromatograms obtained using those 3 fibers, pinpointing the position of the selected peaks. It is interesting to note that BHT was not mentioned in the literature however as it is an anti-oxidant, it was probably added as conservative on the fish. There are no limits set in Europe yet concerning fish products however an ADI (acceptable daily intake) is set at 0,3mg/kg bw (Codex document CX/FA 07/39/9 (Part 1)). Therefore, it was included in the list to see its behavior under normal and vacuum SPME sampling conditions.

Figures 9 shows the chromatographic areas (based on the EIC of the reference ion reported in table 5) of the 18 selected peaks with the three different fibers (CAR/DPMS/DVB, CAR/PDMS, PDMS/DVB) sampled at normal pressure. Two of the selected compounds (2-methyl-butanal and 3-methyl-butanal) showed the highest areas and needed a different graph as their scale was much bigger than the other compounds.

CAR/PDMS/DVB fibers shows higher areas for five out of the 18 selected peaks (2-methyl-butanal, 3-methyl-butanal, 2,4-dimethyl-heptane, 2,6-dimethyl-4-heptanol, benzeneacetaldehyde and BHT). Octanal, nonanal and dodecane were extracted the most with CAR/PDMS or PDMS/DVB fibers (comparable results). Those two fibers showed very similar extraction yield concerning the selected peaks: only tridecane, heptanal and octadecane had a p-value <0,05 in student t-test (H_0: mean difference=0). Tridecane and heptanal were the most extracted with PDMS/DVB and octadecane with CAR/PDMS.

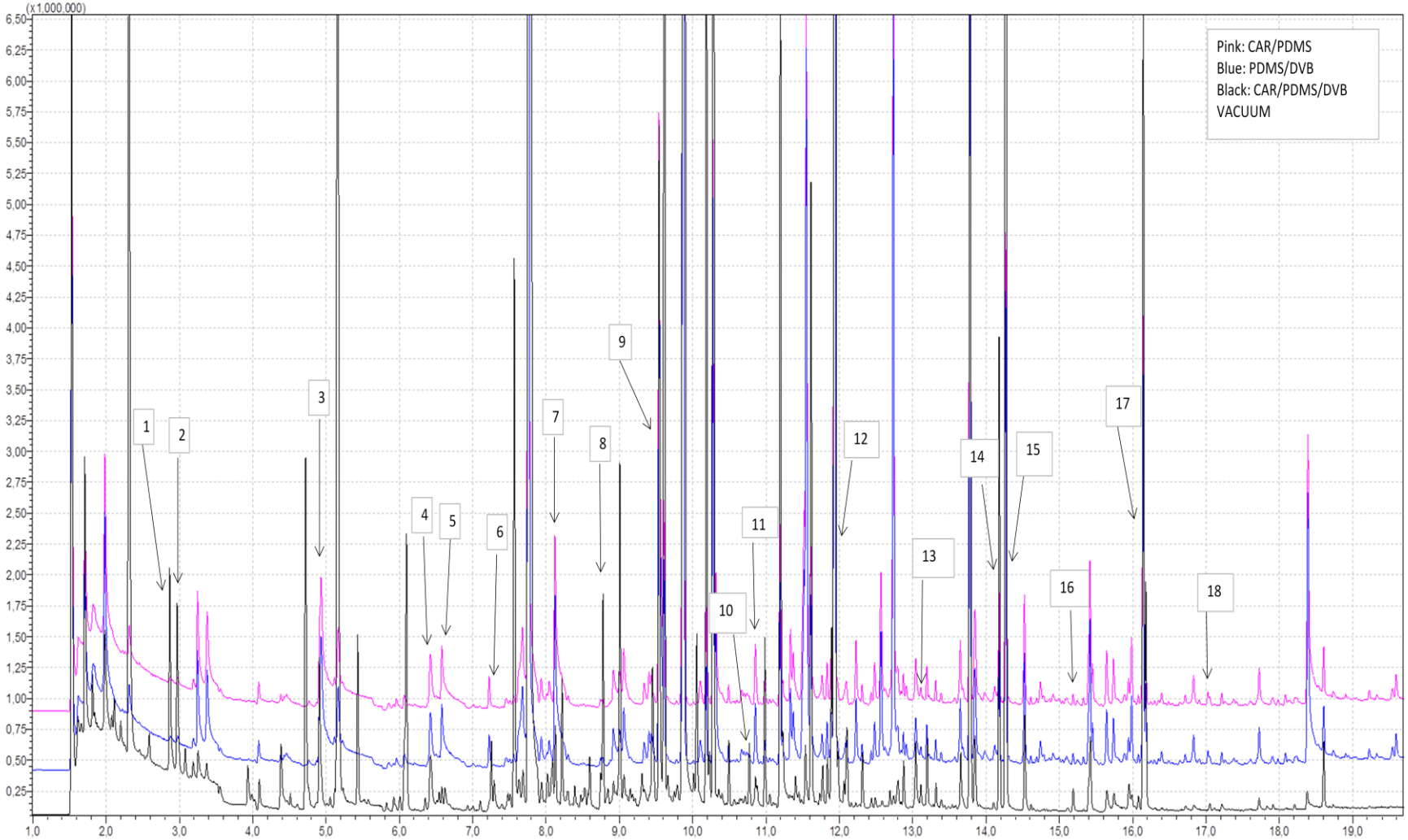

Figure 8: Comparison of the chromatograms obtained with the three different SPME fibers (CAR/PDMS/DVB, CAR/PDMS, PDMS/DVB) under vacuum sampling. Numbers correspond to the compounds list in table 1..

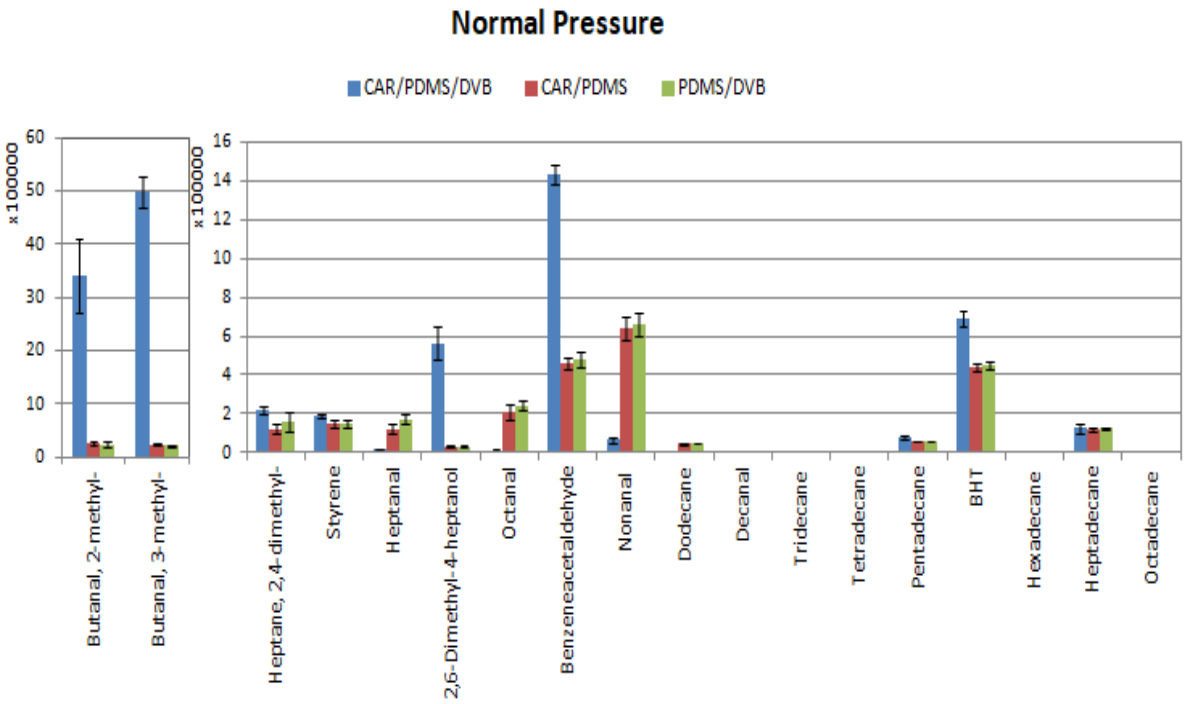

Figure 9: comparison of three fibers extraction efficiency when used at atmospheric pressure.

As vacuum is the parameter of interest, the efficiency of the three fibers was also compared under reduced pressure to see if the trend was similar. Figures 10 shows the areas obtained in those conditions. BHT needed a larger scale and thus a different graph was done.

2-methyl-butanal, 3-methyl-butanal still showed the highest area with CAR/PDMS/DVB but within the range of the other compounds. 2,4-dimethyl-heptane, heptanal, octanal and nonanal were extracted the best with CAR/PDMS and PDMS/DVB. The extraction of nonanal with CAR/PDMS/DVB showed a real improvement with vacuum but it is still not as good as CAR/DPMS and PDMS/DVB. All other compounds were extracted the most with CAR/PDMS/DVB.

The effect of vacuum on the different fibers was evaluated by considering the ration of the extraction under vacuum sampling and under normal pressure condition (Figure 11). If it is equal to 1 (orange line), it means that there is no improvement when using reduced pressure. If the ratio <1, vacuum decreases the extraction of the compound. Finally, if it is >1 then the vacuum increased that compound's extraction. Overall vacuum increased a lot of the selected compounds, reaching a 18-fold increase for dodecane with CAR/PDMS/DVB. Nine compounds, compared to three with CAR/PDMS (and two with PDMS/DVB) increased over a 5-fold with CAR/PDMS/DVB, although the variability increased as well.

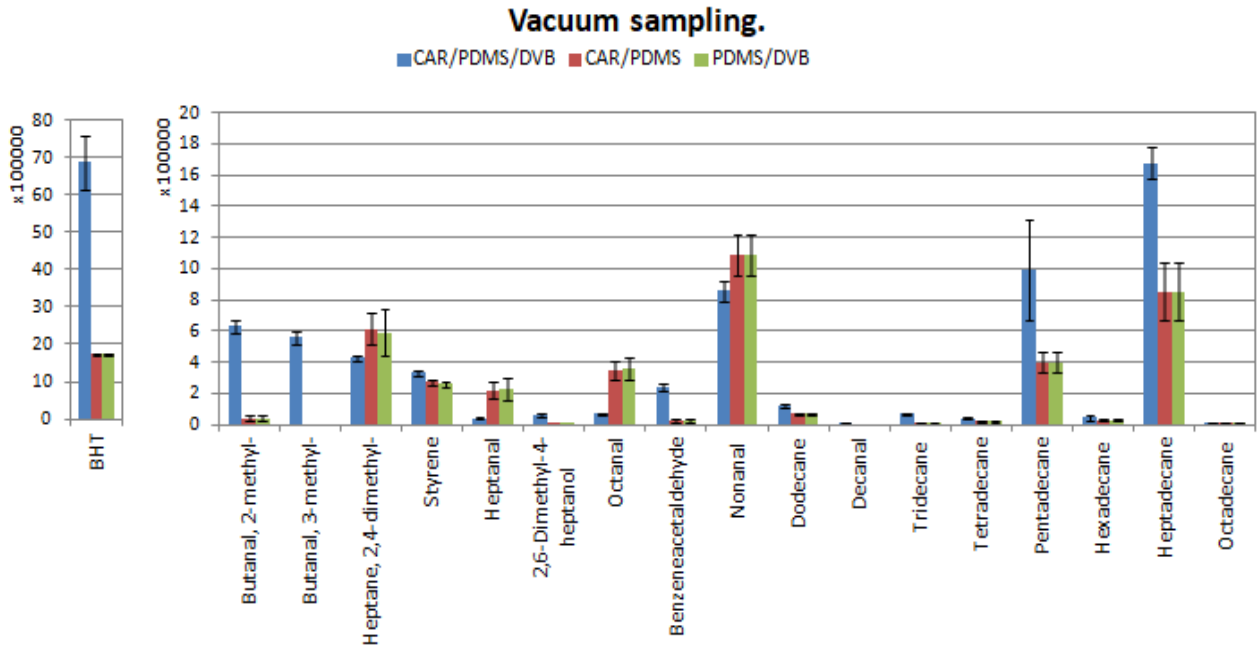

Figure 10: comparison of three fibers extraction efficiency when used with vacuum and according to 18 selected peaks.

Some compounds were less extracted with vacuum: the branched butanals, 2,6-dimethyl-4-heptanol and benzeneacetaldehyde (with a ratio ≤0,2). According to the theory (Psillakis, 2017), more volatiles compounds are less affected by the use of reduced pressure conditions while less volatile compounds show a large improvement under those conditions. It was formulated for aqueous samples that according to their K_H, compounds would react differently to reduced pressure. Compounds with a K_H lower than $1{,}6\times10^{-4}$ atm m^3/mol should have their extraction improved with vacuum. All compounds with K_H above the limit should have either stayed the same or decreased with vacuum. It is important to stress that the theory was formulated for aqueous solutions (as K_H refers to that) and even if fish is composed by 70-80% of water, it contains also a rather high amount of protein (~20%) and lipids (~10%), notwithstanding that it is a solid sample, thus the compounds mobility within the sample is different. At present, no theory has been formulated yet for such complex systems and further investigation should be made to elucidate the process. Although the particular trend related to K_H was not observed as expected, there was a general improvement with the boiling point, although not the unique effect.

CAR/PDMS/DVB was selected for further experiment since it showed the overall best extraction yield both under atmospheric and reduced pressure, being by far the most reactive to the different operational pressures.

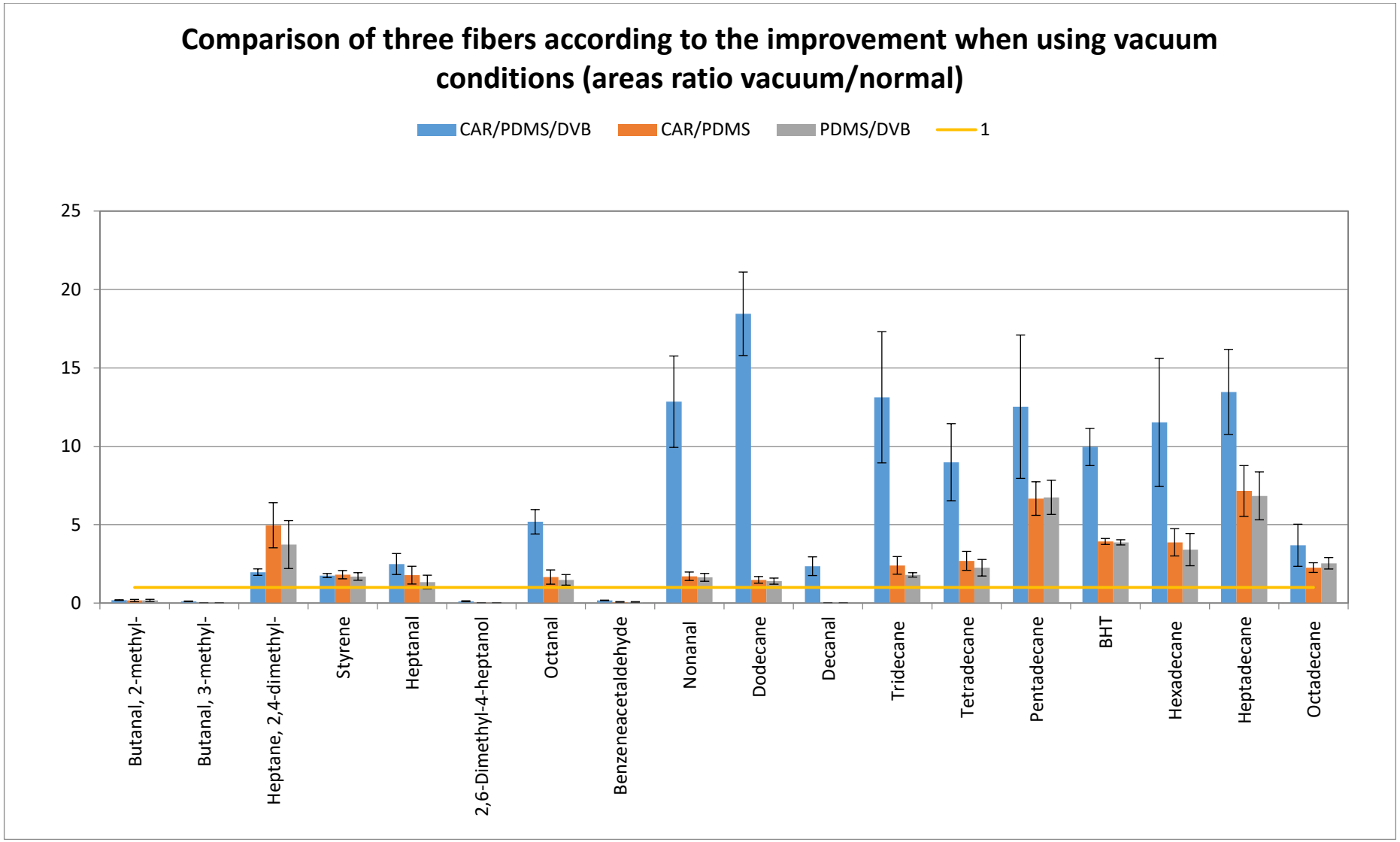

Figure 11: Comparison of three fibers based on the ratio of the areas obtained under vacuum and normal pressure

2. Time profile

The next parameter investigated was time. For this, an extraction time profile (10, 20, 30, 40 and 60 minutes) was investigated at 5, 30 and 40°C under vacuum and regular conditions. The complete results are in annex 5 to 9. In the next section, the general trends observed will be discussed and only one graph per trend will be included to illustrate the point.

2.1 5°C

The global trend is found in figure 13. It represents the ratio of the extraction under vacuum on the areas obtained with normal conditions. As explained above, this ratio translates the improvement brought by vacuum. In general, vacuum improved less the most volatile compounds (the first one eluted) and longer extraction times allow more molecules to be extracted for one compound.

The two butanals showed a very particular trend: the regular sampling extracts them more than under vacuum, as shown on figure 12. Those two compounds are the most volatiles and, according to the theory (see point 3.4 Vacuum in the introduction), it is likely that they are already extracted well under normal conditions and low temperature. With the time increase the extraction shows an overall increase too. However with vacuum, the equilibrium settles so fast that they probably reached the plateau in less than 10 minutes. Therefore, the increase of extraction time is not improving their extraction.

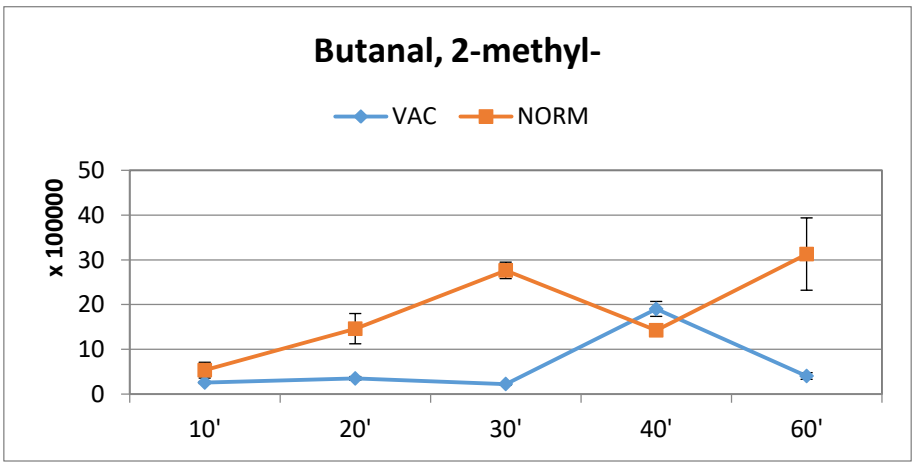

Figure 12: Extraction of 2-methyl-butanal under vacuum and normal conditions at 5°C.

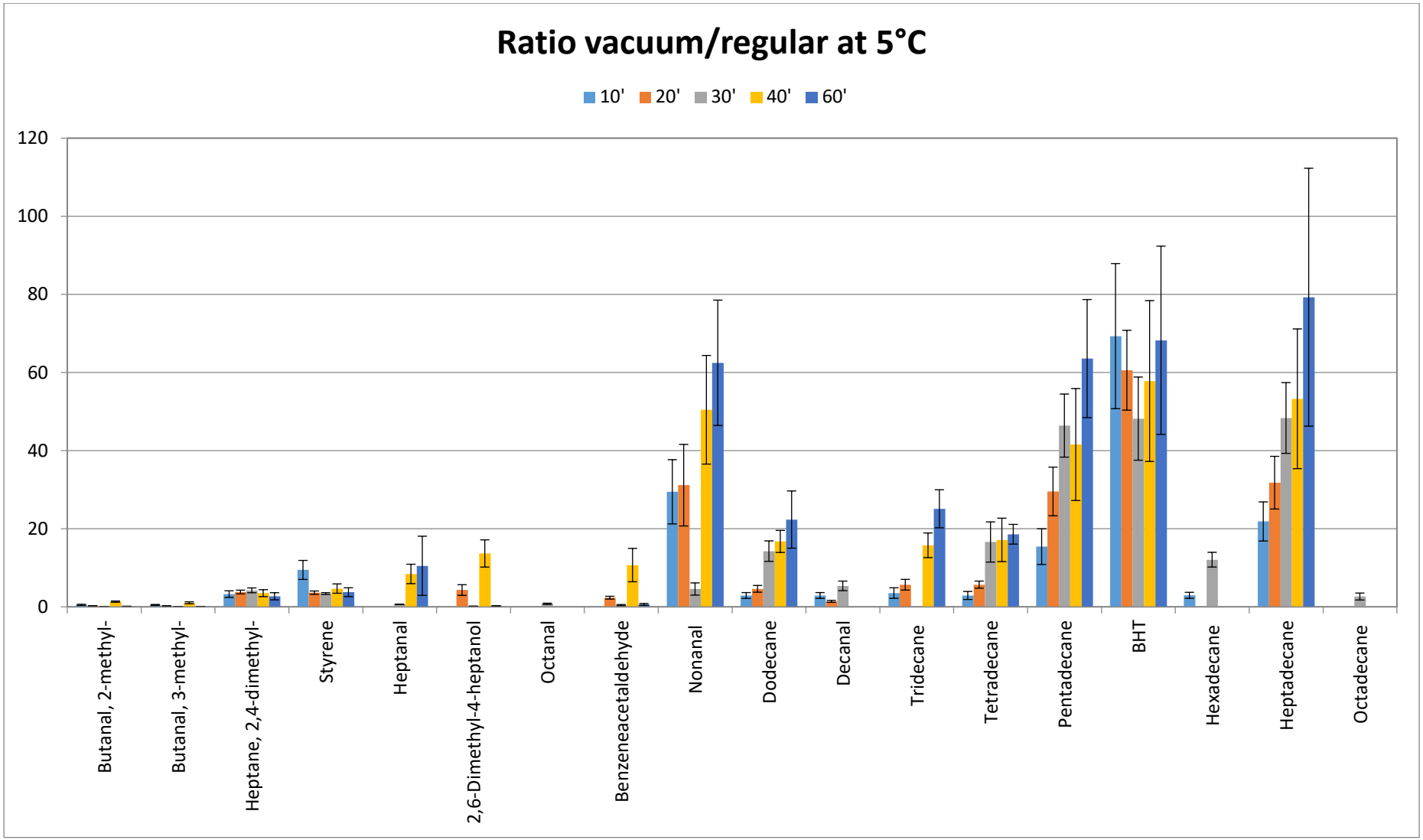

Figure 13: ratio of the extraction under vacuum and normal conditions at 5°C.

Octadecane, the less volatile of all selected compounds, was not extracted a lot at 5°C. However vacuum improves the extraction as shown on figure 14. On figure 13, only the ratio at 30 minutes of extraction is shown as at the other extraction times and under normal conditions nothing was extracted (figure 14). This compound is the less volatile of the selection thus the most difficult to extract, especially at 5°C.

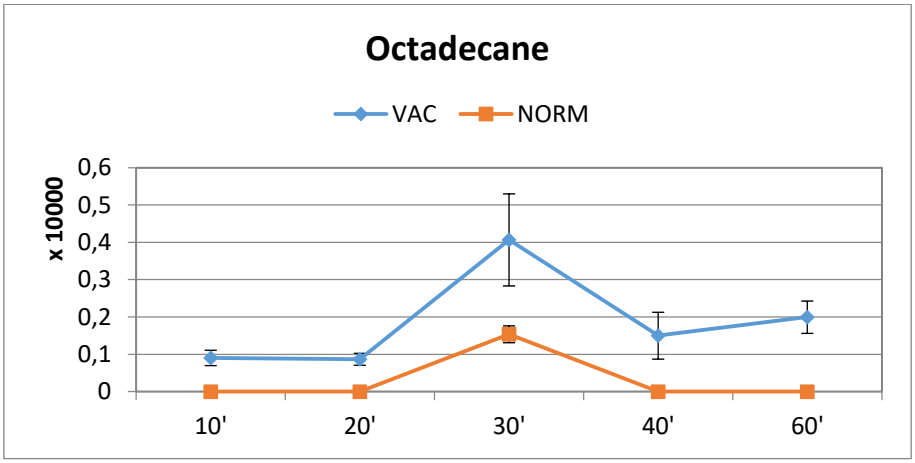

Figure 14: Extraction of Octadecane under vacuum and normal conditions at 5°C.

All other compounds show a general increase with time. Figure 13 can be misleading for some compounds, especially hexadecane. The ratio is small or inexistent but this is due to the fact that the extraction under normal conditions is so small (the average area is null) that the ratio is undetermined (divided by 0). But figure 15 shows how vacuum actually increases the extraction. As for octadecane, this compound is not very volatile so under normal conditions and low temperature, the extraction is not optimized. After 30 minutes of extraction, the curve stabilizes thus indicating that the equilibrium has been reached.

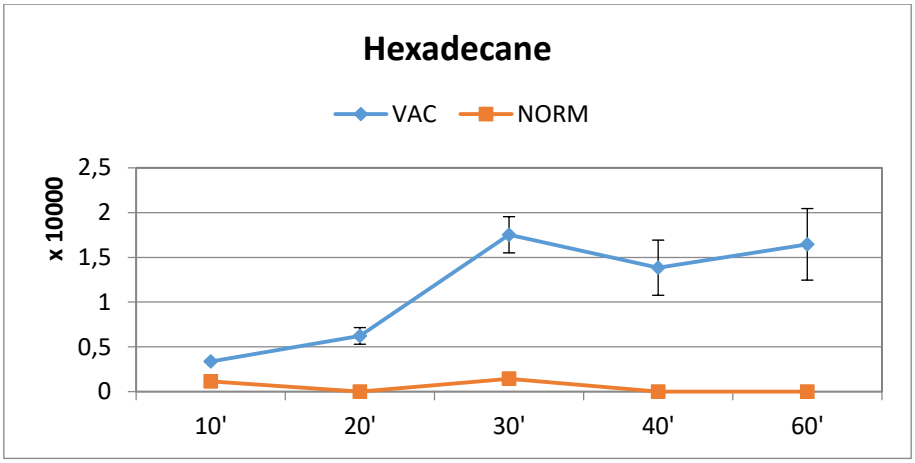

Figure 15: Extraction of hexadecane under vacuum and normal conditions at 5°C.

2,4-dimethyl-heptane, styrene, heptanal, 2,6-dimethyl-4-heptanol, octanal and benzeneacetaldehyde show a similar increase under vacuum and normal conditions. This translates a constant ratio over the different times (figure 13). Figure 16 shows the behavior of styrene. The curves of vacuum and normal extractions are almost parallels. As previously described, at 30 minutes of extraction, the curve reaches its peak.

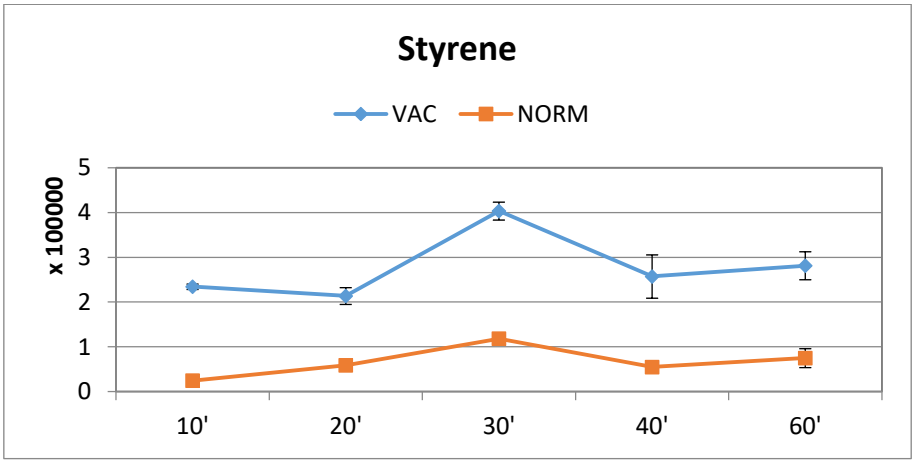

Figure 16: Extraction of styrene under vacuum and normal conditions at 5°C.

Nonanal, dodecane, decanal, tridecane, tetradecane, pentadecane, BHT and heptadecane also show an increase in extraction with time. Under vacuum it increases faster than with normal conditions thus showing ratios getting bigger on figure 13. Figure 17 shows the trend followed by BHT. As for octadecane and hexadecane, normal conditions are not optimized. However for this type of compounds vacuum is highly increased with time.

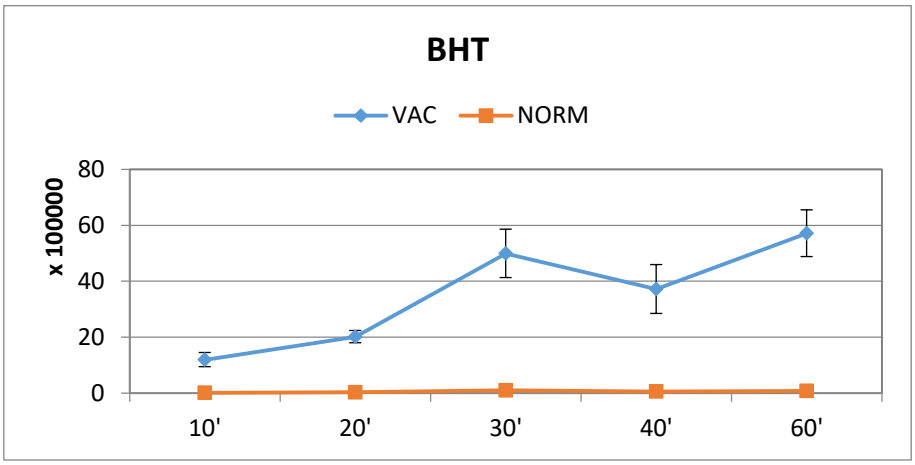

Figure 17: Extraction of BHT under vacuum and normal conditions at 5°C.

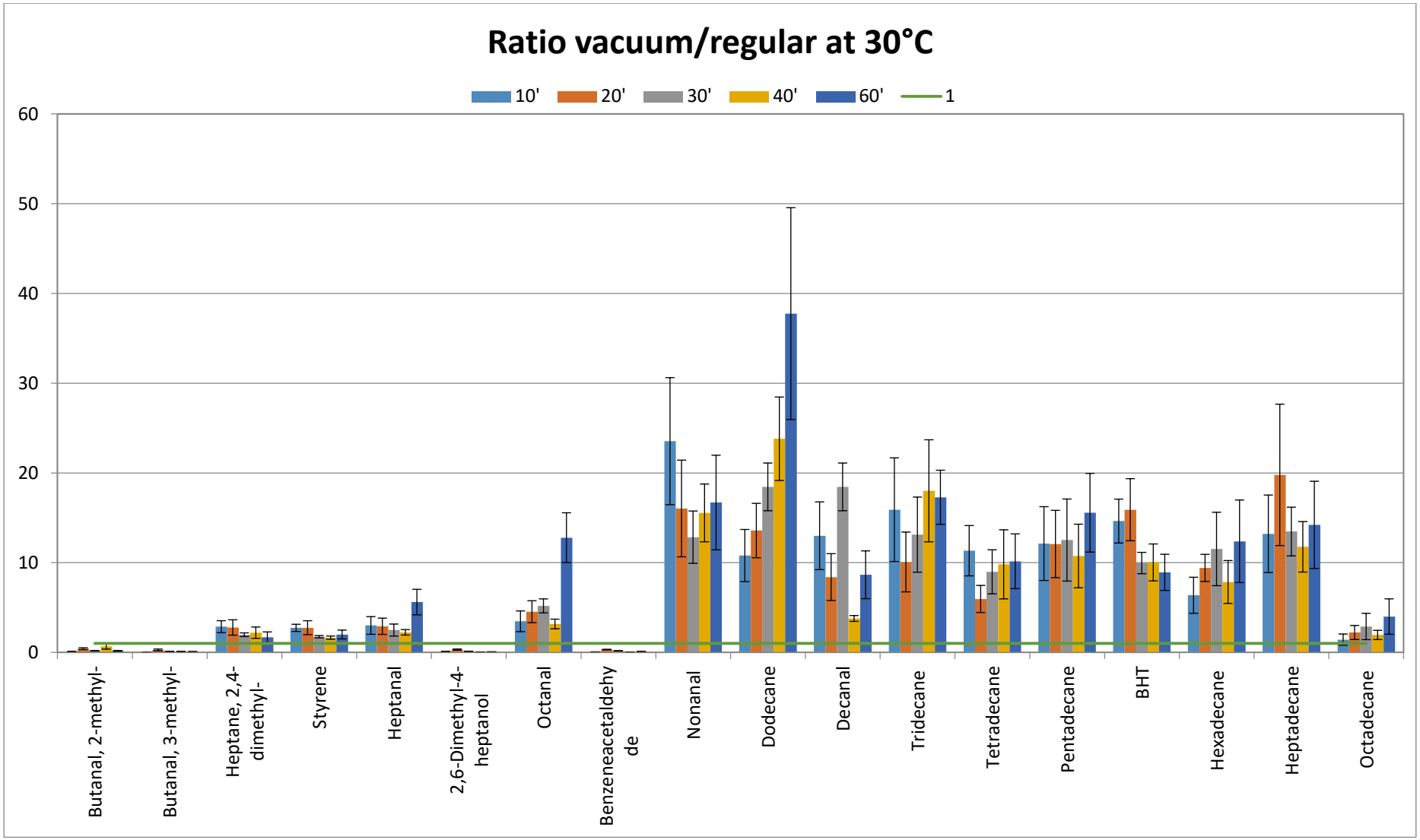

Figure 18: ratio of the extraction under vacuum and normal conditions at 30°C.

2.2 30°C

The global trend regarding the improvement of vacuum at 30°C is presented in figure 18. Compared to figure 13 (which shows the improvement at 5°C), the improvement is smaller. It reached almost a 80-fold increase at 5°C and 60 minutes of extraction for heptadecane. At 30°C the best ratio reach almost 40-fold increase. Moreover, compared to 5°C, the ratio is almost constant throughout the different extraction times for the same compound.

Once again the two butanals are better extracted with normal conditions (ratio <1 on figure 18) (figure 19, on the left). However at 30°C, 2,6-dimethyl-4-heptanol and benzeneacetaldehyde share the same trend (figure 19, on the right). According to table 5, those compounds have low K_H (3-methyl-butanal is slightly higher). As for the fiber choice, the experiments present results that are opposite to the theory (the extraction of compounds with low K_H should be improved with vacuum). This could be due to kinetics improved so much that the equilibrium does not even need 10 minutes to be reached or to displacement. Indeed, the vacuum increases other compounds extraction and, because the fibers works on a competition basis, compounds with higher affinity with the fiber take their place.

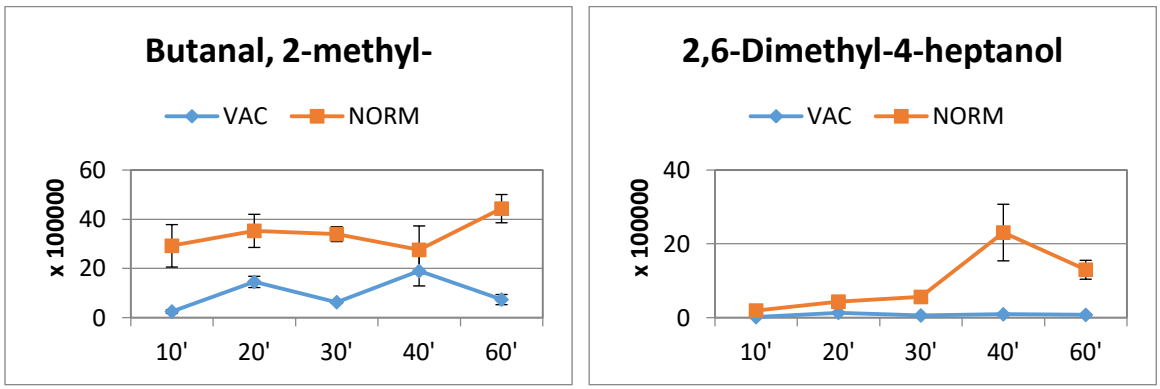

Figure 19: Extraction of 2-methyl-butanal and 2,6-dimethyl-4-heptanol under vacuum and normal conditions at 30°C.

All other compounds were improved with vacuum (ratio >1 on figure 18) however they showed different trends. Some compounds (heptanal, octanal, dodecane and tridecane) showed a ratio increasing with time. As shown on figure 20, under normal conditions the extraction is very low and it takes a very long time for the equilibrium to be reached (not achieved under tested conditions). On the other hand, under vacuum, the exponential phase starts between 20 and 30 minutes of extraction.

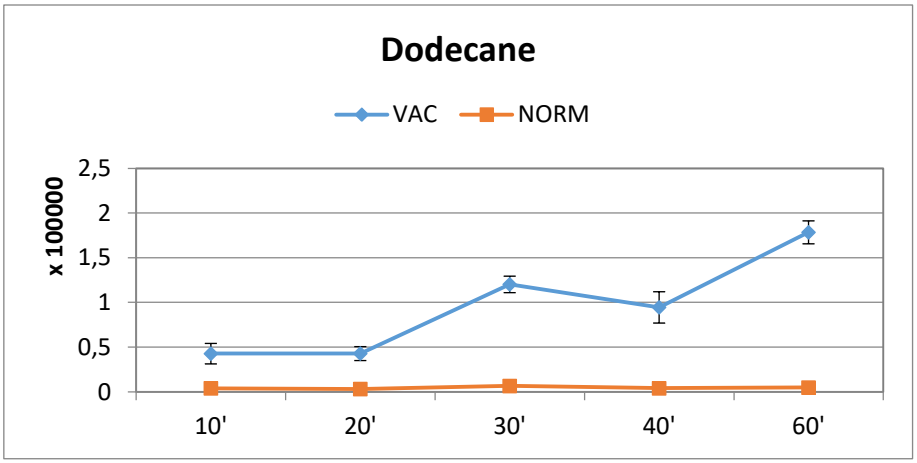

Figure 20: Extraction of dodecane under vacuum and normal conditions at 30°C.

The most followed trend is shown by 2,4-dimethyl-heptane, styrene, nonanal, decanal, tetradecane, pentadecane, BHT, hexadecane, heptadecane and octadecane. For these compounds, the ratio stays constant on figure 18. Six of these compounds (styrene, nonanal, pentadecane, BHT, hexadecane and heptadecane) show an increase with the extraction time under both pressure conditions. This is illustrated on figure 21 for BHT.

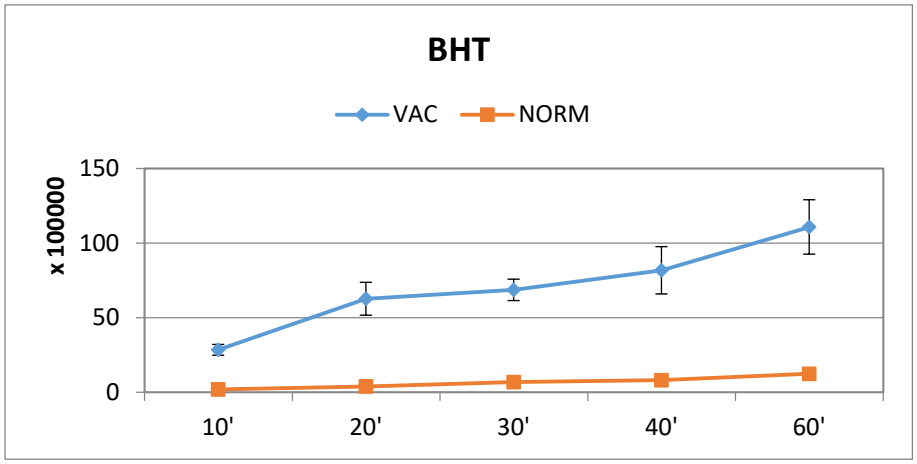

Figure 21: Extraction of BHT under vacuum and normal conditions at 30°C.

Three of the compounds (2,4-dimethyl-heptane, tetradecane and octadecane) also have constant ratio on figure 18 . However, on their profile (figure 22) they have an increase then they reach a plateau (meaning the equilibrium is reached). 2,4-dimethyl-heptane reaches it after 20 minutes and the other two compounds reach the equilibrium after 30 minutes of extraction.

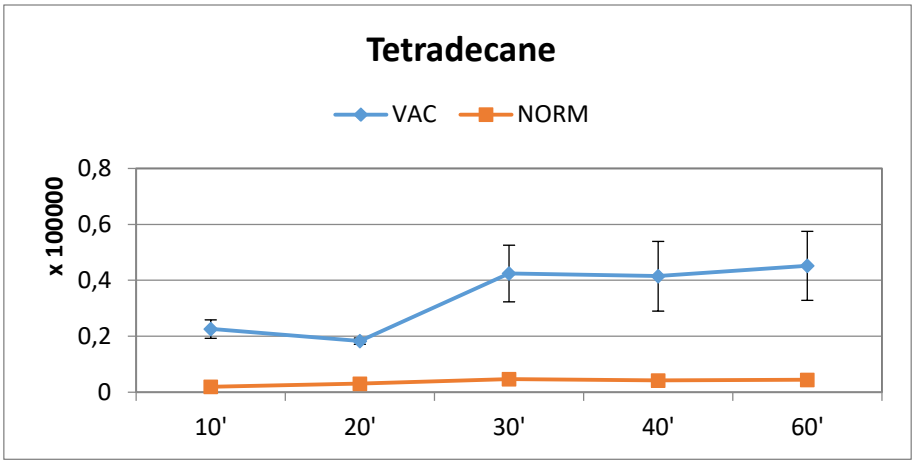

Figure 22: Extraction of tetradecane under vacuum and normal conditions at 30°C.

Finally decanal is the only compound, under vacuum, showing an increase up to 30 minutes of extraction then a decrease, most probably due to a displacement effect (figure 23).

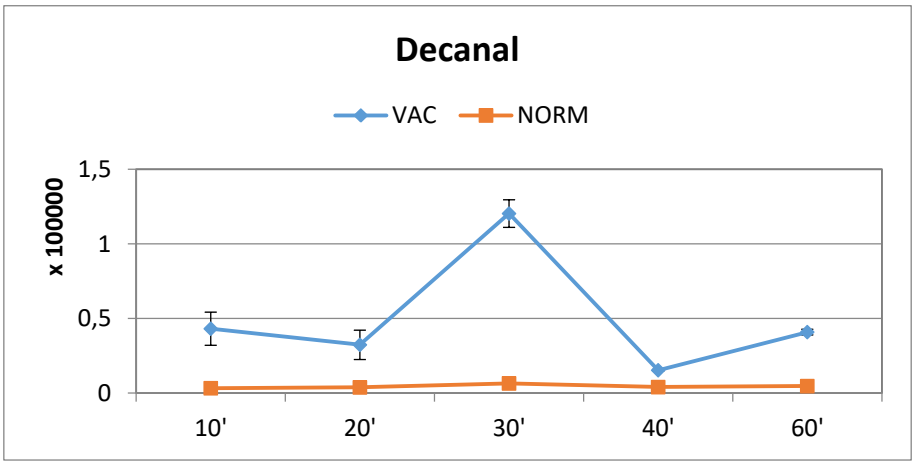

Figure 23: Extraction of decanal under vacuum and normal conditions at 30°C.

2.3 40°C

Figure 24 shows the improvement brought by vacuum at 40°C. Compared to figure 18 the ratios are smaller: the highest does not reach a 30-fold increase. Some compounds show ratios constant over the time profile which means that vacuum and normal condition evolve at the same pace. Some compounds show increased ratios over time, due to a more significant increase on the extraction yield over time when sampling under vacuum conditions rather than on regular conditions. Finally, some show an increase then a decrease. In this case, the improvement brought by vacuum is higher at short extraction time, then the equilibrium is reached and the extraction yield starts to decrease while under normal condition it continues to grow until reaching the equilibrium, thus reducing the impact of vacuum.

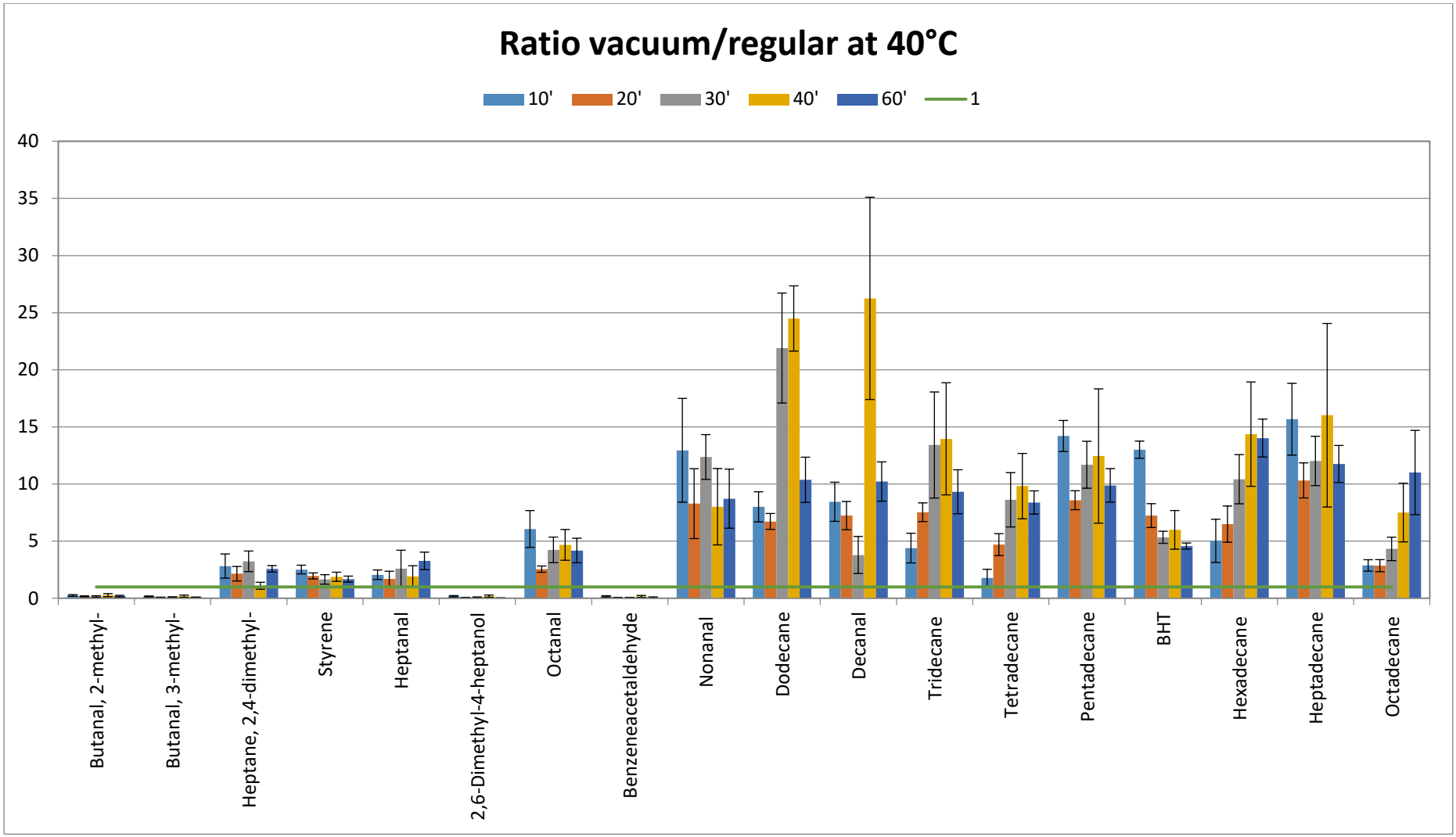

Figure 24: ratio of the extraction under vacuum and normal conditions at 40°C.

The two butanals, 2,6-dimethyl-4-heptanol and benzeneacetaldehyde have a similar trend as at 30°C: vacuum conditions extract them less than sampling under normal pressure. However figure 25 shows a higher scale: those four compounds are more extracted with higher temperature.

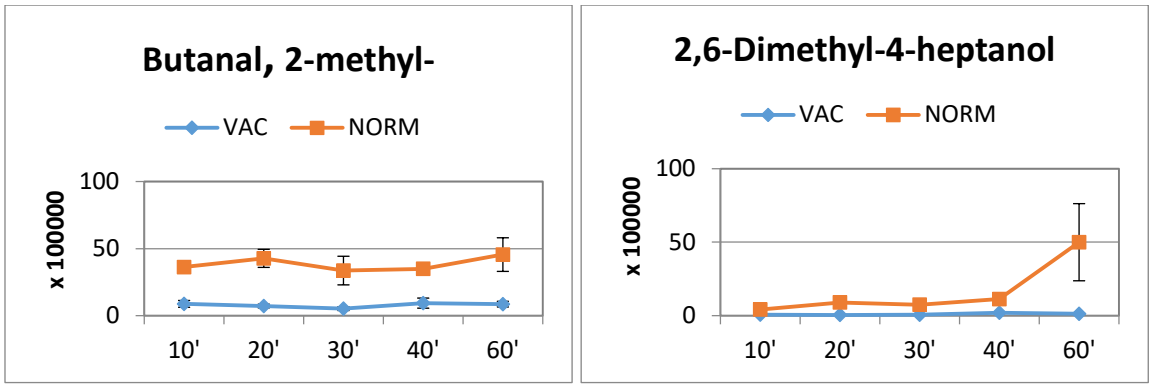

Figure 25: Extraction of 2-methyl-butanal and 2,6-dimethyl-4-heptanol under vacuum and normal conditions at 40°C.

Concerning the profile followed with longer extraction time, heptanal, tetradecane, pentadecane, hexadecane, heptadecane and octadecane show a continuous improvement of the extraction as shown on figure 26.

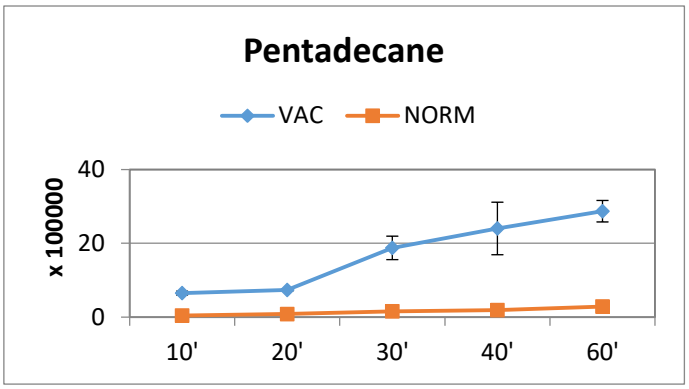

Figure 26: Extraction of pentadecane under vacuum and normal conditions at 40°C.

Octanal, nonanal and tridecane show an increase then they reach a plateau at 30 minutes of extraction (figure 20 on the left). Styrene and BHT reach the plateau after 40 minutes of extraction. Finally, 2,4-dimethyl-heptane is constant through the time profile which means it reaches the plateau in less than 10 minutes (figure 27, on the right).

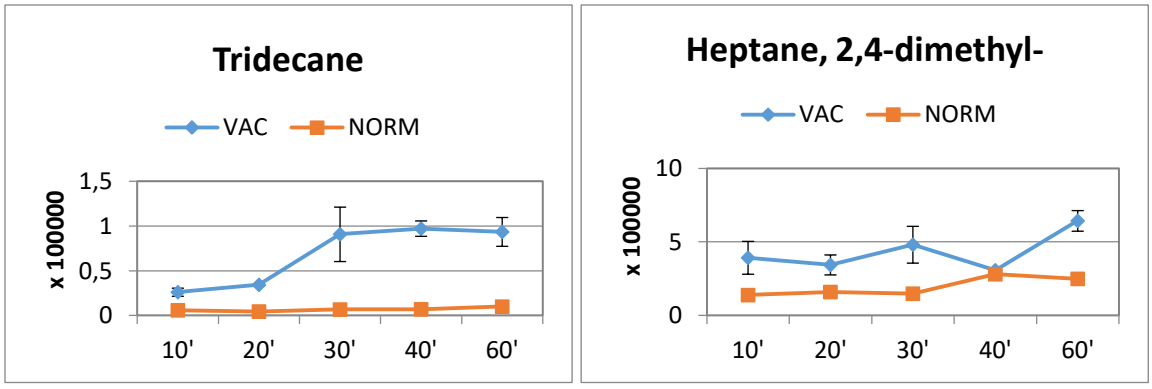

Figure 27: Extraction of tridecane and 2,4-dimethyl-heptane under vacuum and normal conditions at 40°C.

The last trend is followed by decanal (as at 30°C) and dodecane. The extraction reaches a peak at 40 minutes of extraction then decrease due to displacement. The trend is the same as at 30°C (figure 23).

2.4 Comparison of the extraction at 5°C with vacuum and 30 or 40°C with normal conditions

As one of the goals was to work at low temperature with vacuum, it is interesting to compare the results obtained at 5°C under vacuum and those obtained at 30 and 40°C with normal conditions. The trends followed by each compound are fairly similar between the comparison of 5 and 30°C and 5 and 40°C (annex 8 and 9). The rest of the discussion will be done with the comparison of 5 and 40°C as the extreme cases.

The first trend observed is when 40°C with normal conditions extracts better than 5°C with vacuum (*i.e* ratio <1). This happens for the two butanals, 2,6-dimethyl-4-heptanol, benzeneacetaldehyde and octadecane (figure 28). Concerning the four first compounds, it was already observed that vacuum was not improving their extraction because of displacement. However the extraction of octadecane was improved with vacuum at 5, 30 and 40°C. This compound is the less volatile of the selection and it appears that the improvement brought by vacuum is not as important as the improvement brought by high temperature.

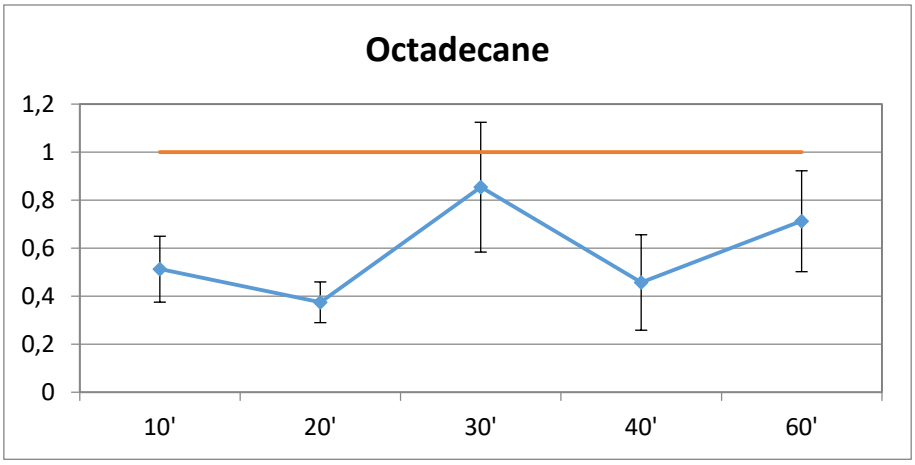

Figure 28: Ratio of the extraction of octadecane at 5°C under vacuum on the extraction at 40°C with normal pressure.

The second trend is observed for 2,4-dimethyl-heptane, styrene, heptanal, octanal, tetradecane, pentadecane, BHT, hexadecane and heptadecane. This time ratio is included between 1 and 4 throughout the time profile. The first four compounds are part of the most volatile compounds and the last 5 are part of the less volatile. Those compounds show two trends with time increase: styrene, octanal and BHT decreased. Styrene even drops under a ratio of 1 for 40 and 60 minutes of extraction (figure 29, on the left). 2,4-dimethyl-heptane, heptanal, tetradecane, pentadecane, hexadecane and heptadecane are more constant. However the 2,4-dimethyl-heptane and the four alkanes ratios peak for 30 minutes of extraction (figure 29, on the right).

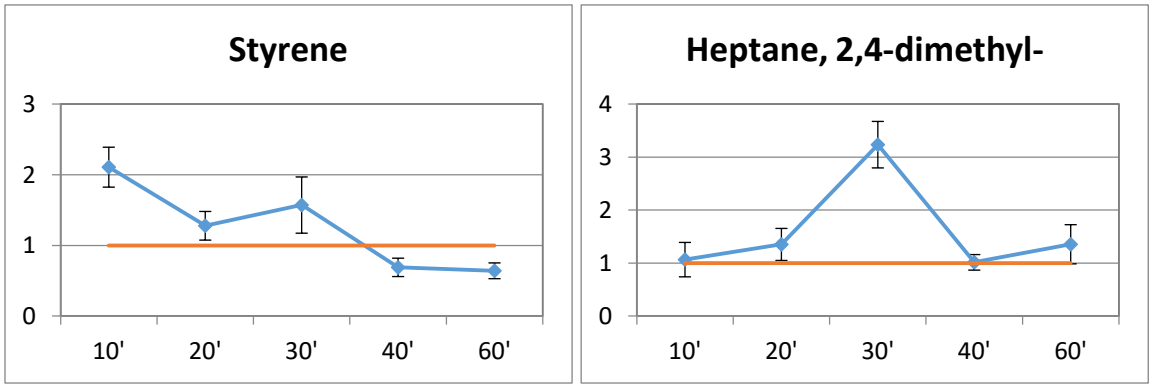

Figure 29: Ratio of the extraction of 2,4-dimethyl-heptane and styrene at 5°C under vacuum on the extraction at 40°C with normal pressure.

Finally, nonanal, dodecane, decanal and tridecane show a significant higher uptake at 5°C under vacuum compared to 40°C under regular sampling. Their ratio is included between 5 and 8. Those compounds are exactly in the middle of the selection's volatility range. Nonanal keeps a constant ratio over the time profile (figure 30, on the left) and the other three reach a peak for 30 minutes of extraction (figure 30, on the right).

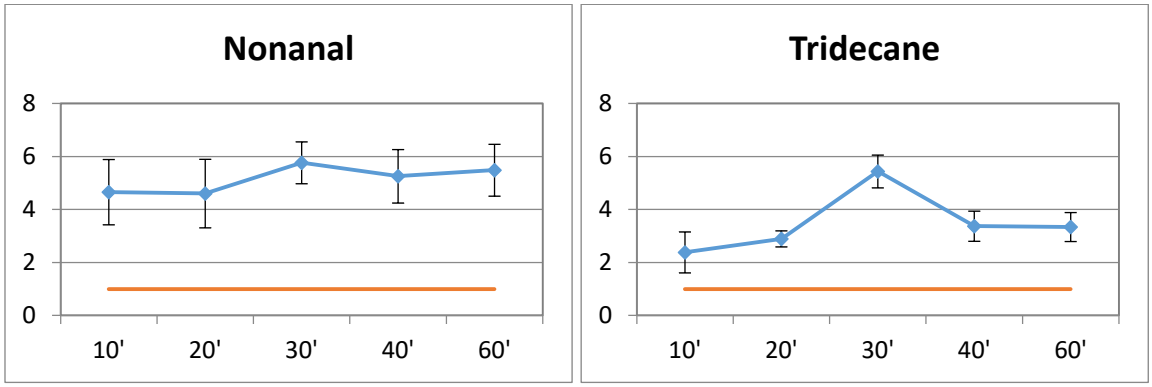

Figure 30: Ratio of the extraction of nonanal and tridecane at 5°C under vacuum on the extraction at 40°C with normal pressure.

In conclusion the trend looks like a Gaussian curve. The very low or high volatile are better extracted at high temperature and normal conditions. The compounds in the middle of the volatility range have a more significant impact on the vacuum sampling rather than the temperature increase. The compounds reaching a peak reached it at 30 minutes of extraction thus this value is chosen for the rest of the project.

3. Temperature profile

The last parameter assessed for the optimization is temperature. 5, 10, 20, 30, 40 and 50°C were investigated with an extraction time of 30 minutes under vacuum and normal pressure. As for the time, the complete results can be found in annex 10 and 11. The next part will discuss the trends found and one example will be chosen to illustrate it.

On figure 31 a trend is clearly evident: decreasing the volatility of the compounds, the vacuum increased the extraction more significantly at lower temperature (except for octadecane, for which the ratio remains almost constant over the different temperature). This means that temperature compensate the better extraction obtained sampling under vacuum conditions. Confirming the main advantage of Vac-HS-SPME which is the possibility to increase the extraction yield under milder conditions.

Different kinds of profiles are found with the increase of temperature. Volatile compounds (2-methyl-butanal, 3-methyl-butanal, 2,4-dimethyl-heptane, styrene, heptanal and octanal) stay constant. As mentioned before, the two first butanals are extracted better under normal conditions (figure 32, on the left) whereas the rest shows improved extraction with vacuum (figure 32, on the right).

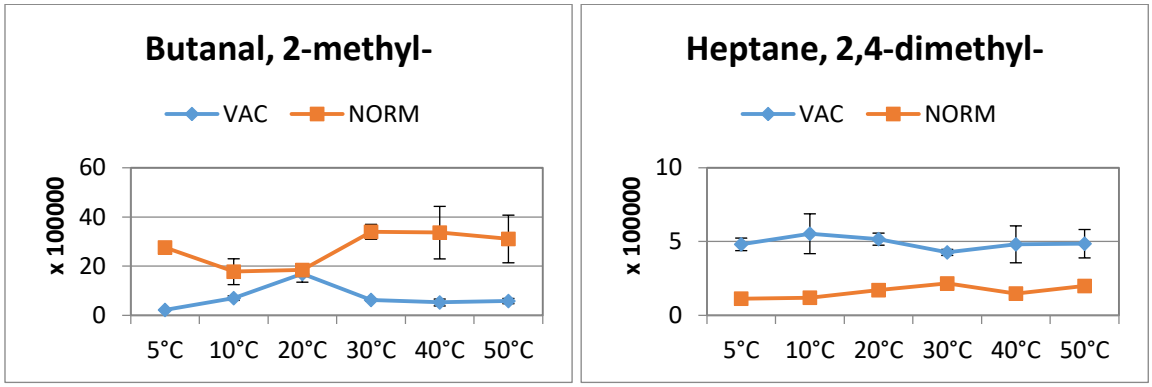

Figure 32: Extraction of 2-methyl-butanal and 2,4-dimethyl-heptane under vacuum and normal condition through a temperature profile (5 to 50°C).

2,6-dimethyl-4-heptanol, benzeneacetaldehyde, nonanal and decanal show an overall increase with temperature. The two first compounds are better extracted with normal conditions (figure 33, on the left) while the other two are improved with vacuum (figure 33, on the right). Dodecane, tridecane and tetradecane show a similar trend but they reach a plateau in the higher temperatures (figure 34, on the left). The last trend is followed by pentadecane, BHT, hexadecane, heptadecane and octadecane. These are the less volatile compounds of the selection and they have an exponential increase with temperature (figure 34, on the right).

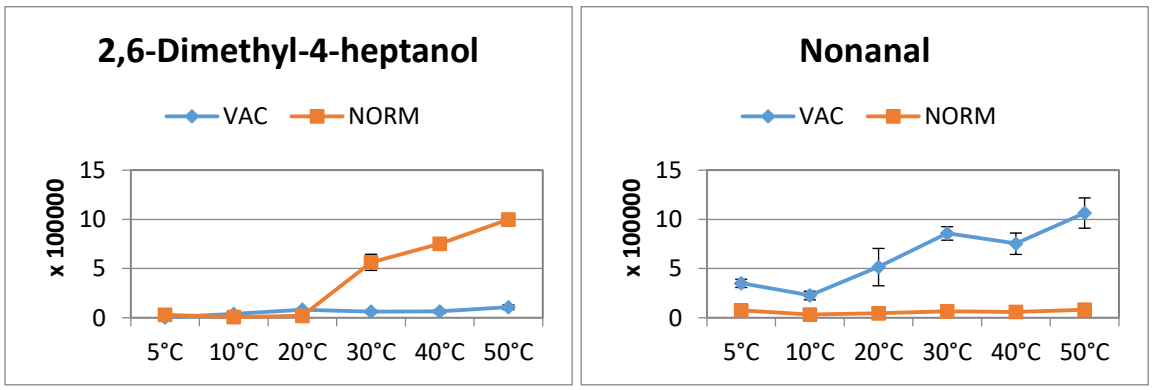

Figure 33: Extraction of 2,6-dimethyl-4-heptanol and nonanal under vacuum and normal condition through a temperature profile (5 to 50°C).

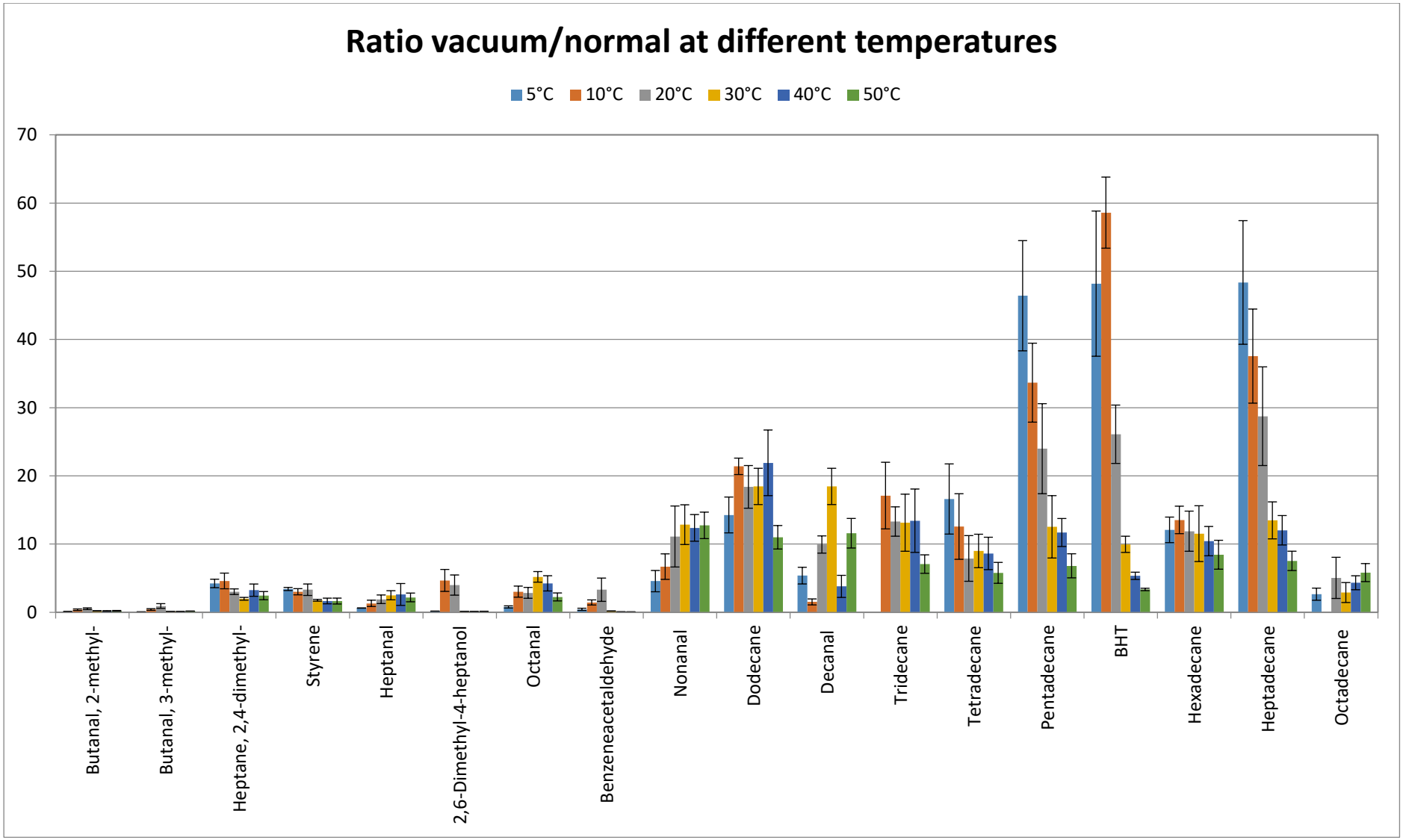

Figure 31: ratio of the extraction under vacuum and normal conditions over increasing temperature (5 to 50°C)

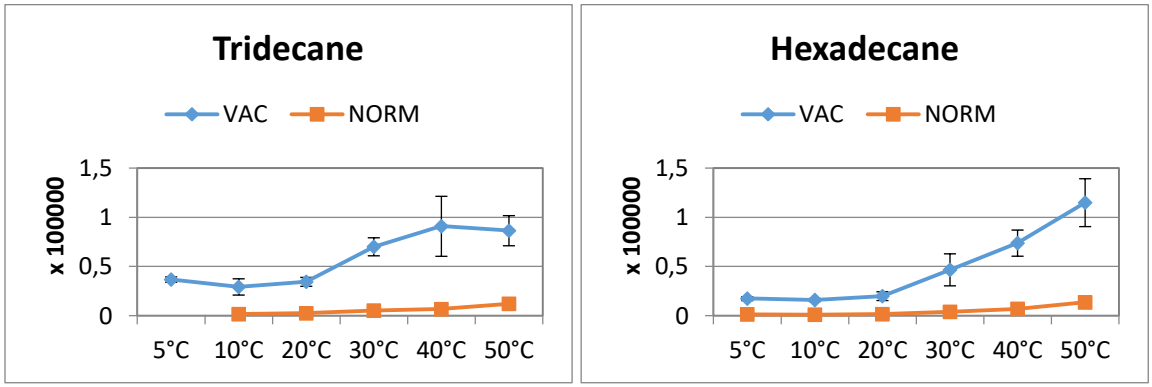

Figure 34: Extraction of tridecane and hexadecane under vacuum and normal condition through a temperature profile (5 to 50°C).

Concerning the ratios of the extraction under vacuum on the extraction with normal conditions, two trends appear. The compounds that are on the extreme of the volatility range (either very volatile or the less volatile) show an overall decrease of the improvement brought by vacuum (figure 35 show one example for each extreme).

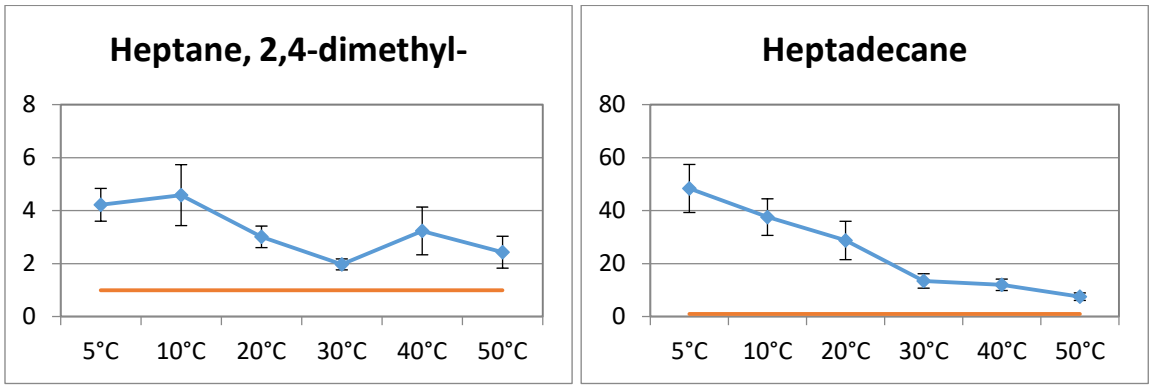

Figure 35: Ratio of the extraction of 2,4-dimethyl-heptane and heptadecane under vacuum and normal condition over increasing temperatures (5 to 50°C)

Compounds in the middle range show either a ratio constant over the temperature increase or an increase with a plateau around 20°C (figure 36).

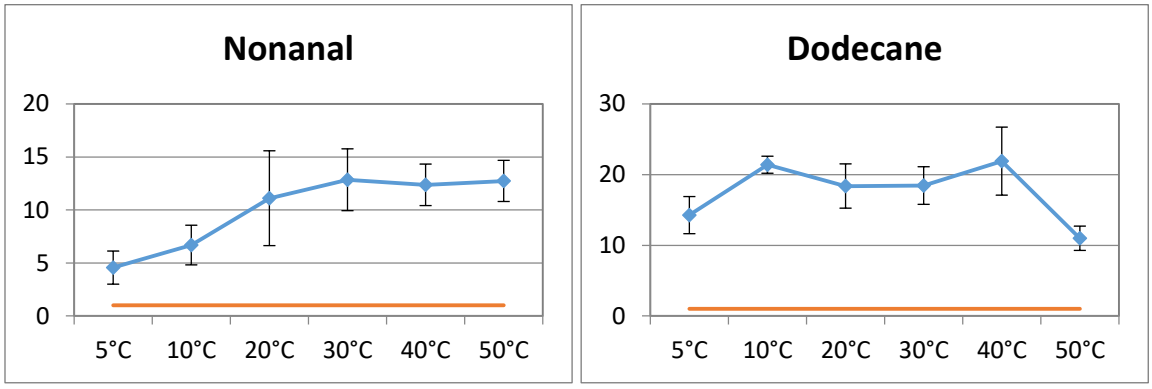

Figure 36: Ratio of the extraction of nonanal and dodecane under vacuum and normal condition over increasing temperatures (5 to 50°C)

Chapter 2: Fish spoilage

The second part of the project was the fish spoilage assessment. The fishes were kept in a regular fridge at 5°C up to five days and three replicates for each kind of fish were analyzed at 5 and 40°C under normal and vacuum conditions each day. The complete set of data can be found in annex 12 to 15 (12: Salmon, 13: Cod; 14: Pollock, 15: Redfish). Herein the discussion is focused on the comparison between 5°C under vacuum and 40°C under normal sampling conditions to emphasize the potentiality of VAC-HS-SPME. The trend of the different compounds will be discussed in the following part and one example for each trend is reported in the main text. Some data is missing due to technical problem with the autosampler. For time reason, it was not possible to repeat the experiments, therefore the final discussion will be mainly focused on the comparison between first (J0) and last (J4) day.

1. Salmon

Only two trends appear in the salmon degradation regarding the 18 selected compounds. No matter the trend followed, both conditions of extraction (5°C vacuum and 40°C normal) show the same profile (figure 30). Regarding the intensity, it depends on the compounds: 2-methyl-butanal, 2,4-dimethyl-heptane and heptadecane show very similar trends. 3-methyl-butanal, 2,6-dimethyl-heptanol and benzeneacetaldehyde are the only three compounds for which 40°C under normal conditions extracted better than 5°C with vacuum (figure 31). All other compounds are better extracted with the latter conditions.

The majority of volatiles decrease with time as shown on figure 37 (on the left). On the other hand, the two butanals, 2,4-dimethyl-heptane, 2,6-dimethyl-heptanol and benzeneacetaldehyde increase in the first days of storage (up to day 1 or 2) then decrease (figure 37, on the right). Octadecane was never detected with the method whereas it was when working on the optimization. This is probably due to the different sample handling: for the optimization, the fish was left for 24h on the lab bench in the middle of summer in order to maximize the intensity and variability of the volatile profile, whereas for the spoilage, the fish was directly put into vials and kept in the fridge.

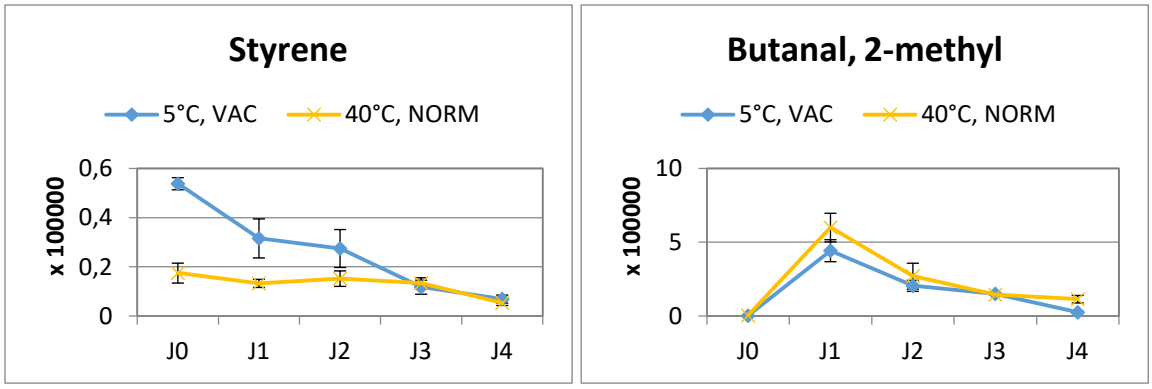

Figure 37: Evolution of Styrene and 2-methyl-butanal over 5 days of storage.

2. Cod

In cod, the trends followed by 5 and 40°C are very similar for a majority of compounds (figure 38). The extraction of decanal and BHT is improved with vacuum conditions and 5°C. Three main trends are found for the cod spoilage. 2-methyl-butanal and styrene decrease over time (figure 38, on the left). Similarly, 3-methyl-butanal, 2,4-dimethyl-heptane and 2,6-dimethyl-heptanol start to decrease in the first days but they show an increase between day 2 and 3 (figure 38, on the right).

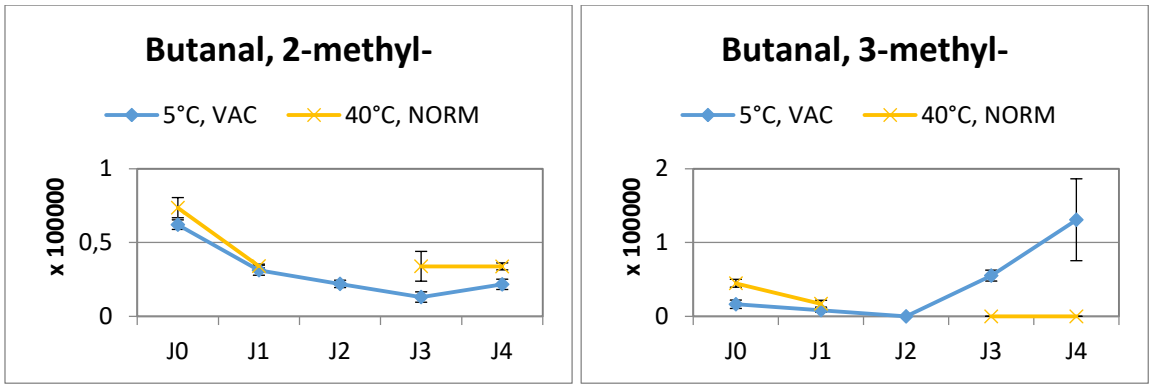

Figure 38: Evolution of 2-methyl-butanal and 3-methyl-butanal over 5 days of storage. (Missing samples due to technical problems)

The rest of the compounds kept a more constant profile over time (figure 39). The last three compounds (hexa-, hepta- and octadecane) were not detected in this sample.

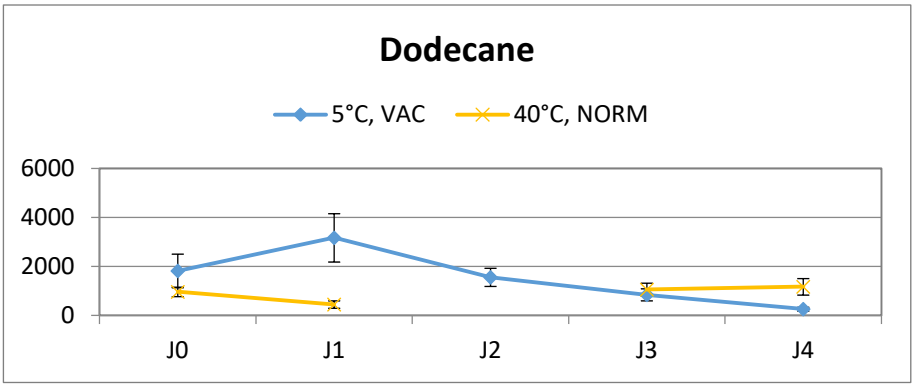

Figure 39: Evolution of dodecane over 5 days of storage. (Missing samples due to technical problems)

3. Pollock

In Pollock, compounds also follow three trends. Only 2-methyl-butanal is increasing over time (figure 40, on the left). Six compounds (3-methyl-butanal, 2,4-dimethyl-heptane, heptanal, 2,6-dimethyl-heptanol, benzeneacetaldehyde and tridecane) stay constant and eight (styrene, octanal, nonanal, dodecane, decanal, tetradecane, pentadecane and BHT) decrease (figure 40, in the middle and on the right respectively). Just as for cod, the last three volatiles are not detected.

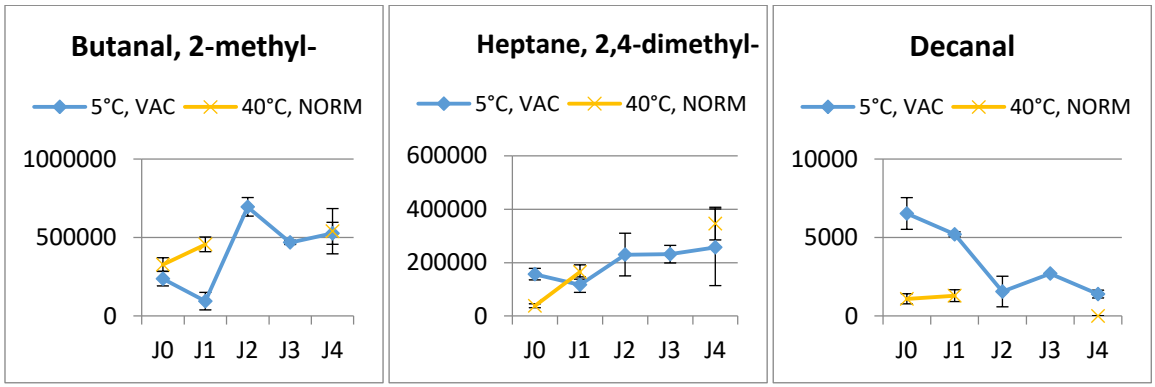

Figure 40: Evolution of 2-methyl-butanal, 2,4-dimethyl-heptane and decanal over 5 days of storage. (Missing samples due to technical problems)

Concerning the difference between 5 and 40°C, the first eight compounds extracted show similar trends. On the other hand, nonanal, dodecane, decanal, tridecane, tetradecane, pentadecane and BHT were extracted better at 5°C under vacuum conditions (figure 40, on the right).

4. Redfish

In the case of redfish, most of the compounds remained constant over the days tested. This behavior is followed by 3-methyl-butanal, styrene, 2,6-dimethyl-heptanol, benzeneacetaldehyde, tridecane and pentadecane (figure 41, on the left). The second trend is when compounds decrease over time as decanal, tetradecane, BHT, hexadecane and octadecane (figure 41, on the right). All these compounds, except BHT and octadecane, show similar extraction trends at 5°C under vacuum and 40°C with normal conditions. Concerning BHT and Octadecane, vacuum conditions extracted them better even at 5°C.

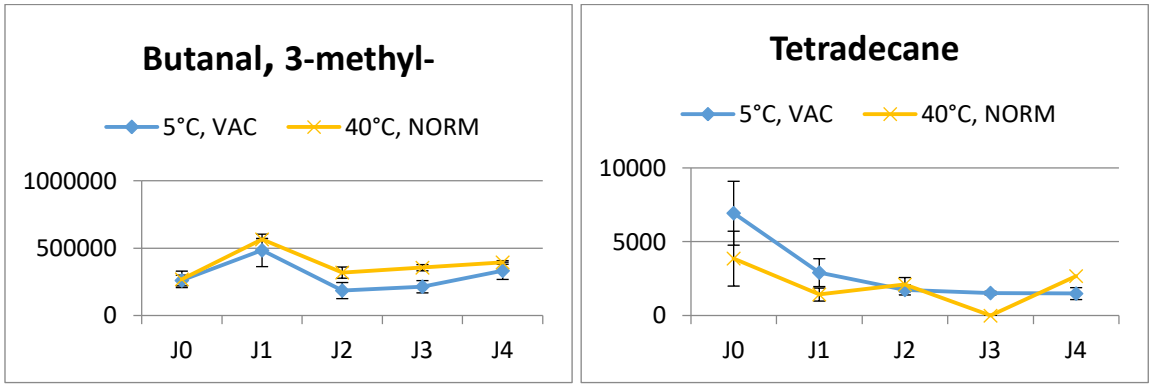

Figure 41: Evolution of 3-methyl-butanal and tetradecane over 5 days of storage.

2,4-dimethyl-heptane, octanal and dodecane show an increasing profile with time (figure 42, on the left). The trend at 40°C increases much faster than at 5°C. Considering that they are oxidation products, it is likely that the increase is getting faster with high temperature compared to real spoilage degradation. Therefore the results obtained at 5°C appear to be more reliable. Finally, heptanal is the only compound showing an increase in the first days then a drop after day 3 (figure 42, on the right).

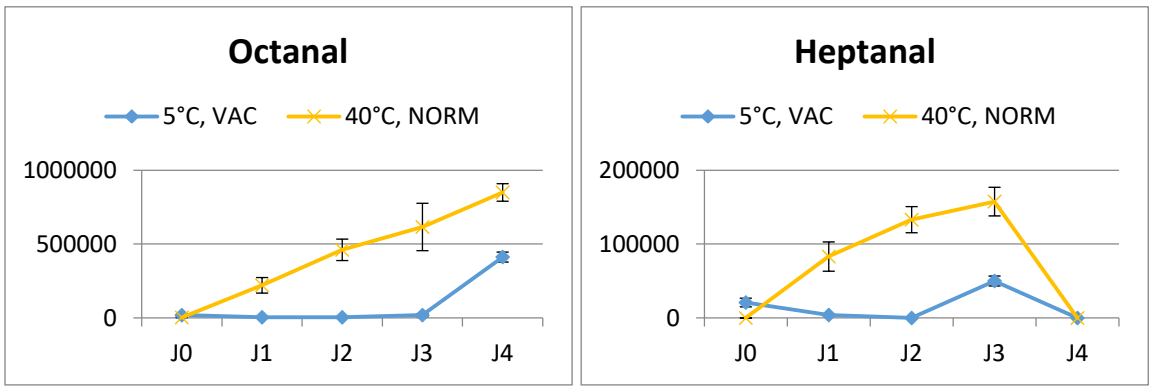

Figure 42: Evolution of octanal and heptanal over 5 days of storage.

5. Comparison

This part of discussion will focus on the comparison of 5°C with vacuum and 40°C with normal conditions. For each condition and each fish, a *t*-test was performed to see if the extracted amount of one compound on the first day (J0) was equal to the amount extracted on the last day (J4). Of course, some compounds were not extracted with some conditions, in this case it was considered "null". Table 6 presents the complete results obtained.

Table 6: Comparison of the extraction at 5°C under vacuum and at 40°C with normal conditions according to the amount extracted on day 0 and on day 4. +: increase, -:decrease, =: constant, 0: compound not detected.

	Salmon		Cod		Pollock		Redfish	
	5°C, Vac	40°C, Norm	5°C, Vac	40°C, Norm	5°C, Vac	40°C, Norm	5°C, Vac	40°C, Norm
Butanal, 2-methyl-	+	+	=	-	-	-	-	0
Butanal, 3-methyl-	=	+	-	-	+	=	=	=
Heptane, 2,4-dimethyl-	=	+	=	=	=	+	+	=
Styrene	-	-	-	-	-	-	=	=
Heptanal	-	0	0	0	0	-	-	-
2,6-Dimethyl-4-heptanol	=	+	=	-	=	-	=	=
Octanal	=	0	+	=	-	+	+	+
Benzeneacetaldehyde	+	+	0	0	0	-	-	=
Nonanal	-	0	0	=	-	=	-	+
Dodecane	=	0	=	=	-	+	=	=
Decanal	-	0	0	0	-	-	-	-
Tridecane	-	0	+	+	-	+	+	+
Tetradecane	-	0	0	=	=	+	-	=
Pentadecane	=	-	+	+	-	+	-	=
BHT	=	-	=	0	-	0	-	0
Hexadecane	-	0	0	0	-	+	-	-
Heptadecane	=	-	0	0	+	0	0	0
Octadecane	-	0	0	0	+	0	-	0

The first observation is that the results obtained at 5°C and 40°C, for the same fish, are not always the same. In fact, for salmon and Pollock, only three compounds have the same trend (2-methly-butanal, styrene and benzeneacetaldehyde for salmon, 2-methly-butanal, styrene and decanal for Pollock). Cod and redfish have more similar results: 12 compounds were extracted similarly with the two conditions for cod and 10 for redfish (table 6).

According to literature, 2-methyl-butanal is used as freshness marker as it is present when the fish is fresh but it increases over time (Duflos &al, 2006). However according to the results in table 2, only salmon shows an increase for this compound. The other species show a decrease with time. The presence of styrene, an aromatic hydrocarbon, could be due to plastic pollution in the ocean or to a transfer from the plastic packaging the fish was kept in.

Nonanal, the most cited compound in the literature was used as freshness marker as it is found both in very fresh fish and in spoiled fish (Duflos &al, 2010). However, in table 2, the results obtained at 5°C and 40°C are different for all four tested species. At 5°C with vacuum, it was found to decrease in all fishes whereas at 40°C the results are constant or increasing. Considering the efficiency of extraction at 5°C for other compounds, this discrepancy might be caused by artifacts formation due to the extraction temperature. Indeed, in the paper indicating nonanal as a marker, an extraction temperature of 50°C for 40 minutes was used. As nonanal derives from oxidation of oleic acid (Pratama &al, 2108), the conditions used may increase the rate of the reaction. Moreover after almost 1h (40 minutes of extraction + extra 10 minutes of incubation) at 50°C a fish cannot be considered fresh and raw anymore. 5°C under vacuum may be a method to avoid the production of such artifacts.

Special attention was brought on BHT as it presents a very particular trend. In cod, pollock and redfish it is present in smaller amount than in salmon (10x less for Pollock and 100x less for cod and redfish) and it is only extracted under vacuum in those species. Indeed, even at 40°C, normal conditions do not extract any BHT whereas with vacuum it was always detected. In salmon, the amount present is so high that all tested conditions (5 and 40°C with normal pressure and vacuum) could extract it. This brings new interest for the VAC-HS-SPME as it may help extracting trace compounds. Therefore, it may be applied in the field of food contaminants and additives.

Chapter 3: Perspectives and conclusion

The first part of this work focuses on the study of different parameters, namely fiber type, temperature and time, for the analysis of raw fish samples under normal and reduced pressure conditions. 18 compounds were selected for this purpose. From the fiber comparison, CAR/PDMS/DVB was selected as it was the best performing over the selected compounds and the one that was improved the most with vacuum. Different temperature and time profiles were investigated (5, 10, 20, 30, 40 and 50 °C with 30 of extraction and 10, 20, 30, 40 and 60 minutes of extraction at 5, 30 and 40°C). 5°C and 40°C under normal and vacuum conditions were selected and used during the fish spoilage assessment. Their results were compared in the second part of the thesis.

When comparing 5°C with vacuum conditions to 40°C with normal conditions, two trends appeared: the first one was the extraction being improved for many compounds at 5°C. The improvement was the best for compounds that have middle range volatility. The second trend was followed by very high or low volatiles that were better extracted with normal conditions and high temperature. It is possible that the matrix (in this case a solid with high content of fat and proteins) plays a significant role in the behavior towards the improvement brought by vacuum. However further work should be conducted in this area with similar matrix (for example other fish species or food products such as different meat) to bring more data and thus formulate a theory.

Another interesting result is the behavior of some compounds towards vacuum. For example, BHT followed a very particular trend: when it was present as trace in the sample, it could only be detected when it was extracted with vacuum. This proves that VAC-HS-SPME can also be used for trace analysis and is more efficient than regular HS-SPME.

Concerning fish species and their spoilage, the results presented in this thesis are only a start in order to obtain a comprehensive list of markers. For the work presented, only the 18 compounds, selected for the optimization, were followed. Some trends could already be highlighted however fishes present very complex aroma profiles and untargeted analysis should be considered to have a more complete view of all compounds. Two goals could be achieved with a complete profile: freshness and spoilage markers as well as species specific markers could be highlighted. This, in turn could be used as quality control. Nonetheless, the extraction at 5°C under vacuum gave similar or better results than 40°C and normal

conditions. So in addition of using milder conditions that do not produce artifacts, the extraction yield was better. Moreover, the compounds found in the literature were mostly extracted with high temperature (40-60°C) and long extraction time. It would be very interesting to work on the same fishes, at lower temperature and vacuum conditions to check if the trends are similar or if some compounds were artifacts.

In a practical aspect, on the chromatograms some compounds were co-eluting, affecting sometimes the integration repeatability. Therefore it would be interesting to couple the VAC-HS-SPME with a stronger separating instrument such as GC×GC. Concerning the fibers, they reacted differently to vacuum and more work could be done to further understand how vacuum affects the extraction. Finally, to be used regularly and to prove that the results are reliable, the method needs to be validated.

In conclusion, VAC-HS-SPME is a strong method for volatile profiling. There is much work to be done to fully understand the theory that lies behind the experiments and it is likely that in the near future many more improvements will be discussed. Moreover VAC-HS-SPME keeps all the advantages of SPME: ease of use, no need for solvents and fast analysis. For all these reasons, it is highly likely that VAC-HS-SPME will be more and more used in different fields.

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