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# 2-Step Multi-Volatile Method (2-Step MVM) for Characterization of Aroma Compounds in Bread

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## Introduction

Headspace gas chromatography (HS-GC) is frequently used for the analysis of aroma compounds in food due to its practical advantages of simplicity, amenability to full automation, less contamination from non-volatile constituents and elimination or reduction of solvent use. There are several established HS techniques, e.g. static headspace (SHS), dynamic headspace (DHS), and head space solid phase micro-extraction (HS-SPME). However, these techniques are more selective for volatile and/or hydrophobic compounds and result in a partial chromatogram with an under-representation of hydrophilic and/or low vapor pressure aroma compounds.

A full evaporation DHS (FEDHS) method, based on a classical full evaporation technique (FET) [1] was demonstrated for uniform enrichment of aroma compounds in several sample types [2, 3]. FEDHS of 10-100 µL of samples at 80ºC enables near complete vaporization and uniform recovery of aroma compounds with hydrophilic and/or low vapor pressure characteristics, while leaving most of the low volatile matrix behind. In 2014, a multi-volatile method (MVM) with sequential DHS sampling using different individual trapping conditions on the same sample was developed for uniform extraction and enrichment of a wide range of aroma compounds in aqueous samples [4]. The feasibility and benefits of using the MVM approach have been demonstrated by the determination of key aroma compounds (spanning the range from highly volatile acetaldehyde to much less volatile vanillin) in brewed coffee. There are several important aspects to consider when developing an MVM procedure: (1) Proper water management for successful GC/MS analysis, (2) choice of adsorbent trap to enable targeting of specific compound ranges, (3) an appreciation of the risk of breakthrough of (very) volatile compounds, (4) thermal desorption efficiency for polar and/or low vapor pressure compounds, (5) recovery of hydrophilic and/or low vapor pressure compounds from aqueous sample. In order to satisfy these considerations, the MVM procedure consists of three different DHS sampling steps performed at increasing temperatures, including a final full evaporation DHS (FEDHS) method. This 3-Step MVM approach provides a representative image of the overall volatile fraction of an aqueous sample, but one limitation is the application to fermented food samples, including alcoholic beverages, which have high concentrations of Ethanol (% levels). A carbon based multi-bed adsorbent trap (Carbopack B/Carbopack X/Shincarbon-X) used in the 1$^{st}$ and 2$^{nd}$ steps in the 3-Step MVM has a very high affinity to ethanol and it is difficult to eliminate ethanol from the trap with the dry purge conditions used.

In this study, we developed a 2-Step MVM approach, which enables the uniform enrichment of aroma compounds with vapor pressures (VP) <20 kPa in fermented food samples, while eliminating high

concentrations of Ethanol as well as water. The 2-Step MVM consists of two different DHS method parameter sets. In the first DHS sampling step at 25ºC, a carbon based dual-bed adsorbent trap (Carbopack B/Carbopack X) is used, which targets volatile compounds with moderate vapor pressure (1-20 kPa). The second DHS sampling step is performed using a Tenax TA trap at 80ºC targeting volatile compounds with low vapor pressure (<1 kPa) and/or hydrophilic characteristics. The performance of the 2-Step MVM was illustrated with analysis of a wide variety of aroma compounds in two different bread samples made with butter or shortening.

## Experimental

*Instrumentation*. Analyses were performed using a 7890 B GC equipped with a 5977 Mass Selective Detector (Agilent Technologies), Thermal Desorption Unit (TDU), PTV inlet (CIS 4), and MPS with DHS option (all GERSTEL), Figure 1.


*Figure 1. GERSTEL MPS-DHS-TDU Agilent 7890/5977 GC-MSD system.*

Two subsequent extractions were performed from the same sample HS-vial using different DHS conditions and traps. Afterwards, both traps were thermally desorbed in the Thermal Desorption Unit and the analytes collected in the CIS inlet serving as a Cryotrap. Finally, the collected analytes from the two extractions were injected for one single GC-MS run (Figure 2). This methodology is termed “2-Step Multi-Volatile Method” or 2-Step MVM. After this procedure, only non-volatile residues are left in the sample.

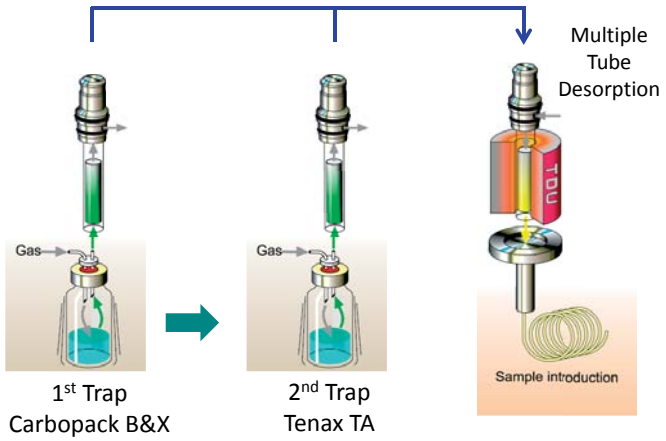


*Figure 2. Schematic procedure for 2-Step MVM analysis with sequential DHS sampling.*

*Sample*. Butter (Échiré doux; France), shortening (palm oil shortening; Colombia), and strong wheat flour (Haruyutaka blend, Japan) were purchased in a local store in Tokyo. Butter, shortening, and two kinds of homemade bread were used for the analysis. The procedure used for bread making is the following:

1 Mix 250 g of flour, 3 g of salt, 10 g of sugar, 150 mL of water, and 5 g of dry yeast,
2 add 15 g of butter or shortening to the mixture,
3 the 1st fermentation is performed for 40 min at 40ºC,
4 after degassing, the 2nd fermentation is performed for 40 min at 40ºC,
5 After shaping, baking is performed for 15 min at 210ºC.

100 mg of sample (butter and heated butter, shortening and heated shortening, bread with crust and non-crust interior) were placed for analysis in 10 mL headspace vials.

*Analysis conditions DHS Trap1 (Ethanol management).*

| | |
|---|---|
| Trap: | Carbotrap B / Carbopack X |
| Incubation: | 25°C (5 min) |
| Sampling: | Trap 30°C |
| | Volume 650 mL |
| | Flow 100 mL/min |
| Dry Purge: | Trap 30°C |
| | Volume 300 mL |
| | Flow 50 mL/min |

*Analysis conditions DHS Trap2 (FEDHS).*

| | |
|---|---|
| Trap: | Tenax TA |
| Incubation: | 80°C (0 min) |
| Sampling: | Trap 40°C |
| | Volume 3000 mL |
| | Flow 100 mL/min |
| Dry Purge: | n.a |

*Analysis conditions.*

| | |
|---|---|
| TDU : | splitless, desorption flow 50 mL/min |
| | Trap 1: 40°C (0.5 min); 720°C/min; 300°C (3 min) |
| | Trap 2: 40°C (0.5 min); 720°C/min; 240°C (3 min) |
| PTV: | Tenax TA liner, |
| | split 1:10 |
| | Trap 1: -50°C (1.5 min); 12°C/s; 240°C (hold) |
| | Trap 2: 10°C (held during desorption) |
| Column: | 30 m DB-Wax (Agilent) |
| | $d_i$ = 0.25 mm $d_f$ = 0.25 µm |
| Pneumatics: | He, constant flow = 1 mL/min |
| Oven: | 40°C (3 min); 10°C/min; 240°C (hold) |
| MSD: | Scan, 28.7 - 300 amu |

*Data analysis.* MSD ChemStation ver. F.01.03. 2357 (Agilent), Mass Hunter ID browser ver. B.0500 (Agilent), Mass Profiler Professional ver. 12.5 (Agilent), Automated Mass Spectral Deconvolution and Identification system (AMDIS) ver. 2.70 Build 130.53 (National Institute of Standard and Technology, Gaithersburg, MD, USA), and Aroma Office $^2$D database ver. 4.00 (Gerstel K.K.) were used for data analysis.

## Results and discussion

*Evaluation of 2-Step MVM.* In order to evaluate the recovery and linearity of aroma compounds with vapor pressure >1 kPa in an Ethanol rich sample, a spiked Water sample containing 5 % Ethanol was analyzed using the 1$^{st}$ set of DHS sampling condition (1$^{st}$ Trap). Table 1 shows recovery and linearity (5-100 µg/mL) of eight test compounds in 100 µL of a 5 % Ethanol – 95 % Water sample spiked at 10 µg/mL. Furan (VP: 79 kPa) and Propanal (VP: 42 kPa) which have the highest and the second highest vapor pressure showed very low recovery (< 11 %) and poor linearity with $r^2$ < 0.954 due to their low breakthrough volume for the 1$^{st}$ trap. In contrast, the rest of the test compounds with vapor pressure <21 kPa showed good recovery (>79 %) and linearity ($r^2$ >0.996), except for the recovery of 2,3-Butanedione (27 %). 2,3-Butadione has a VP value (9 kPa) similar to the VP value of Butanal (14 kPa), which showed 79 % recovery, but the log $K_{o/w}$ value is quite low at -1.34 compared to that of Butanal (log $K_{o/w}$: 0.82). It is possible that the presence of 5 % Ethanol in the aqueous sample increases the solubility of 2,3-Butanedione, resulting in the lower 27 % recovery under the 1$^{st}$ set of DHS sampling condition.

*Table 1. Test aroma compounds studied and corresponding vapor pressure, log $K_{o/w}$, recovery and linearity obtained for DHS-GC-MS analysis using the 1$^{st}$ DHS sampling condition (1$^{st}$ Trap) of spiked water (with 5 % Ethanol).*

| Compound | VP [kPa] | log $K_{o/w}$ | Rec. [%] | $r^2$ |
|---|---|---|---|---|
| Furan | 79 | 1.36 | 5.3 | 0.865 |
| Propanal | 42 | 0.33 | 11 | 0.954 |
| 2-Methyl furan | 21 | 1.91 | 120 | 0.997 |
| Butanal | 14 | 0.82 | 79 | 0.999 |
| 2,3-Butanedione | 9 | -1.34 | 27 | 0.996 |
| Pentanal | 4.2 | 1.31 | 81 | 0.999 |
| Dimethyl disulfide | 3.1 | 1.87 | 94 | 0.999 |
| Pyrrole | 1.3 | 0.88 | 89 | 0.999 |

Figure 3 shows a comparison of the total ion chromatograms (TICs) obtained from a bread sample using two different sets of 1st step DHS sampling conditions. A very broad Ethanol peak was eluted in the TIC obtained from the original 1st DHS sampling condition using Carbopack B/Carbopack X/Shincarbon X (for 3-Step MVM, Figure 3a). The column was overloaded and the MS detector saturated, resulting in serious co-elution with 2,3-Butanedione and poor peak shape of 1-Propanol and iso-Butanol. In contrast, well defined peaks for Ethanol, 1-Propanol, and iso-Butanol were eluted in the TIC obtained from the new 1st step DHS sampling condition using Carbopack B/Carbopack X (for 2-Step MVM, Figure 3b). Also, the important bread aroma compound 2,3-Butanedione was clearly detected, whereas this peak was totally distorted in the broad Ethanol peak shown in Figure 3a. under the conditions previously used. It was clearly shown that the newly developed 1st step DHS sampling parameters enable management of both Ethanol and Water in fermented food samples, such as bread, while recovering important volatile aroma compounds such as 2,3-Butanedione.

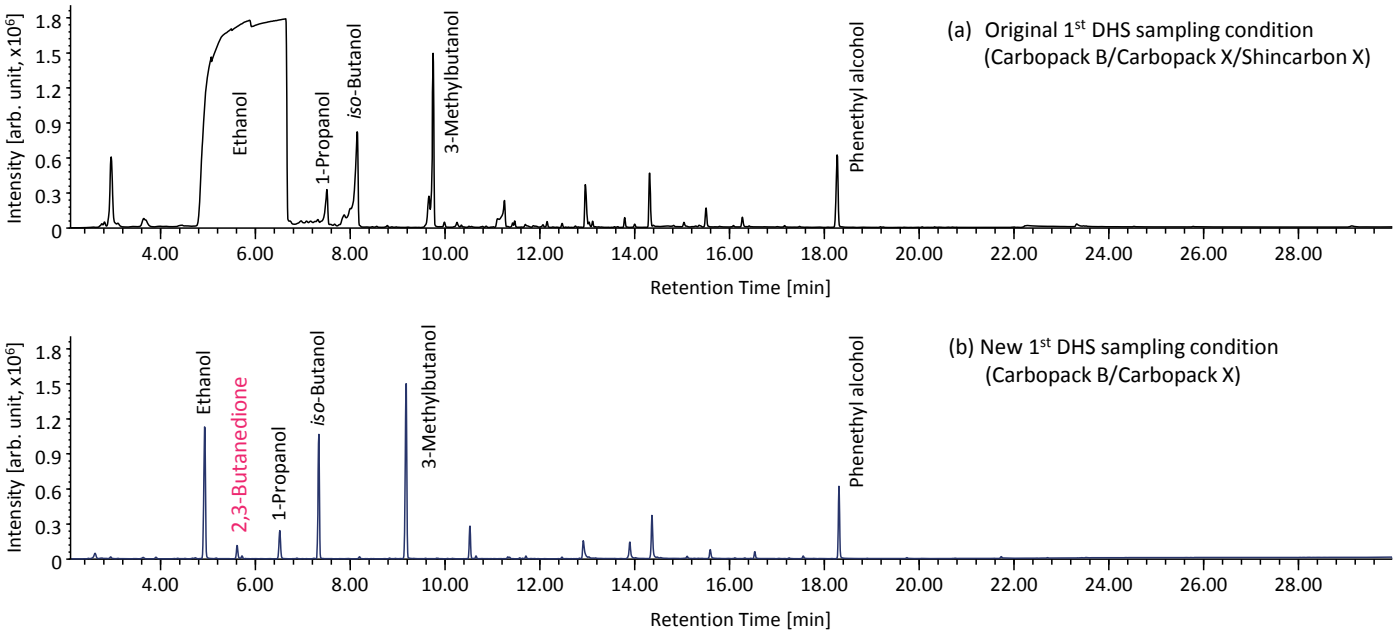


*Figure 3. Comparison of total ion chromatogram (TIC) obtained from two different 1st DHS sampling conditions of bread sample.*
*(a) Original 1st DHS sampling condition using Carbopack B/Carbopack X/Shincarbon X.*
*(b) New 1st DHS sampling condition using Carbopack B/Carbopack X.*

*Screening of Aroma Compounds in Butter, Shortening, and Bread.* In order to screen potential aroma compounds, which contribute to the flavor of the final bread product, butter, heated butter, shortening, and heated shortening were initially analyzed with triplicate sampling by 2-Step MVM-GC-MS."Shortening" shortens gluten strands in flour and is 100% vegetable fat as compared to 80% animal fat in butter. The use of shortening increases the tenderness of baked goods. For heated samples, butter and shortening were heated for 30 min at 150ºC in a HS vial before the analysis. Figure 4 shows a comparison of TICs obtained from 2-Step MVM-GC-MS of butter (a) and shortening (b). One remarkable difference is the detection of more late eluting peaks (retention time (Rt): 18-26 min) in TIC (a). Numerous late eluting compounds including characteristic aroma compounds (milky/creamy/nutty/fruity) found in dairy products, e.g. C8-C14 δ-Lactones, γ-Undecalactone, and γ-Dodecenolactone are clearly detected in the heated butter sample. Also, typical Maillard reaction related compounds such as Maltol (burnt sugar) and 5-Hydroxy methyl furfural (HMF) (spicy/sweet) are more intense in TIC (a).

For screening of the aroma compounds in the TICs, mass spectral deconvolution with the NIST AMDIS was initially performed. The candidate lists were filtered with a Mass Hunter ID Browser using the following conditions: (1) a candidate should have a spectral match score of more than 50, (2) the candidate should consist of C, H, and N/O/S (without any other elements), (3) the candidate name should not be replicated, (4) peaks detected in the blank run should be excluded. From the butter, heated butter, shortening, and heated shortening data files 483, 583, 383 and 552 candidate compounds were obtained respectively. The increased number of candidates from both heated butter and shortening clearly reflects the effect of heat-induced compound formation. The Aroma Office database (Gerstel K.K.), which is integrated in the MSD ChemStation was then applied for additional filtering of the aroma compounds from the candidate lists. Aroma Office is an integrated software approach, which allows automated processing of retention index- and mass spectral data for improved identification of flavor compounds. It contains the most comprehensive data base of flavor compounds available. Aroma Office is also a searchable data base with retention index and aroma character information on >10,000 compounds from greater than 100,000 entries from a wide range of literature references [5]. The number of aroma compounds found during screening was 60 for butter, 87 for heated butter, 44 for shortening, and 60 for heated shortening, respectively.

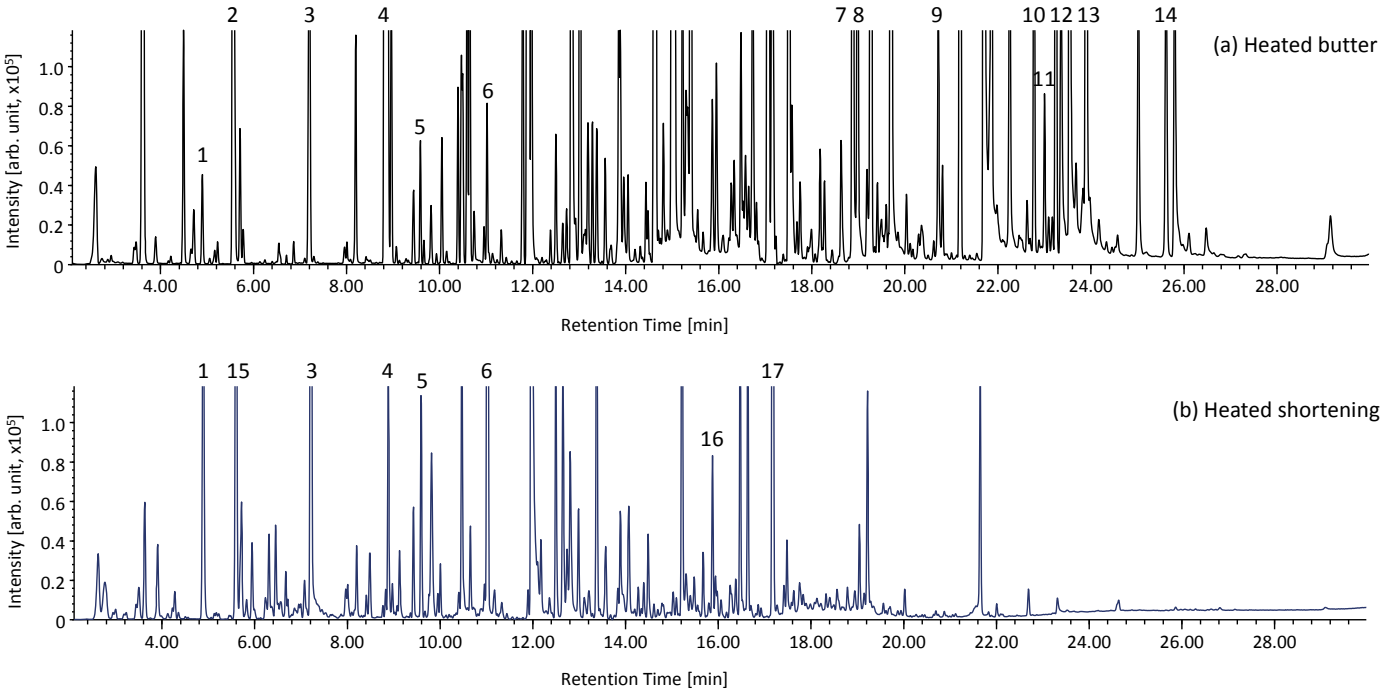


*Figure 4. Comparison of total ion chromatograms (TIC) obtained from 2-Step MVM-GC-MS of heated butter (a) and shortening (b).*

*1. Ethanol, 2. 2-Heptanone, 3. 1-Hexanol, 4. 2-Heptanone, 5. 2-Pentyl furan, 6. 2-Heptanal, 7. Maltol, 8. γ-Octalactone, 9. δ-Decalactone, 10. γ-Undecalactone, 11. γ-Dodecalactone, 12. δ-Dodecalactone, 13. HMF, 14. δ-Tetradecalactone, 15. Pentanal, 16. 2,4-Nonadienal, 17. 2,4-Decadienal*

For bread samples, the separate elements of non-crust interior and crust were analyzed with triplicate sampling by 2-Step MVM-GC-MS. Figure 5 illustrates a comparison of TIC of non-crust interior of bread made with butter (a) and that made with shortening (b). Compared to the TICs obtained from the heated butter and shortening (Figure 4), the TICs obtained from the related bread samples showed similar profiles. Screening of aroma compounds in bread samples were performed with the same procedure for butter and shortening samples (deconvolution using NIST AMDIS followed by cross searching using Aroma Office database). The number of aroma compounds found during screening was 60 for non-crust interior (butter), 65 for crust (butter), 60 for non-crust interior (shortening), and 62 for crust (shortening), respectively.

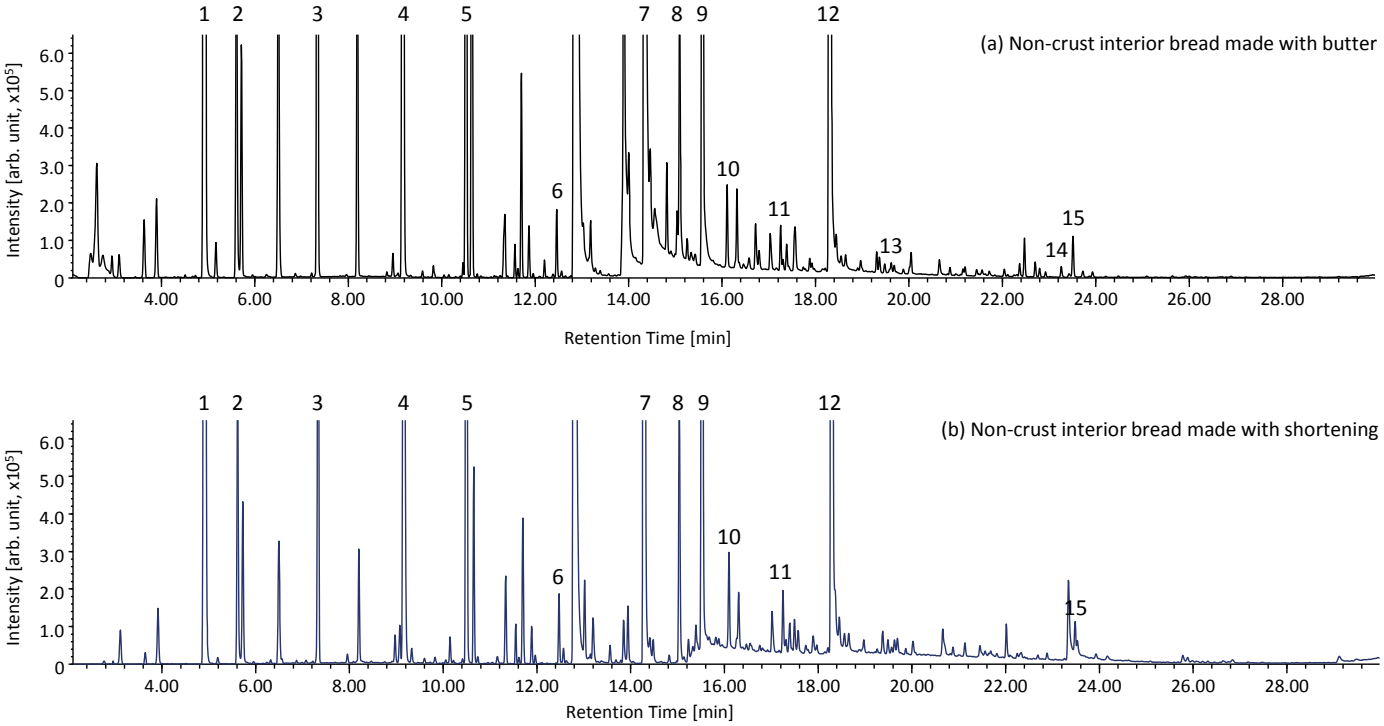


*Figure 5. Comparison of total ion chromatograms (TIC) obtained from 2-Step MVM-GC-MS of non-crust interior bread made with butter (a) or shortening (b).*
*1. Ethanol, 2. 2-Pentanone, 3. Isobutyl alcohol, 4. Isoamyl alcohol, 5. Acetoin, 6. Ethyl octanoate, 7. Propanoic acid, 8. Butanoic acid, 9. Isovaleric acid, 10. Methionol, 11. Phenetyl acetate, 12. Phenethyl alcohol, 13. δ-Nonalactone, 14. δ-Dodecalactone, 15. Indole.*

*Principal Component Analysis of Aroma Compounds in Butter, Shortening, and Bread.* Principal component analysis (PCA) was applied to all samples (in triplicate analysis) to obtain a simplified view of the relationship between butter, heated butter, shortening, and heated shortening, and the relationship between non-crust interior and crust of bread samples, as well as two different bread samples made with butter and shortening, respectively. Figure 6 shows the PCA score plot using PC1 and PC2 and the corresponding loading plot for butter, heated butter, shortening, and heated shortening. The two principal components (PC) account for 80.54 % of the total variance of the data (PC1: 44.78%, and PC2: 35.76%, respectively). This score plot clearly differentiates all butter and shortening samples. From the loading plot, several aroma compounds can be characterized according to the strength of the contribution to each PC. Maltol (burnt sugar) shows higher contribution to the positive PC 1 and is correlated with the heat-induced effect (Maillard reaction) from milk-proteins and lactose. Two dienals such as 2,4-Nonadienal and 2,4-Decadienal show higher contribution to the negative PC 2 and are correlated with the heat stress on lipid peroxide.

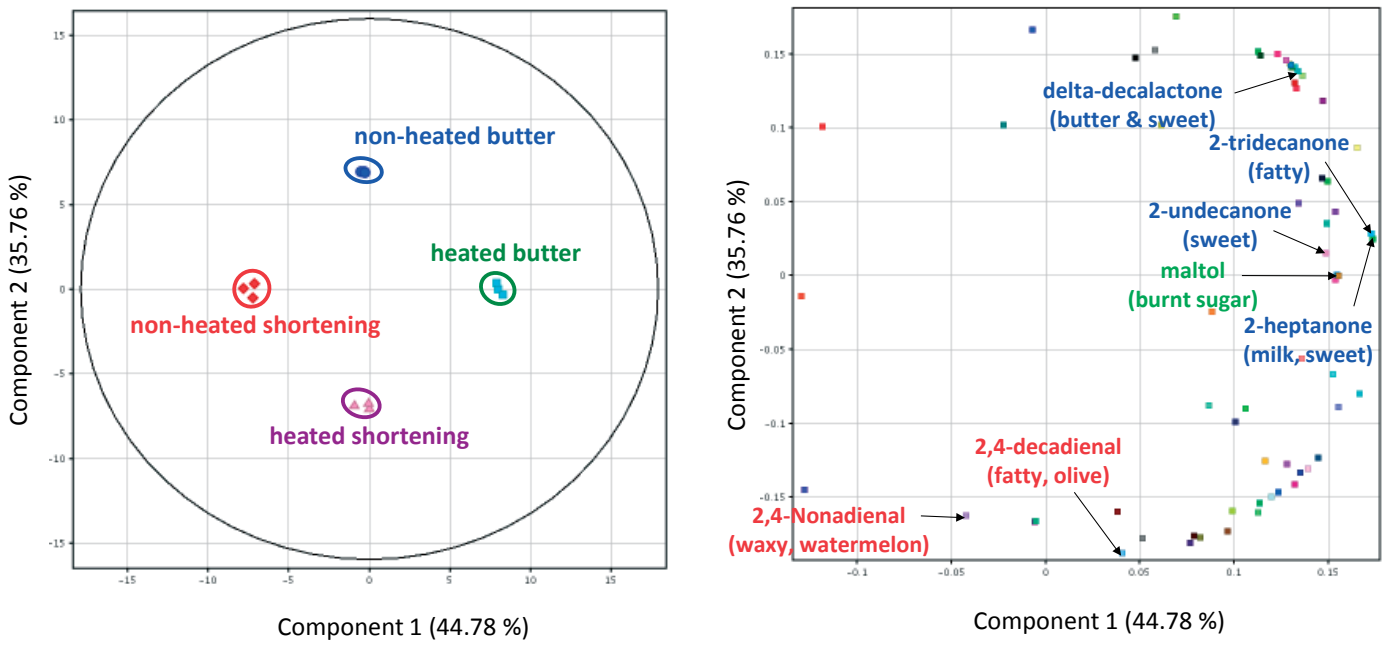


*Figure 6. PCA score plot using PC1 and PC2 and the corresponding loading plot for butter, heated butter, shortening, and heated shortening.*

Figure 7 illustrates the PCA score plot using PC 1 and PC2 and the corresponding loading plot for non-crust interior made with butter, crust made with butter, non-crust interior made with shortening, and crust made with shortening. The two principal components (PC) account for 71.24 % of the total variance of the data (PC1: 50.81%, and PC2: 20.43%, respectively). PC1 differentiates between non-crust interior and crust and PC 2 differentiates between non-crust interior samples, while showing small difference between crust samples. Several aroma compounds such as 2,3,5-Trimethyl pyrazine (roasted, nutty), 2,5-Dimethyl pyrazine (roasted), 2-Acetyl pyrrole (roasted), 2-Furanmethanol (caramel, toasted), and Maltol (burnt sugar) shows higher contribution to the positive PC1 and are correlated with heat effects in the baking process. Several characteristic aroma compounds in dairy products such as 2-Heptanone (milky, sweet), 2-Undecanone (sweet), 2-Tridecanone (fatty), and δ-Decalactone (butter, sweet) show higher contribution to the negative PC2, while typical lipid peroxide related compounds such as 2,4-Nonadienal (fatty, cucumber) and 2,4-Decadienal (waxy, watermelon) show higher contribution to the positive PC 2. Figure 8 illustrates relative responses of aroma compounds which contribute to classification of bread samples.

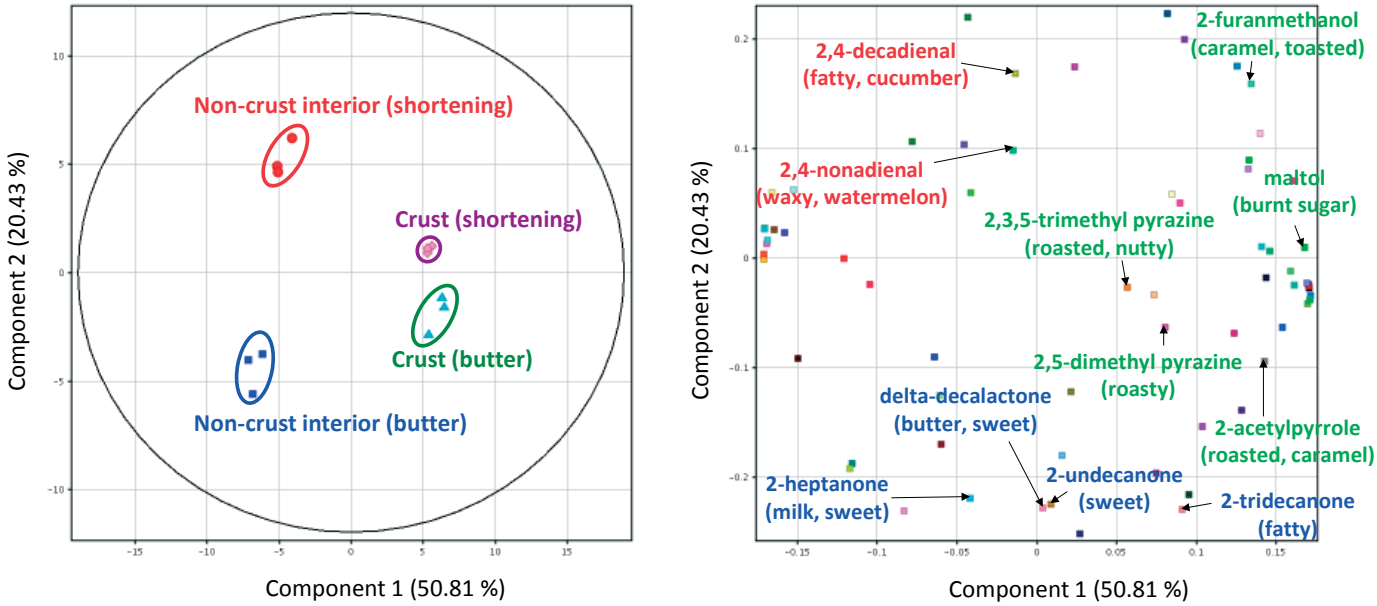


*Figure 7. PCA score plot using PC1 and PC2 and the corresponding loading plot for non-crust interior and crust made with butter and shortening*

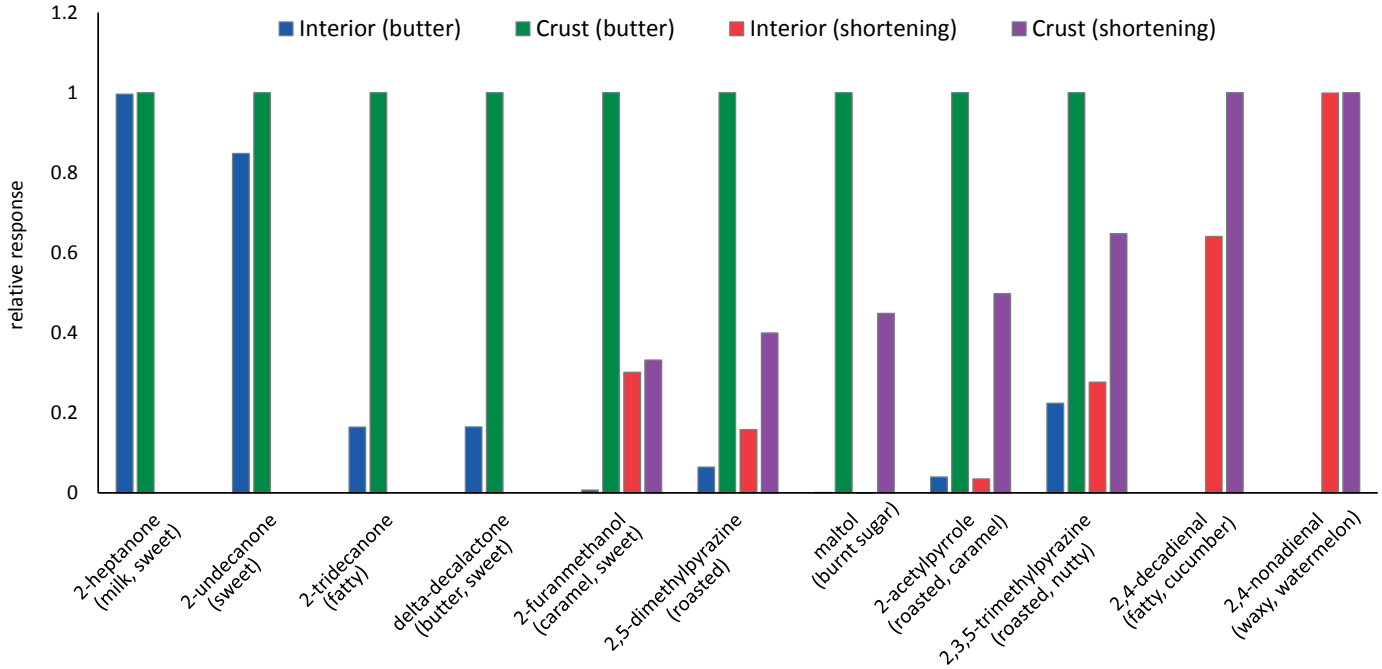


*Figure 8. Relative response of aroma compounds which contribute to classification of bread samples. Relative response was normalized with the highest response.*

## Conclusion

A 2-Step MVM has been developed for analysis of aroma compounds in fermented food samples. Two different DHS sampling parameter sets are sequentially performed for both volatile compounds and hydrophilic and/or polar compounds, while minimizing or eliminating ethanol and water. The performance of the method was demonstrated by analyzing aroma compounds in butter, shortening, and two different bread samples which were made with butter or shortening.

The combination of 2-Step MVM and a powerful set of software packages such as NIST AMDIS deconvolution, Aroma Office database and Mass Profiler Professional offers highly effective synergies for characterization of bread sample flavor profiles.

## References

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