Vacuum-assisted headspace-solid phase microextraction for determining volatile free fatty acids and phenols. Investigations on the effect of pressure on competitive adsorption phenomena in a multicomponent system

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Abstract

This work proposes a new vacuum headspace solid-phase microextraction (Vac-HSSPME) method combined to gas chromatography-flame ionization detection for the determination of free fatty acids (FFAs) and phenols. All target analytes of the multicomponent solution were volatiles but their low Henry's Law constants rendered them amenable to Vac-HSSPME. The ability of a new and easy to construct Vac-HSSPME sampler to maintain low-pressure conditions for extended sampling times was concurrently demonstrated. Vac-HSSPME and regular HSSPME methods were independently optimized and the results were compared at all times. The performances of four commercial SPME fibers and two polymeric ionic liquid (PIL)-based SPME fibers were evaluated and the best overall results were obtained with the adsorbent-type CAR/PDMS fiber. For the concentrations used here, competitive displacement became more intense for the smaller and more volatile analytes of the multi-component solution when lowering the sampling pressure. The extraction time profiles showed that Vac-HSSPME had a dramatic positive effect on extraction kinetics. The local maxima of adsorbed analytes recorded with Vac-HSSPME occurred faster, but were always lower than that with regular HSSPME due to the faster analyte-loading from the multicomponent solution. Increasing the sampling temperature during Vac-HSSPME reduced the extraction efficiency of smaller analytes due to the enhancement in water molecule collisions with the fiber. This effect was not recorded for the larger phenolic compounds. Based on the optimum values selected, Vac-HSSPME required a shorter extraction time and milder sampling conditions than regular HSSPME: 20 min and 35 ºC for Vac-HSSPME *versus* 40 min and 45 ºC for regular HSSPME. The performance of the optimized Vac-HSSPME and regular HSSPME procedures were assessed and Vac-HSSPME method proved to be more sensitive, with lower limits of detection (from 0.14 to 13 $\mu g \cdot L^{-1}$), and better intra-day precision (relative standard deviations values <10% at the lowest spiked level) than regular HSSPME for almost all target analytes. The proposed Vac-HSSPME method was successfully applied to quantify FFAs and phenols in milk and milk derivatives samples.

Keywords: headspace solid-phase microextraction, vacuum-assisted headspace solid-phase microextraction, gas chromatography, volatile free fatty acids, phenols, milk samples.

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1. Introduction

A number of methods exist for the analysis of food aroma, typically involving a preconcentration step prior to gas chromatographic (GC) analysis [1,2]. Headspace solid-phase microextraction (HSSPME) is currently the method of choice, having the inherent advantages of eliminating organic solvent consumption, combining extraction and preconcentration into one step and high enrichment factors [3-5]. SPME sampling from the headspace above the sample is a multi-stage process with analytes partitioning between the sample, headspace and fiber [6]. Volatile analytes typically possess high Henry's Law constant (K_H) values and are extracted faster than less volatile analytes or compounds with lower K_H values, since transfer of the latter from the sample into the headspace is a rate-limiting step [7,8]. The extraction kinetics for these compounds can be accelerated by applying agitation, ensuring a large sample/headspace interface [8,9], increasing the sampling temperature [10,11], adopting the cold fiber HSSPME approach [12,13], or lowering the sampling pressure.

The positive effect of low-pressure conditions on HSSPME extraction kinetics was first reported for the extraction of headspace volatiles from aqueous standards and raw turkey meat homogenates [14]. In 2012, aqueous samples were introduced for the first time into pre-evacuated sample containers followed by HSSPME sampling and the resulting method was termed vacuum-assisted HSSPME (Vac-HSSPME). According to the theory, non-equilibrium Vac-HSSPME can be particularly beneficial for compounds with a K_H value close or below the reported threshold value for low K_H compounds, since mass transfer resistance for these analytes is concentrated in the gas-phase [7]. At equilibrium, the theory predicts that Vac-HSSPME will behave similarly to regular HSSPME, since the final amount of analyte extracted by the fiber is not affected by the sampling pressure conditions [7]. The majority of past Vac-HSSPME investigations focused on the extraction of semi-volatile compounds with

low K_H values where lowering the sampling pressure was typically reported to yield high extraction efficiencies and very good sensitivities within short sampling times and at mild sampling temperatures compared to regular non-equilibrium HSSPME sampling [7,8,15-20]. Volatile analytes were also studied in the past, though the results were not related to the K_H values of the target analytes [14,21]. In these studies, HSSPME sampling at low pressure conditions yielded a notable improvement in HSSPME sensitivity and, depending on the matrix, increased the number of identified matrix components [14,21].

For Vac-HSSPME, ensuring constant low-pressure conditions throughout sampling is critical. To this end, considerable research efforts have been placed on designing and testing new gas-tight samplers ranging in size and number of available gas-tight ports [8,15-17,21]. Recently, we proposed a new experimental setup consisting of a specially designed O-ring seal screw cap offering gas-tight seal to commercial headspace vials. With this sampler, all operations were performed through a single port (air evacuation of the sampler, sample introduction and HSSPME sampling) [17]. Here, we report an alternative version of this device that maintains all key-features but is easier to construct since it does not require molding by a manufacturer. The device consists of a commercial crimp-top glass vial and a crimp-top Mininert® valve that was in-house modified to overcome the problems of high leak rates under low pressure conditions we reported in the past for the original valve [17].

A new Vac-HSSPME method combined to GC-flame ionization detection (GC-FID) is proposed here and used as a tool for milk (and milk derivatives) analysis. The aroma of milk and milk derivatives is the result of the presence of a high number of volatile and semi-volatile compounds. Among them, short- and medium-chain free fatty acids (FFAs) are important compounds of the volatile fraction. They are primarily formed in milk and dairy products by the enzymatic breakdown of glycerides and have strong sensory properties rendering them important compounds in the flavor and aroma [22]. Moreover, short-chain

FFAs act as precursors of other important aroma components such as methylketones, alcohols, phenols, aldehydes, and esters [1,22]. Important sources of these compounds are milks with a high fat content, such as goat milk [22]. Phenols are another interesting group of compounds, with recognized antioxidant activity [23]. They consist of thermal degradation or microbial decomposition products of phenolic acids detected in food and contribute to their flavor [2]. The multicomponent mixture considered in this study consisted of seven FFAs and four phenols, most of which were previously monitored by regular HSSPME in cheese-related studies [11,22]. All target phenols were also monitored in milk and dairy products [11,24].

A comprehensive comparative study between Vac-HSSPME and regular HSSPME was carried out. The ability of the new Vac-HSSPME sampler to maintain low-pressure conditions for extended sampling times was concurrently demonstrated. The performance of four commercial SPME fibers and two polymeric ionic liquid (PIL)-based SPME fibers was evaluated. Different experimental parameters (salt content, GC desorption time, extraction time and temperature) were also controlled and optimized under each pressure condition. The results were compared and discussed on the basis of previous theoretical and experimental findings, thereby advancing existing fundamental knowledge related to SPME. Finally, the performance of the optimized Vac-HSSPME and regular HSSPME methods were assessed and real milk and milk derivative samples were analyzed with Vac-HSSPME to evaluate the applicability of the method.

2. Experimental

2.1. Chemicals, materials, and samples

Guaiacol (2-methoxyphenol, 2-MOP, 98.0%) and eugenol (4-allyl-2-methoxyphenol, 4-Al-2-MOP, 99.0%) were supplied by Fluka (Buchs, Switzerland). Phenol (Ph, 99.0%) was purchased from Merck (Darmstadt, Germany) and 2-ethylphenol (2-EP, 99.5%) was obtained from Dr. Ehrenstorfer GmbH (Ausburg, Germany). Individual solutions of phenols at 2000 $mg \cdot L^{-1}$ in acetonitrile (≥ 99.9%) were supplied by VWR International Eurolab S.L. (Barcelona, Spain).

The standard mix ref. 46975-U of volatile FFAs was obtained from Supelco (Bellefonte, PA, USA) and contained 10 $mmol \cdot L^{-1}$ of *iso*-butyric acid (*i*-C_4), *n*-butyric acid (*n*-C_4), *iso*-valeric acid (*i*-C_5), *n*-valeric acid (*n*-C_5), *iso*-hexanoic acid (*i*-C_6), *n*-hexanoic acid (*n*-C_6), and *n*-heptanoic acid (*n*-C_7) in water. Three intermediate solutions were prepared for extractions: an aqueous solution of FFAs each at a concentration of 1.2 $mmol \cdot L^{-1}$, obtained by dilution of the standard mix of FFAs; and two intermediate solutions containing each phenol (2-MOP, Ph, 2-EP and 4-Al-2-MOP) at a concentration level of 300 $mg \cdot L^{-1}$ and 10 $mg \cdot L^{-1}$ respectively, obtained by dilution of the individual solutions in acetonitrile. All solutions were stored at 4 ºC. All aqueous solutions used for extraction were prepared by dilution of the intermediate solutions in ultrapure water, keeping the total content of acetonitrile at 0.33 % (v/v). Ultrapure water (18.2 MΩ·cm) was obtained from a Milli-Q water purification system (Millipore, Watford, UK). NaCl (≥99.5%) and acetonitrile (≥99%) were obtained from Sigma-Aldrich (Steinheim, Germany). During method optimization all experiments were carried out using aqueous solutions at a concentration level of 0.022 $mmol \cdot L^{-1}$ for each FFA (*i.e.* 0.19 $mg \cdot L^{-1}$ for *i*-C_4 and *n*-C_4, 0.22 $mg \cdot L^{-1}$ for *i*-C_5 and *n*-C_5, 0.26 $mg \cdot L^{-1}$ for *n*-C_6, and 0.29 $mg \cdot L^{-1}$ for *n*-C_7) and 1 $mg \cdot L^{-1}$ for each phenol. The calibration curves of HSSPME-GC-FID and Vac-HSSPME-GC-FID methods were obtained using aqueous solutions with concentrations ranging between 0.005 to 14 $mg \cdot L^{-1}$ for the analytes tested.

Four commercial SPME fibers and two PIL-based SPME fibers were used here. The commercial SPME fibers of 1 cm of length were: carboxen/polydimethylsiloxane (CAR/PDMS, 75 µm of film thickness), divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm of film thickness), polydimethylsiloxane (PDMS, 100 µm of film thickness) and polyacrylate (PA, 85 µm of film thickness). They were obtained from Supelco. Two different crosslinked PIL-based SPME fibers of 1.3 cm of length, termed as PIL-1 and PIL-2, were prepared. The crosslinked PILs were covalently attached to derivatized nickel-titanium wires using the spin-coating method proposed by Cagliero *et al.* [25] and included onto the Supelco assembly. PIL-1 (~40 µm of film thickness) was composed by the IL monomer 1-hexadecyl-3-vinylimidazolium bis[(trifluoromethyl)sulfonyl)]imide (C_{16}Vim-NTf_2) and the dicationic IL crosslinker 1,12-di(3-vinylimidazolium)dodecane bis[(trifluoromethyl)sulfonyl)]imide ($(ViIm)_2C_{12}$–$2NTf_2$), whereas PIL-2 (~35 µm of film thickness) was developed using the IL monomer 1-hexadecyl-3-vinylbenzylimidazolium bis[(trifluoromethyl)sulfonyl)]imide (C_{16}VBim-NTf_2) and the dicationic IL crosslinker 1,12-di(3-vinylbenzylimidazolium)dodecane bis[(trifluoromethyl)sulfonyl)]imide ($(ViBIm)_2C_{12}$–$2NTf_2$). All IL monomers and crosslinkers were synthetized an d fully characterized following previous procedures [10,26-29].

The raw goat milk sample was obtained from a local farmer in Tenerife (Spain) and was stored at 4 ºC. A liquid yogurt (with fruits flavor) was obtained at a local supermarket (Tenerife) and was also stored at 4 ºC until analysis. Prior extraction, the salinity of each sample was measured using a CM Crison conductimeter from Crison Instruments SA (Barcelona, Spain). Assuming that the salt content in the samples was due to the presence of NaCl, the total content of salt on both samples was then adjusted to 25% (w/v) by dissolving the appropriate amount of NaCl. The organic content was also adjusted to 0.33% (v/v) by adding acetonitrile, so as to mimic the conditions utilized with the calibration curves.

2.2. Modification of the crimp-top Mininert® valve

Figure 1 shows in detail the new device consisting of an in-house modified crimp-top Mininert® valve (Sigma-Aldrich) and a 10 mL crimp-top glass vial. The Mininert® valve was modified at the Laboratory of Aquatic Chemistry, School of Environmental Engineering, Technical University of Crete and the top part (push/pull button and septum) of the crimp-top Mininert® valve was removed and a hole was drilled that could tightly accommodate a cylindrical Thermogreen® LB-1 septum with half-hole (6 mm diameter × 9 mm length; Supelco). To ensure leak-tight seal to the vial, an O-ring with a 10 mm internal diameter was also fitted in the lower part of the modified valve.

2.3. Vac-HSSPME and regular HSSPME procedures

For Vac-HSSPME, the air inside the sampling device containing a magnetic polytetrafluoroethylene (PTFE) stir bar (15 mm × 4.5 mm; Sigma-Aldrich) was evacuated prior introducing the aqueous sample. To achieve this, the barrel of a 5 mL plastic medical syringe equipped with a detachable 22 gauge hypodermic needle was tightly secured to the tubing of a VP 2 Autovac pumping unit with vacuum and speed control (7 mbar ultimate vacuum without gas ballast) manufactured by Vacuubrand (Wertheim, Germany). The needle was then inserted through the septum and the device was air-evacuated for 1.5 min. A 5 mL of aqueous sample containing target analytes and a set amount of NaCl was introduced in the device through the septum using the gastight syringe and the sampler was then placed on a magnetic stir plate (RCT basic IKA-Werke, Staufen, Germany) and a Selecta P heating bath (Barcelona, Spain). The needle of the SPME fiber/holder assembly (Supelco) was introduced

into the device by piercing the septum and HSSPME sampling was performed for a preset period of time and temperature and under a 400 rpm agitation speed. The fiber was then retracted and transferred for thermal desorption to a GC-FID. The sample container was emptied and cleaned after piercing the septum with a disposable syringe needle so as to equilibrate the pressure with atmospheric. The septum was changed after 5 extractions to prevent losses of vacuum inside the vial due to successive piercings. Each experiment was repeated in triplicate (n = 3).

The Vac-HSSPME procedure under optimum conditions for milk and milk derivatives requires the use of the CAR/PDMS fiber, 25% (w/v) of NaCl, 20 min of extraction at 35 ºC, and 2 min GC desorption time at 280 ºC.

For regular HSSPME, sampling was performed using the 10 mL vials and caps and PTFE/butyl septa supplied by Supelco. The procedure was similar to that used for Vac-HSSPME, the only difference being that the step of air evacuation was omitted. Each experiment was repeated in triplicate (n = 3).

The HSSPME procedure under optimum conditions requires the use of the CAR/PDMS fiber, 25% (w/v) of NaCl, 40 min of extraction at 45 ºC, and 2 min GC desorption time at 280 ºC.

2.4. GC-FID analysis

A Varian 450 model CP-3800 GC-FID was used. The separation was achieved using a CP-FFAP CB column (25 m × 0.32 mm I.D., 0.30 μm of film thickness) from Agilent Technologies (Amstelveen, Holland). A flow rate of 1 $mL \cdot min^{-1}$ of nitrogen (carrier gas) was required for the separation of the analytes. The experiments were performed under *splitless* injection mode, using a straight tapered 0.75 mm ultra-inert inlet liner from Agilent

Technologies. Different desorption temperatures were tested to prevent decomposition of the SPME fibers in the injector and 280 ºC was the selected desorption temperature for CAR/PDMS, PDMS and PA, 270 ºC for DVB/CAR/PDMS, and 250 ºC for PIL-based SPME fibers (PIL-1 and PIL-2). The GC oven temperature program was the following: initially isothermal for 2 min at 40 ºC, then increased at 20 ºC·min^{-1} up to 100 ºC, holding this temperature during 20 min, then increased at 5 ºC·min^{-1} up to 165 ºC, and finally increased at 25 ºC·min^{-1} up to 240 ºC, holding this temperature during 2 min. The temperature of the FID was kept at 280 ºC, using an air flow of 300 mL·min^{-1}, a make-up flow of nitrogen of 30 mL·min^{-1}, and a hydrogen flow of 30 mL·min^{-1}.

3. Results and discussion

The main physico-chemical properties of the seven FFAs and four phenols considered here are given in Table ESM-1 of the electronic supplementary material (ESM). The high vapor pressures combined with the high water solubility values resulted in K_H values below the threshold for low K_H compounds (~1.2·10^{-5} atm m^3 mol^{-1}). Accordingly, air evacuating the sample container prior to HSSPME was expected to improve evaporation rates and accelerate non-equilibrium HSSPME sampling when compared to regular HSSPME.

3.1. Optimization of Vac-HSSPME and regular HSSPME procedures

The first objective of the present study was to optimize HSSPME under vacuum and regular pressure conditions using a factor-by-factor approach. All optimization studies were performed using aqueous solutions because of the aqueous nature of milk and milk derivatives. The results obtained during optimization studies were also used to discuss and

evaluate the effect of pressure on competitive adsorption phenomena in a multicomponent system when sampling with porous SPME fibers.

3.1.1. Vac-HSSPME and regular HSSPME sampling using commercial and PIL-based SPME fibers

Four commercial coatings (CAR/PDMS, DVB/CAR/PDMS, PDMS and PA) and two PIL-based SPME fibers (PIL-1 and PIL-2) were evaluated for Vac-HSSPME and regular HSSPME sampling of multicomponent solutions. At this evaluation stage, extraction time was set at 30 min and sampling temperature at 40 ºC to ensure that a sufficient amount of analytes would be extracted under each pressure condition. A 25% NaCl (w/v) content was also selected to favor transfer of the analytes from the sample solution to the headspace and a 6 min-long GC desorption time was applied to prevent *carry-over* effects.

Figure ESM-1 of the ESM shows the extraction efficiencies of each fiber obtained with Vac- and regular HSSPME, expressed as chromatographic analyte peak areas. As seen, reducing the sampling pressure generally improved extraction kinetics and the best overall results were obtained with Vac-HSSPME sampling whilst using the CAR/PDMS fiber.

A more detailed analysis of the results shows that under each pressure condition, CAR/PDMS and DVB/CAR/PDMS yielded the most intense signals compared to the rest of the fibers, confirming the strong adsorbing ability of Carboxen porous material [14,30-32]. A comparison of the Vac-HSSPME/HSSPME peak area ratios obtained with CAR/PDMS or DVB/CAR/PDMS (Table 1), showed that for each fiber, reducing the sampling pressure improved the extraction of the less volatile analytes and the best improvement was recorded for 4-Al-2-MOP, the least volatile analyte tested here. For the more volatile *i*-C_4, *n*-C_4 and *i*-

C_5, lowering the total pressure in the headspace did not improve extraction when sampling with CAR/PDMS, as Vac-HSSPME/HSSPME peak area ratios were close to 1 (Table 1). In the case of the DVB/CAR/PDMS coating, these peak area ratios were below 1 indicating decreased mass loading under vacuum conditions (Table 1). In one of our previous reports [1], *i*-C_4, *n*-C_4 and *i*-C_5 reached an equilibrium level during HSSPME sampling with CAR/PDMS faster than *n*-C_5 and longer chain FFAs. We also reported that prolonged sampling times resulted in decreased extraction efficiencies for these three analytes [1], pointing towards competitive adsorption and displacement [10]. The design of DVB/CAR/PDMS allows larger analytes to interact with the outer DVB layer and small analytes to diffuse relatively fast through the DVB layer so as to reach the inner Carboxen layer [31,32]. Assuming Carboxen as the predominant adsorptive phase for these three small analytes, saturation and consequent inter-analyte competitive adsorption from the Carboxen coating is likely to occur faster in DVB/CAR/PDMS than in CAR/PDMS due to the smaller Carboxen phase thickness (30 and 75 μm coating thickness respectively) and as such less active sites available for adsorption [32]. Here, reducing the sampling pressure accelerated the extraction kinetics of *i*-C_4, *n*-C_4 and *i*-C_5 and enhanced their headspace concentrations. Therefore, it was assumed that saturation and consequent analyte displacement in the DVB/CAR/PDMS fiber occurred faster under vacuum compared to atmospheric pressure conditions, thus explaining the relative decrease in mass loading upon reducing the sampling pressure. The assumption of competitive displacement and the ability of smaller FFAs to reach fast an equilibrium level on a CAR/PDMS layer under each pressure condition will be further supported and discussed in a latter section dealing with the effect of extraction time on Vac- and regular HSSPME sampling.

Figure ESM-1 of the ESM also shows that the rest of the fibers tested here exhibited low extraction efficiencies under both pressure conditions. The commercially available PA and

PDMS fibers are absorbent-type phases where non-competitive partitioning is the extraction mechanism, as opposed to the competitive extraction observed in the proximity of saturation conditions for adsorbent coatings [30,34]. Moreover, a previous report investigating the extraction mechanism of various classes of PIL-based coatings concluded that analytes are extracted via a non-competitive partitioning mechanism regardless of the extent of crosslinking [35]. The beneficial effect of reduced pressure conditions on pre-equilibrium HSSPME sampling of low K_H compounds using commercial absorbent-type phases has been demonstrated in the past [7,8,15]. In addition, the theoretical prediction that the final amount of analyte extracted at equilibrium by the SPME fiber is not affected by the sampling pressure has been experimentally verified in all cases where the fiber acted as a zero sink for target analytes [15].

A closer investigation of the effect of pressure on the absorbent-type phases (Table 1) showed that for the moderately polar PA, the presence of an air-evacuated headspace yielded enhancements up to 1.8 times compared to atmospheric pressure (for 30 min extractions at 40 ºC and 400 rpm). The only exception was *n*-C_4 where the Vac-HSSPME/HSSPME peak area ratio was close to 1 presumably because this analyte was close to equilibrium under each pressure condition. As expected [30,36], the PA absorbent coating improved extraction under each pressure condition compared to the non-polar PDMS phase which exhibited the lowest affinity for all target analytes compared to the rest of tested fibers (Figure ESM-1 of the ESM). For the latter, there was also no enhancement in extraction efficiencies upon reducing the sampling pressure, possibly indicating that 30 min of sampling at 40 ºC and 400 rpm were sufficient to attain equilibrium conditions under each pressure condition (Table 1). The extraction efficiencies obtained with PIL-1 fiber were found higher than those obtained with PIL-2 and PDMS. Under each pressure condition, PIL-1 was also found to improve the extraction of most target FFAs when compared to PA. Vac-HSSPME sampling using the PIL-

based SPME fibers only improved the extraction of 4-Al-2-MOP. For the rest, Vac-HSSPME/HSSPME peak area ratios were close to 1 for a 30 min sampling time and in some extreme cases (*i*-C_4, *n*-C_4, *i*-C_5 and *n*-C_5) these ratios were below 1 (Table 1). Increasing the sampling temperature during Vac-HSSPME analysis of water samples was reported to gradually decrease mass loading of analytes for commercial absorbent-type fibers SPME fibers due to the increased humidity in the headspace. At the same time, higher extraction temperatures were previously reported to decrease extraction efficiencies of PIL-based SPME fibers during regular HSSPME [11]. Although the present findings point towards an adverse effect of humidity on the characteristics of the PIL-based SPME fibers tested here when sampling under a low sampling pressure, the limited experimental data available did not permit an absolute conclusion to be made. This line of inquiry is out of the scope of the present work; however, it is acknowledged that the observed phenomenon merits more investigations.

Based on the results obtained here, it was decided to use the CAR/PDMS fiber for both Vac- and regular HSSPME.

3.1.2. Effect of salt and GC desorption time on Vac-HSSPME and regular HSSPME

Adding salt into the aqueous sample was previously reported to increase the amount of target analytes extracted with regular HSSPME due to the salting out effect [1,37,38]. Here, the effect of salt on Vac- and regular HSSPME sampling was investigated within the concentration range 0–25% w/v NaCl. The results, depicted in Figure ESM-2 of the ESM, showed that adding salt had a positive effect on the extraction of almost all target analytes under each sampling pressure condition. The only exception was *i*-C_4 where an unexpected and important decrease in signal was recorded with Vac-HSSPME when increasing the ionic

strength of the aqueous solution (Figure ESM-2(B) of the ESM). It was assumed that this signal decrease was the result of competitive displacement by the increased amount(s) of other analyte(s) present in the multicomponent mixture having a higher affinity for the adsorbent. Such competition effects were expected to be more intense during Vac-HSSPME, since saturation and competitive displacement for *i*-C_4 was expected to occur faster due to the substantial increase of analytes' headspace concentrations in the presence of an air-evaluated headspace and high salt solutions. This is the first time a negative effect of salt is reported for the HSSPME sampling of *i*-C_4. In all previous FFA studies, phenols were not included [1,38], highlighting the importance of studying multicomponent solutions when using an adsorbent-type coating phase.

A side point to note is that a signal decrease with increased salt content was not recorded for *n*-C_4, demonstrating that molecular mass alone is insufficient to explain the ability of an adsorbent to retain analytes. It has been suggested that the strength of an adsorbent to retain analytes is irreversibly proportional to analyte size, and branching in *i*-C_4 results into a smaller size as well as hinders interactions with the adsorbent phase when compared to *n*-C_4 [30]. Another report, suggested that next to molecular weight, vapor pressure could also be considered to explain the competitive extraction by the CAR/PDMS fiber [34] and, to this end, *i*-C_4 has a lower vapor pressure than *n*-C_4 (Table ESM-1 of the ESM). Overall, based on the results obtained here, the optimum salt content value was set at 25% (w/v) NaCl for both pressure conditions.

Next, the GC desorption time was evaluated within the range 2–6 min and the results are given in Figure ESM-3 of the ESM. As seen, 2 min were sufficient for desorbing all target analytes extracted with Vac- or regular HSSPME sampling. Longer desorption times did not result in significant changes in extraction efficiency. Moreover, *carry over* effects were not

recorded with 2 min of desorption. Thus, this value was selected as optimum for both Vac- and regular HSSPME.

3.1.3. Effect of extraction time on Vac-HSSPME and regular HSSPME

Figure 2 shows three typical extraction time profiles (*n*-C_5, *n*-C_7 and 4-Al-2-MOP) obtained with Vac- and regular HSSPME. The extraction time profiles of the rest of the target analytes can be found in Figure ESM-4 of the ESM. At all times, the sampling temperature was set at 45 °C to enhance mass transfer and investigate in more depth analyte behavior in multicomponent solutions. The results showed that with Vac-HSSPME the presence of an air-evacuated headspace had a dramatic effect on extraction kinetics compared to regular HSSPME as all analytes reached an equilibrium level within the sampling times tested. In particular, with Vac-HSSPME, uptake of most FFAs increased with sampling time before reaching a maximum at 20 min. For *n*-C_7, 40 min were required to attain equilibrium level, which was somewhat expected based on the physico-chemical properties of this analyte. At the same time, almost all phenols reached a maximum within approximately 40 min of sampling. The only exception was the least volatile and more hydrophobic 4-Al-2-MOP where approximately 80 min were needed. For regular HSSPME, longer sampling times were necessary to attain, whenever possible, an equilibrium level. In particular, *i*-C_4 and *i*-C_5 reached a maximum after sampling the headspace for 40 min. The rest of FFAs reached an equilibrium level at 60 min or even 80 min in the case of *n*-C_6. The only exception was *n*-C_7 where the amount of extracted analyte continued to increase with increasing sampling times. In the case of phenolic compounds, regular HSSPME sampling times as long as 100 min were not sufficient for reaching an equilibrium level.

In accordance with our previous report [1], a decrease in adsorbed analyte with time was recorded after reaching an adsorption maximum, indicating that competition and displacement of low molecular weight analytes took place. This observation was more obvious with Vac-HSSPME since all target analytes reached a maximum fast, leaving enough time within the time frame tested to record signal decreases. An important point to note here is that for those analytes that reached an equilibrium level under both pressure conditions within the time frame tested (*i*-C_4, *n*-C_4, *i*-C_5, *n*-C_5, *i*-C_6, and *n*-C_6), the local maximum of adsorbed analyte with Vac-HSSPME occurred faster but was always lower than that with regular HSSPME. This indicates that the adsorption process is even more competitive under vacuum conditions and lower amounts of these analytes are adsorbed because loading of the rest becomes also fast. A similar observation was reported in the past whilst investigating the effect of air velocity during air sampling with porous SPME fibers [33]. In this study, the extraction time profiles of toluene at different air velocities showed a local maximum that occurred faster and was lower when increasing air velocity. The authors concluded that at higher air velocities, the thickness of the boundary layer was smaller resulting in an adsorption process that was even more competitive due to the faster loading of other analytes present in the gas mixture. The present findings extend the existing fundamental knowledge related to sampling/sample preparation with SPME and corroborate the use of short sampling times with porous SPME coatings when sampling under reduced pressure conditions so as to minimize the effect of competitive adsorption. Moreover, they provide further evidence that conferring about reaching equilibrium is not appropriate when using Carboxen fibers [39].

Based on the results obtained here, a 20 min sampling was selected as the optimum extraction time for Vac-HSSPME and 40 min for regular HSSPME with the purpose to reduce the extraction time.

3.1.4. Effect of sampling temperature

Sampling temperature was recognized as one of the most important parameters in regular HSSPME sampling with liquid or porous solid SPME coatings [33]. Hitherto, the positive effects of pressure and temperature on Vac-HSSPME could be combined only when sampling dry soil sample *i.e*. in the absence of water [20]. For Vac-HSSPME, sampling of aqueous samples or solid samples containing water as a modifier at higher extraction temperatures was found to change the fiber's characteristics and decrease extraction efficiencies. The adverse effect of temperature on Vac-HSSPME was recorded whilst using different liquid and porous SPME coatings (other than CAR/PDMS) for the extraction of semi-volatile analytes [15,17,18,20], though the effect was more pronounced when using absorbent- rather than adsorbent-type SPME fibers [17].

Here, the effect of sampling temperature on Vac-HSSPME and regular HSSPME was investigated using the optimum extraction time found for each pressure condition. The results, given in Figure 3, revealed that with the exception of *i*-C_4, the extraction efficiencies of FFAs increased when heating the sample up to an optimum temperature and then signals decreased for a further increase in temperature. The optimum temperatures recorded were 25 ºC for *i*-C_4, *i*-C_5 and ~30 ºC for *n*-C_4, 35 ºC for *n*-C_5 and *i*-C_6 and 45 ºC for *n*-C_6 and ~45 ºC for *i*-C_7. For regular HSSPME (Figure 3) maximum extraction efficiencies for free fatty acids were obtained at a higher sampling temperature, namely 35 ºC for *i*-C_4 and 45 ºC for the rest.

Increasing the temperature increases the vapor pressure of water and as such the amount of water present in the headspace. To this end, water molecules were previously reported to compete with other volatile molecules and occupy a portion of active sites on the adsorbent coating surface, leaving fewer sites available for volatiles to adsorb [33]. During Vac-HSSPME sampling at a given temperature, we have postulated that the fiber coating will

uptake gas molecules much faster relative to regular HSSPME, since the portion of molecules in an air-evacuated headspace colliding with the fiber will be much larger than that in the presence of air [15]. This enhancement in water molecule collisions when reducing the sampling pressure may result in fewer binding sites available for FFAs and the effect will be more pronounced when heating the sample (*i.e.* increasing the amount of water molecules in the headspace), thus explaining the lower optimum temperature values recorded for FFAs with Vac-HSSPME compared to regular HSSPME sampling.

Regarding phenols, the majority of peak areas recorded after Vac-HSSPME or regular HSSPME sampling increased with increasing temperature. This is the first experimental evidence of the positive combined effect of reduced pressure and temperature on HSSPME sampling from water samples, and demonstrates the strong adsorbent ability of CAR/PDMS for these analytes. The only exception was Ph where signals obtained with Vac-HSSPME remained more or less the same when heating the sample. For the 20 min Vac-HSSPME sampling time used here and for temperatures at least up to 45 ºC, Ph is expected to be in a pre-equilibrium stage. Moreover, Ph is the second smallest analyte investigated here and is therefore expected to compete for active sites that can retain small FFAs and water molecules. It was therefore assumed that two opposite effects were taking place and although heating the sample under vacuum conditions increased the pre-equilibrium amount of Ph in the headspace, Ph molecules also had to compete with an increasing amount of water molecules for the same active surface sites.

Based on the above discussion, 35 ºC was selected as the optimum sampling temperature for Vac-HSSPME and 45 ºC for regular HSSPME, representing compromise temperatures for the extraction of both FFAs and phenols.

3.1.5. Analytical implications of the optimum conditions found for Vac-HSSPME and regular HSSPME

Table 2 summarizes the optimum values selected for both Vac-HSSPME and regular HSSPME whilst using the CAR/PDMS fiber and highlights the advantages of using the Vac-HSSPME approach. To help better understand the benefits of sampling under reduced pressure conditions, a new series of experiments was carried out representing two different sets of extraction conditions under each pressure condition (denoted as conditions *(i)* and *(ii)* in Figure 4), where the "Optimum HSSPME" and "Optimum Vac-HSSPME" bars in Figure 4 refer to experiments performed using the optimum conditions found earlier for regular HSSPME and Vac-HSSPME. The "Non-optimum HSSPME" bars in the figure refer to experiments carried out under the optimum conditions found for Vac-HSSPME, and the *vice versa* applies for the "Non-optimum Vac-HSSPME" bars. Figure 4 clearly shows that Vac-HSSPME under optimum conditions yielded the highest extraction efficiencies for all analytes, as peak areas were 1.8 to 28 times higher than those obtained with regular HSSPME under optimum conditions. Even under non-optimum conditions, Vac-HSSPME sampling yielded extraction efficiencies that were higher or similar to those obtained with optimum HSSPME. As expected, regular HSSPME under the mild optimum conditions found for Vac-HSSPME ("Non-optimum HSSPME" bars) yielded the lowest extraction efficiencies found here. This comparison highlights the great benefits of sampling low K_H compounds under low pressure conditions, since adopting the Vac-HSSPME approach results in faster and more sensitive methods that eliminate the need of heating the sample at higher temperatures.

3.2. Analytical performance of the optimized Vac-HSSPME and regular HSSPME procedures

The analytical performances of the two optimized procedures were evaluated and the main quality analytical parameters are given in Table 3. As seen, the obtained calibrations curves had a wide linearity range, and correlation coefficients (R) ranged from 0.991 to 0.999 with regular HSSPME-GC-FID, and from 0.990 to 0.999 with Vac-HSSPME-GC-FID. Furthermore, the sensitivity of the methods, expressed as the slopes of the calibration curves, ranged from (0.14 ± 0.01)×10^6 for *n*-C_4 to (2.37 ± 0.04) ×10^6 for 2-MOP with regular HSSPME and from (0.12 ± 0.01) ×10^6 for *i*-C_4 and *n*-C_4 to (9.8 ± 0.2) ×10^6 for 2-EP with Vac-HSSPME. For most analytes, sensitivity was higher with Vac-HSSPME and the only exceptions were the more volatile *i*-C_4, n-C_4 and *i*-C_5, for which sensitivity was similar with both methods. The limits of detection (LOD) and limits of quantification (LOQ) were estimated as three and ten times the signal-to-noise ratio, respectively, and were verified by performing extractions at those levels. Table 3 shows that for almost all target analytes, lower LOD and LOQ values could be obtained with the proposed Vac-HSSPME procedure.

Table 4 shows the results on intra-day precision study for both methods, estimated as the relative standard deviation (RSD) at two different spiked levels (n = 3). For the low spiking level, the obtained RSD values ranged from 1.4% for Ph to 9.0% for for *i*-C_4 when using HSSPME sampling, and between 0.3% for 2-EP to 10% for *n*-C_7 for Vac-HSSPME. It is noteworthy that with Vac-HSSPME lower RSD values were obtained for almost all target analytes.

3.3. Analysis of milk and milk derivatives samples using Vac-HSSPME

The applicability of the Vac-HSSPME method to the analysis of milk samples was evaluated by analyzing raw goat milk and a milk derivative (a liquid yogurt with fruits flavor), and at optimum Vac-HSSPME conditions (see Table 2). Figure 5 depicts representative

chromatograms obtained for the analysis of each sample and Table 5 includes the estimated values of FFAs and phenols in each sample. 2-EP was not detected in any of the samples, while *i*-C_4 and 4-Al-2-MOP were not detected in the liquid yogurt. 2-MOP and Ph were detected but not quantified in the goat milk, whereas *i*-C_6 and 2-MOP were detected but not quantified in the liquid yogurt. For the remaining analytes, the obtained values ranged between 0.1 ± 0.2 mg·L^{-1} for *i*-C_4 and 4.7 ± 0.4 mg·L^{-1} for *n*-C_4 when goat milk was analyzed, and from 0.10 ± 0.04 mg·L^{-1} for 4-Al-2-MOP to 2.6 ± 0.2 mg·L^{-1} for *i*-C_6 when liquid yogurt was analyzed. In the past, Amer *et al.* determined short chain FFAs in raw milk after derivatization and analysis via GC-MS. The obtained values ranged between 1.8 and 20 mg·L^{-1} [40]. Rincón *et al.* also determined FFAs in goat cheese (which normally present higher contents of FFAs), obtaining an average content for the analogue FFAs of 57 mg·kg^{-1} via multiple HSSPME-GC-FID, and 78 mg·kg^{-1} via solid phase extraction (SPE)-GC-FID [1].

4. Conclusions

The present study is of particular importance to flavor aroma analysis as it involved a thorough investigation and discussion of extraction conditions for best analyte coverage under low pressure conditions and ultimately results in a fast and sensitive Vac-HSSPME procedure at a mild sampling temperature. At the concentration levels used here, competitive displacement became more intense for smaller and more volatile analytes when lowering the sampling pressure due to the faster fiber-loading of all analytes present in the multicomponent solution. For practical rapid sampling with adsorbent-type SPME fibers, the appropriate sampling time for which the mass uptake is still linear should be first estimated and used for Vac-HSSPME. Further evidence is provided here, that conferring about reaching equilibrium is not appropriate when using Carboxen fibers. The first experimental evidence of the positive

combined effect of reduced pressure and temperature on HSSPME sampling from water samples was recorded for the larger analytes of the multicomponent system, and confirmed the strong adsorbent ability of CAR/PDMS. Increasing the temperature was also assumed to enhance water molecule collisions with the fiber and resulted in fewer binding sites for the smaller and more volatile analytes. Despite the intensification of competitive adsorption phenomena when lowering the sampling pressure, the careful selection of optimum extraction conditions resulted in a Vac-HSSPME method that was more sensitive and yielded better intra-day precision than regular HSSPME for almost all target analytes.

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Figure Captions

Figure 1. Schematic representation and image of each of the parts of the novel device for Vac-HSSPME **(A)**, and of the assembled device **(B)**.

Figure 2. Extraction time profiles for *n*-C_5, *n*-C_7 and 4-Al-2-MOP obtained (i) under reduced (Vac-HSSPME; filled symbols) and (ii) atmospheric (Regular HSSPME; open symbols) pressure conditions. Other experimental conditions: CAR/PDMS fiber; 5 mL ultrapure water containing 25% (w/v) NaCl, 0.022 mmol·L^{-1} of each FFA and 1 mg·L^{-1} of each phenol; 45 ºC sampling temperature; 400 rpm agitation rate; 2 min GC desorption time at 280 ºC.

Figure 3. Extraction temperature profiles obtained under **(A)** reduced (Vac-HSSPME) and **(B)** atmospheric (Regular HSSPME) pressure conditions. Other experimental conditions (n = 3): CAR/PDMS fiber; 5 mL ultrapure water containing 25% (w/v) NaCl, 0.022 mmol·L^{-1} of each FFA and 1 mg·L^{-1} of each phenol; 40 min extraction time; 400 rpm agitation rate; 2 min GC desorption at 280 ºC.

Figure 4. Extraction efficiency, expressed as chromatographic peak area of each analyte, obtained performing experiments at two different conditions (termed as (i) and (ii)) using both HSSPME and Vac-HSSPME with CAR/PDMS. Other experimental conditions (n = 3): 5 mL ultrapure water containing 25% (w/w) NaCl, 0.022 mmol·L^{-1} of each FFA and 1 mg·L^{-1} of each phenol, 400 rpm agitation rate, 2 min GC desorption at 280 ºC.

Figure 5. Representative chromatograms obtained after the analysis of **(A)** goat milk and **(B)** a milk derivative (liquid yogurt) using the optimum Vac-HSSPME-GC-FID method.

Table 1. Changes in extraction efficiency expressed as Vac-HSSPME/HSSPME peak area ratios for the four commercial and two PIL-based SPME fibers upon reducing the total pressure. Experimental conditions (n = 3): 5 mL ultrapure water containing 25% (w/v) NaCl, 0.022 mmol·L^{-1} of each FFA and 1 mg·L^{-1} of each phenol; 30 min extraction time; 40 ºC sampling temperature; 400 rpm agitation rate; 6 min GC desorption time at 280 ºC. Experiments using Vac-HSSPME and regular HSSPME were performed using these conditions.

Analyte	CAR/PDMS	DVB/CAR/PDMS	PA	PDMS	PIL1	PIL2
i-C_4	0.9	0.6	1.7	1.0	0.7	0.6
n-C_4	1.1	0.8	0.9	1.2	0.7	0.8
i-C_5	0.9	0.6	1.2	0.8	0.8	0.8
n-C_5	1.5	1.2	1.2	0.7	0.8	0.8
i-C_6	1.4	1.2	1.2	0.8	0.9	1.0
n-C_6	2.2	1.7	1.3	0.8	1.0	1.1
2-MOP	3.1	2.1	1.2	1.0	1.0	1.1
n-C_7	3.0	2.7	1.6	0.8	1.1	1.2
Ph	3.0	2.0	1.3	0.9	0.9	1.0
2-EP	2.8	2.3	1.6	0.9	1.0	1.1
4-Al-2-MOP	4.1	3.9	1.8	1.1	1.4	1.3

Table 2. Summary of the optimum values selected for HSSPME and Vac-HSSPME whilst using CAR/PDMS. The sample agitation speed was 400 rpm and the GC desorption temperature 280 ºC.

Factor	Studied range	Optimum value	
		HSSPME	Vac-HSSPME
NaCl content (%, w/v)	0–25	25	25
Extraction time (min)	20–100	40	20
Extraction temperature (ºC)	25–55	45	35
Desorption time (min)	2–6	2	2

Table 3. Analytical performance of the optimized regular HSSPME and Vac-HSSPME procedures using CAR/PDMS (n = 7 calibration levels).

Analyte	Regular HSSPME method with CAR/PDMS						Vac-HSSPME method with CAR/PDMS					
	Calibration range ($mg \cdot L^{-1}$)	(Slope ± SD[a])·10^{-6}	R[b]	($S_{y/x}$[c]) ·10^{-6}	LOD[d] ($\mu g \cdot L^{-1}$)	LOQ[e] ($\mu g \cdot L^{-1}$)	Calibration range ($mg \cdot L^{-1}$)	(Slope ± SD[a])·10^{-6}	R[b]	($S_{y/x}$[c]) ·10^{-6}	LOD[d] ($\mu g \cdot L^{-1}$)	LOQ[d] ($\mu g \cdot L^{-1}$)
i-C_4	0.4 – 5.8	0.147 ± 0.005	0.997	0.02	18	61	0.1 – 5.7	0.12 ± 0.01	0.994	0.03	13	43
n-C_4	0.6 – 7.6	0.14 ± 0.01	0.991	0.07	19	62	0.6 –5.7	0.12 ± 0.01	0.990	0.04	13	44
i-C_5	0.4 – 8.5	0.43 ± 0.01	0.997	0.07	6.0	20	0.1 – 6.6	0.39 ± 0.02	0.994	0.10	4.0	13
n-C_5	0.7 – 11	0.43 ± 0.02	0.993	0.23	1.7	5.8	0.1 – 6.6	0.53 ± 0.02	0.997	0.10	2.9	9.8
i-C_6	0.5 – 13	0.81 ± 0.04	0.993	0.47	1.5	4.9	0.1 – 12.6	0.82 ± 0.03	0.994	0.44	1.4	4.5
n-C_6	0.07 – 12.6	0.98 ± 0.03	0.998	0.34	1.6	5.4	0.1 – 12.6	1.13 ± 0.03	0.998	0.42	1.0	3.3
2-MOP	0.04 – 1.00	2.37 ± 0.04	0.999	0.04	0.68	2.3	0.001 – 1.0	3.4 ± 0.1	0.993	0.15	0.33	1.1
n-C_7	0.8 – 14	1.66 ± 0.01	0.999	0.18	2.5	8.2	0.1 – 14	2.35 ± 0.09	0.995	1.4	0.27	0.91
Ph	0.005 – 1.00	0.98 ± 0.01	0.999	0.01	4.2	14	0.001 – 1.0	1.28 ± 0.06	0.992	0.07	0.50	1.7
2-EP	0.005 – 1.00	6.9 ± 0.1	0.999	0.06	0.15	0.50	0.001 – 1.0	9.8 ± 0.2	0.998	0.26	0.14	0.46
4-Al-2-MOP	0.005 – 1.00	1.79 ± 0.06	0.996	0.07	0.61	2.0	0.001 – 1.0	2.82 ± 0.05	0.999	0.06	0.44	1.5

[a] Standard deviation of the slope.
[b] Correlation coefficient.
[c] Standard deviation of the residuals (or error of the estimate).
[d] Limit of detection, calculated as 3 times the signal-to-noise ratio.
[e] Limit of quantification, calculated as 10 times the signal-to-noise ratio.

Table 4. Intra-day precision of the proposed regular HSSPME and Vac-HSSPME procedures.

Analyte	HSSPME method with CAR/PDMS				Vac-HSSPME method with CAR/PDMS			
	Low spiked level		Intermediate spiked level		Low spiked level		Intermediate spiked level	
	Spiked level ($mg \cdot L^{-1}$)	RSD[a] (%)	Spiked level ($mg \cdot L^{-1}$)	RSD[a] (%)	Spiked level ($mg \cdot L^{-1}$)	RSD[a] (%)	Spiked level ($mg \cdot L^{-1}$)	RSD[a] (%)
i-C_4	0.86	8.1	3.8	7.7	0.77	3.3	3.8	8.0
n-C_4	0.86	5.5	3.8	7.9	0.77	3.3	3.8	6.1
i-C_5	1.0	9.0	4.4	6.3	0.89	1.9	4.4	7.2
n-C_5	1.0	8.5	4.4	5.4	0.89	2.6	4.4	5.4
i-C_6	1.1	8.0	5.0	6.7	1.0	3.3	5.0	5.8
n-C_6	1.1	7.1	5.0	6.4	1.0	2.8	5.0	6.1
2-MOP	0.080	2.7	0.30	3.9	0.060	2.7	0.30	7.0
n-C_7	1.3	5.3	5.6	9.5	1.1	10	5.6	8.3
Ph	0.080	1.4	0.30	2.9	0.060	3.3	0.30	6.1
2-EP	0.080	2.4	0.30	5.5	0.060	0.3	0.30	7.5
4-Al-2-MOP	0.080	3.0	0.30	8.0	0.060	2.6	0.30	8.9

[a] Relative standard deviation (n = 3).

Table 5. Analysis of real milk and milk derivative samples using the proposed Vac-HSSPME method.

Analyte	Concentration found ± SD[a] ($mg \cdot L^{-1}$)	
	Goat milk	Liquid yogurt
i-C_4	0.1 ± 0.2	N.D.[c]
n-C_4	4.7 ± 0.4	1.5 ± 0.3
i-C_5	0.1 ± 0.2	2.6 ± 0.2
n-C_5	0.3 ± 0.1	0.2± 0.1
i-C_6	0.1 ± 0.4	>LOD, <LOQ[b]
n-C_6	0.1 ± 0.3	0.3 ± 0.3
2-MOP	>LOD, <LOQ[b]	>LOD, <LOQ[b]
n-C_7	0.4 ± 0.5	0.4 ± 0.5
Ph	>LOD, <LOQ[b]	0.10 ± 0.04
2-EP	N.D.[c]	N.D.[c]
4-Al-2-MOP	0.18 ± 0.02	N.D.[c]

[a] Standard deviation (n = 3).

[b] Detected but non-quantified.

[c] Non-detected.

*Graphical Abstract

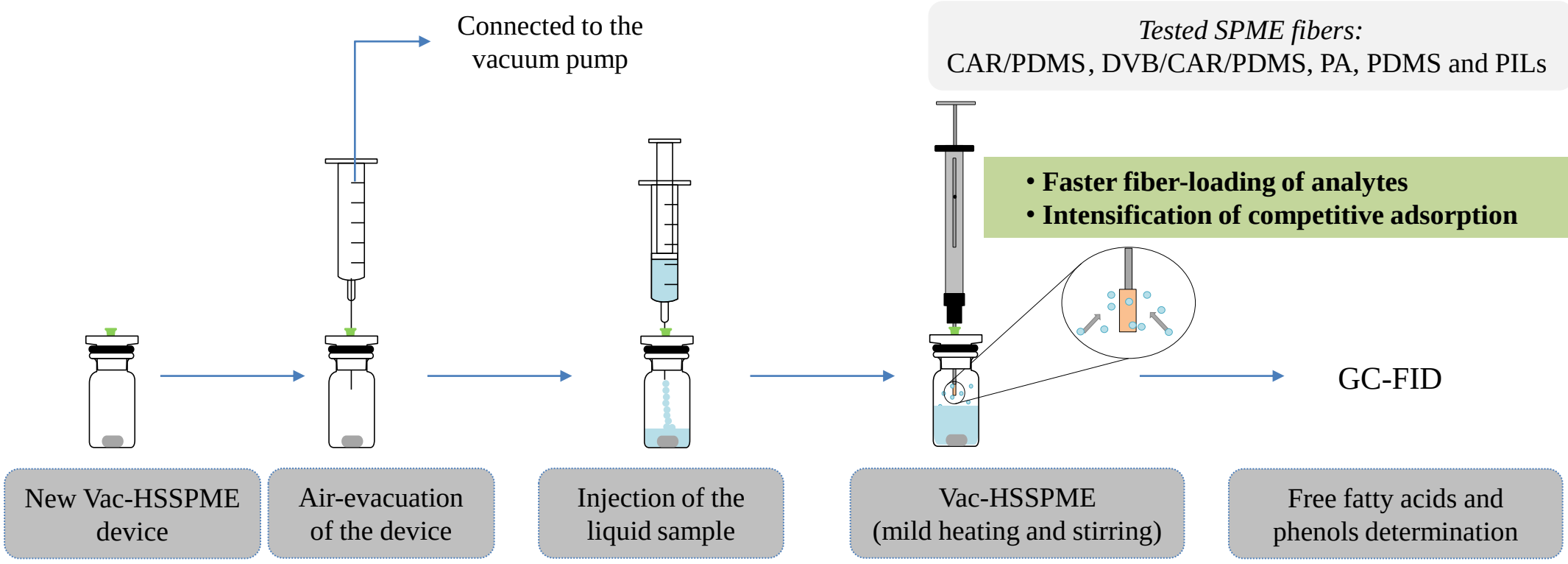


Figure 1

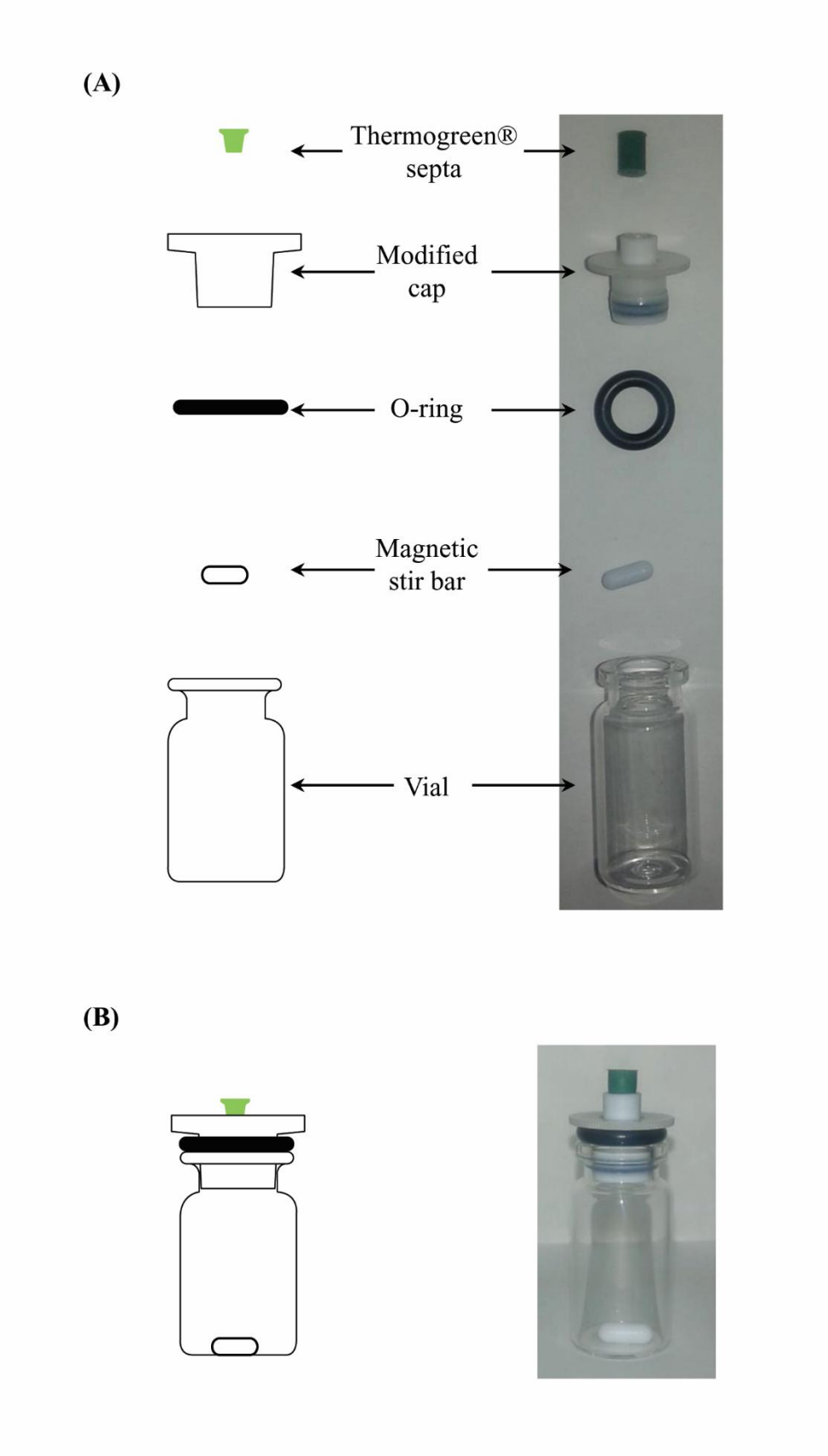
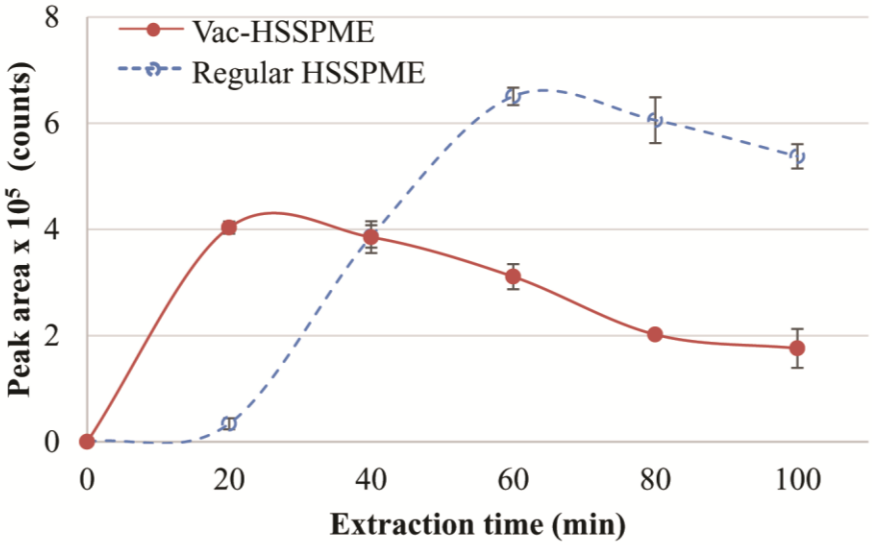
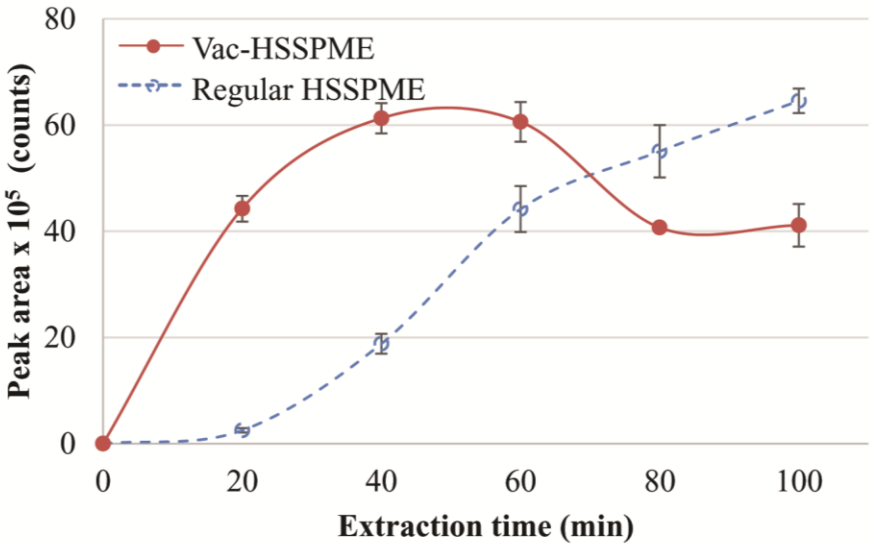

(A)
Thermogreen® septa
Modified cap
O-ring
Magnetic stir bar
Vial
(B)

Figure 2

(A) *n*-C_5



(B) *n*-C_7



(C) 4-Al-2-MOP

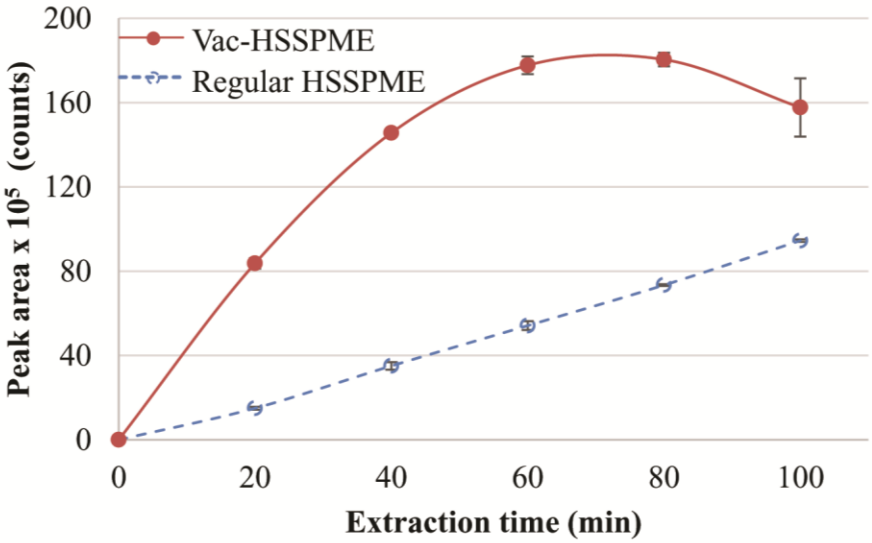


Figure 3

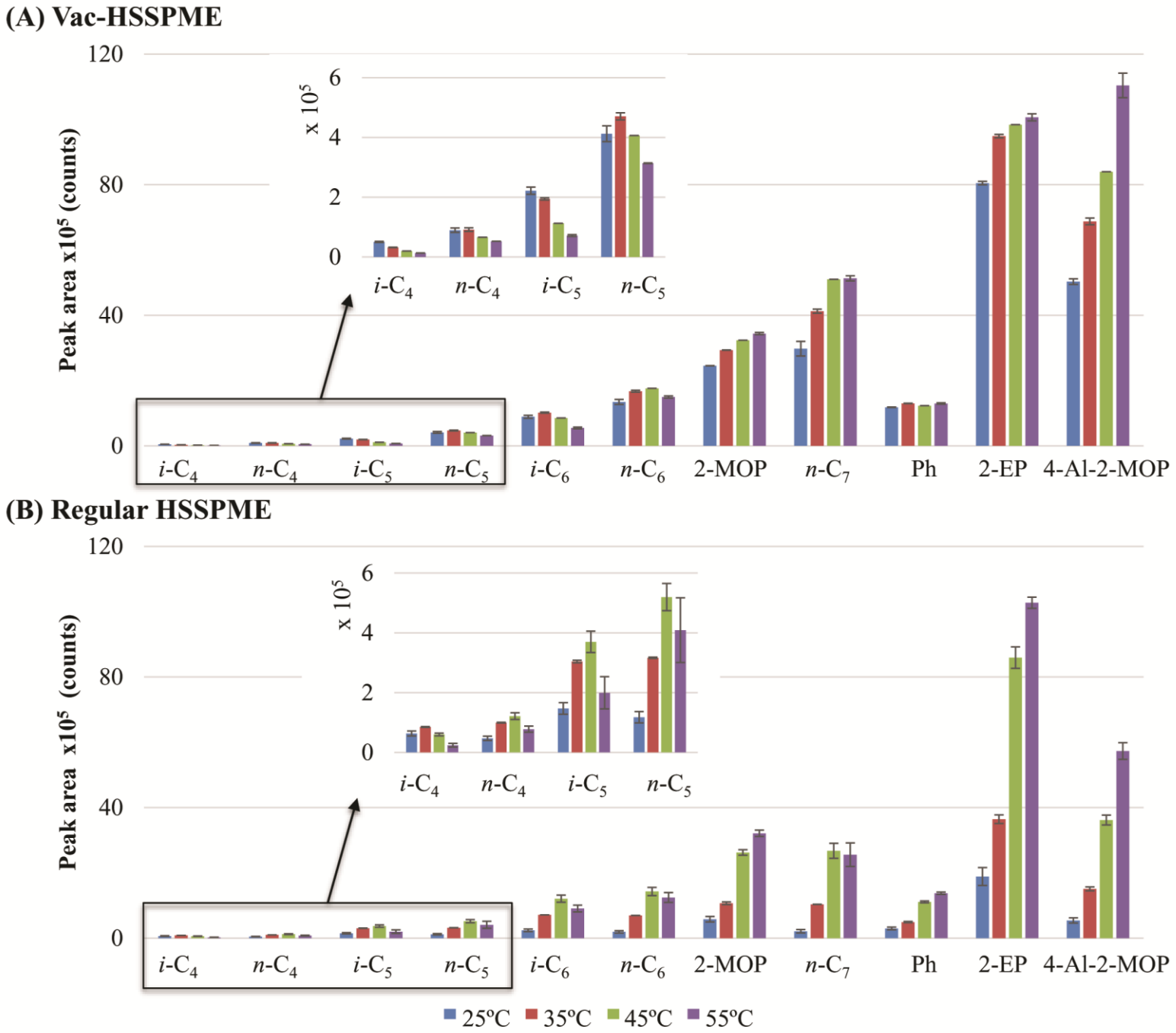

(A) Vac-HSSPME
Peak area x10⁵ (counts)
120
80
40
0
x 10⁵
6
4
2
0
i-C4
n-C4
i-C5
n-C5
i-C6
n-C6
2-MOP
n-C7
Ph
2-EP
4-Al-2-MOP
(B) Regular HSSPME
Peak area x10⁵ (counts)
25ºC
35ºC
45ºC
55ºC

Figure 4

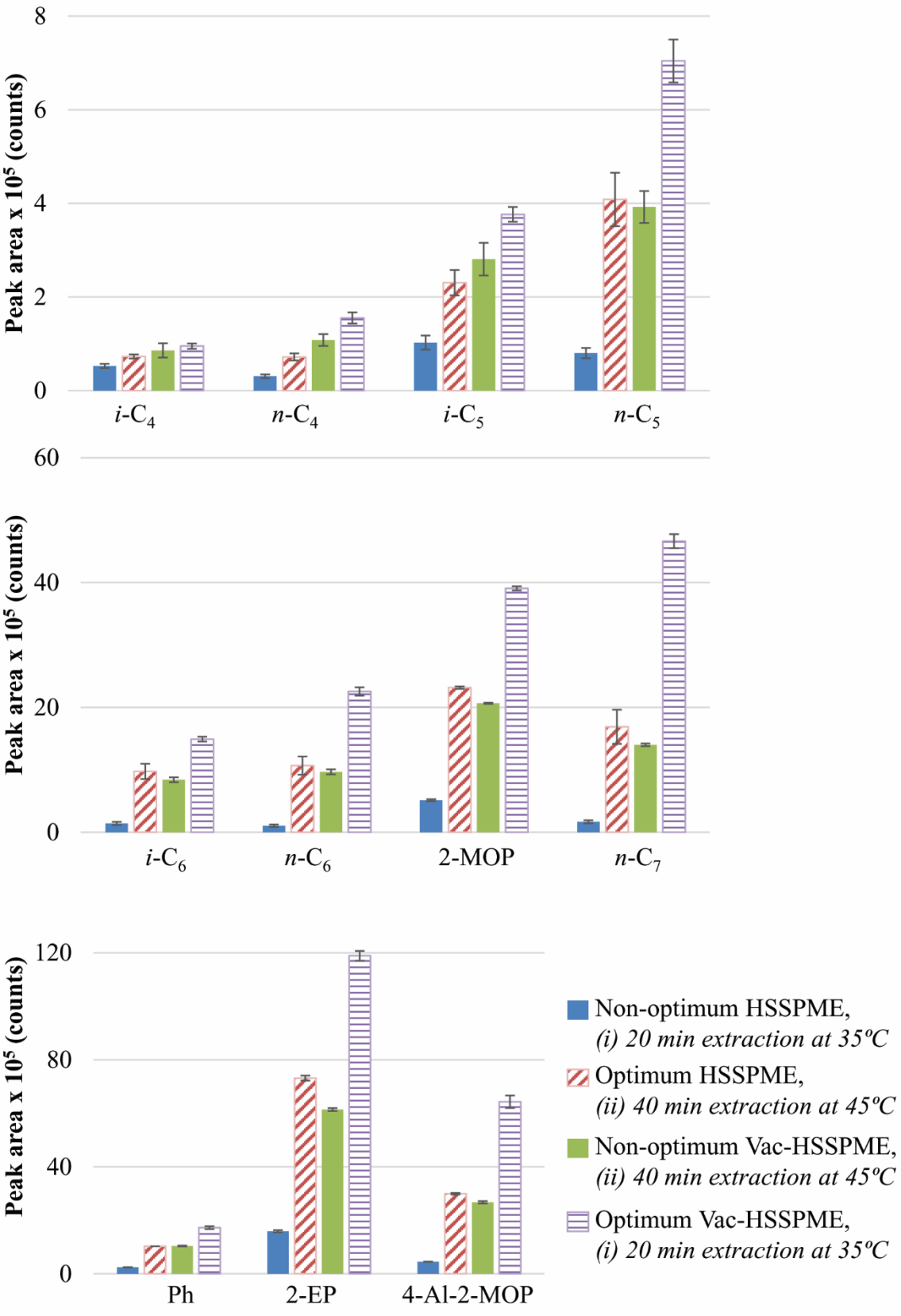
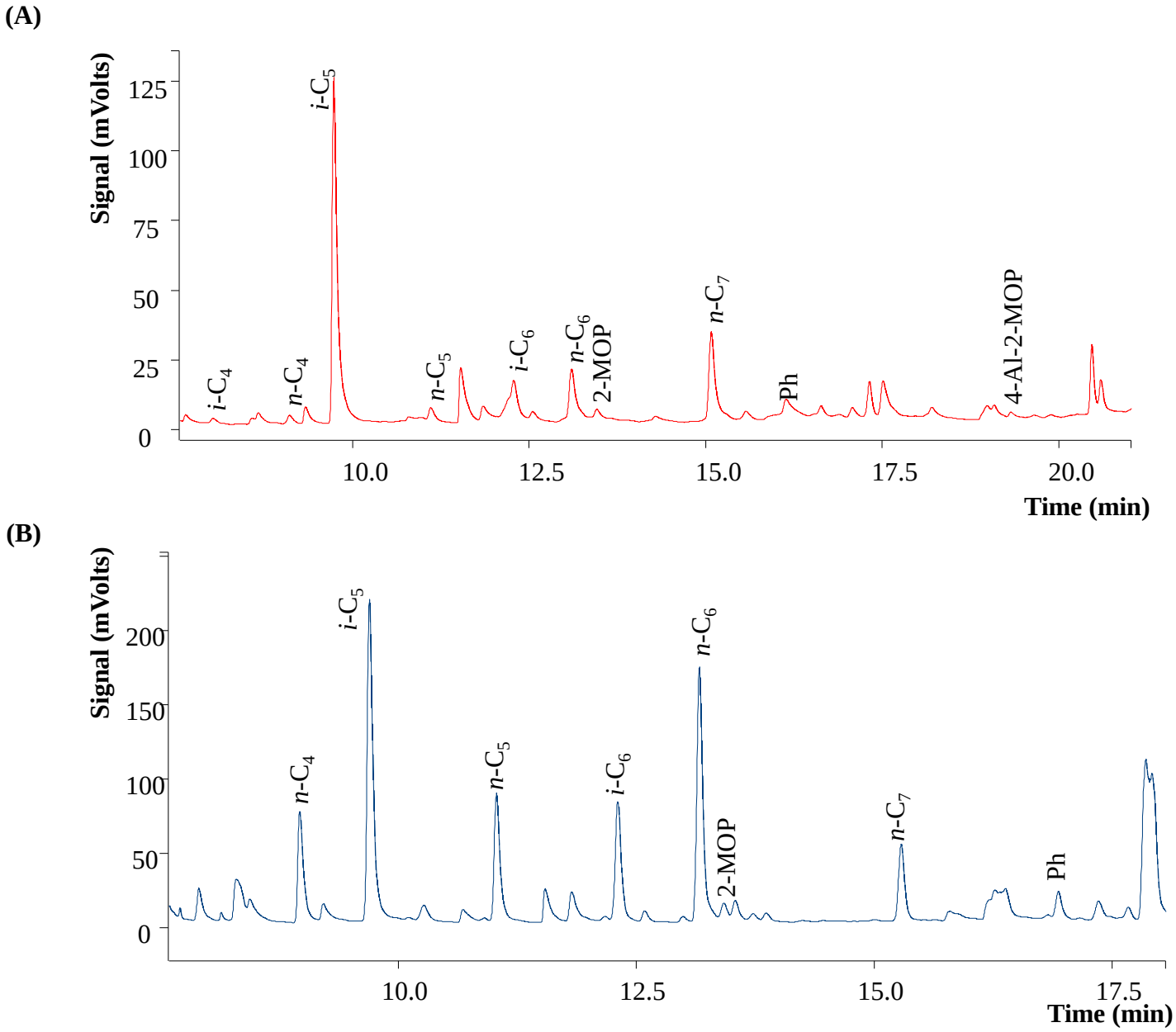

Peak area x 10⁵ (counts)
8
6
4
2
0
i-C4
n-C4
i-C5
n-C5
60
Peak area x 10⁵ (counts)
40
20
0
i-C6
n-C6
2-MOP
n-C7
120
Peak area x 10⁵ (counts)
80
40
0
Ph
2-EP
4-Al-2-MOP
Non-optimum HSSPME, (i) 20 min extraction at 35ºC
Optimum HSSPME, (ii) 40 min extraction at 45ºC
Non-optimum Vac-HSSPME, (ii) 40 min extraction at 45ºC
Optimum Vac-HSSPME, (i) 20 min extraction at 35ºC

Figure 5


(A)
Signal (mVolts)
125
100
75
50
25
0
i-C4
n-C4
i-C5
n-C5
i-C6
n-C6
2-MOP
n-C7
Ph
4-Al-2-MOP
10.0
12.5
15.0
17.5
20.0
Time (min)
(B)
Signal (mVolts)
200
150
100
50
0
n-C4
i-C5
n-C5
i-C6
n-C6
2-MOP
n-C7
Ph
10.0
12.5
15.0
17.5
Time (min)