Application Note

Food and Beverage Testing

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Comparing the Chemical Profiles of Plant-Based and Traditional Meats Using GC/MS-Based Metabolomics

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Abstract

As the consumer interest and market for plant-based meat alternatives grows, understanding the nutritional differences between alternative and traditional meats is essential. This application note describes an untargeted GC/MS-based metabolomics approach to comparing the chemical profiles of a popular plant-based meat alternative and grass-fed ground beef that uses an Agilent 7890 gas chromatograph (GC) system coupled to an Agilent 5977 GC/MSD. Samples were derivatized to simplify chromatography and render polar metabolites more volatile for GC/MS analysis. Statistical and multivariate analysis of the acquired and processed GC/MS data revealed that that 90% of the annotated compounds differed between the plant-based alternative meat and grass-fed ground beef samples. The ground beef and plant-based products each contained several compounds that were found in much smaller quantities, or not at all, in the other product. These results indicate differences in organic composition even though the nutritional labels on the back of the products were similar.

Heat maps, PCA score plots, VIP plots, and clustering of compounds into metabolite classes provided further insights into the differences between the types of meat products. The biological significance of the comparative data was studied using online databases and pathway analysis tools.

Introduction

Meeting the dietary requirements of a growing global population while addressing the health concerns and sustainability issues associated with the consumption of meat has increased consumer and scientific interest in plant-based alternatives. As the popularity of these alternatives grows, it is important to understand whether they are nutritionally adequate substitutes for traditional meats, and if they provide lesser, equal, greater, or even complementary nutritional value.

A nutritional facts panel (NFP) is required on packaged foods in many countries; it is intended to communicate a food's nutritional value by listing factors such as calorie count, and amounts of sugar, fat, vitamins, and minerals. The NFPs of commercially available plant-based meat alternatives and ground beef products are nearly identical.[1] However, studies have shown that foods are complex and contain a wide variety of nutrients not listed on NFPs including phenols, antioxidants, peptides, amino acids, fatty acids, and biogenic amines that play a role in health.[2]

Discovery metabolomics—also known as untargeted metabolomics—using hyphenated mass spectrometry (MS) techniques is an approach to measuring the large numbers of nutrients and other compounds present in food matrices. Combined with an appropriate sample preparation method, gas chromatography/mass spectrometry (GC/MS) in particular provides a robust solution for in-depth profiling of complex samples. Various nutrients—including amino acids, phenols, vitamins, unsaturated fatty acids, and dipeptides with potentially important physiological, anti-inflammatory, and immunomodulatory roles—can be analyzed using GC/MS. After GC/MS analysis, the data are processed to determine the differences between sample sets. Compounds of interest are identified using tools such as spectral libraries and chemical databases.

This application note describes the use of a 7890 GC system coupled to a 5977 GC/MSD for in-depth determination of the chemical differences between grass-fed ground beef and a popular plant-based meat alternative. Compound identification was facilitated using a custom library built on the Agilent Fiehn GC/MS Metabolomics RTL Library.

The complete workflow, including sample preparation, GC/MS, and data analysis methods, was developed and described by Van Vliet, *et al.* in their report "A metabolomics comparison of plant-based meat and grass-fed meat indicates large nutritional differences despite comparable Nutrition Facts panels."[3]

Experimental

An overview of the experimental workflow is provided in Figure 1.

Sample preparation and derivatization

Eighteen 113 g (4 oz) samples each of commercially available packaged plant-based meat alternative (PB) and grass-fed ground beef (GB) were analyzed. As presented by Van Vliet, *et al.*, patties were cooked in a nonstick skillet to 71 °C and one-gram microcore samples were taken, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. The microcores were powdered under liquid N_2 and homogenized in 50% aqueous acetonitrile containing 0.3% formic acid. Sample homogenates (100 µL) were then transferred into 1.5 mL autosampler vials. The proteins in the homogenates were then crash precipitated with

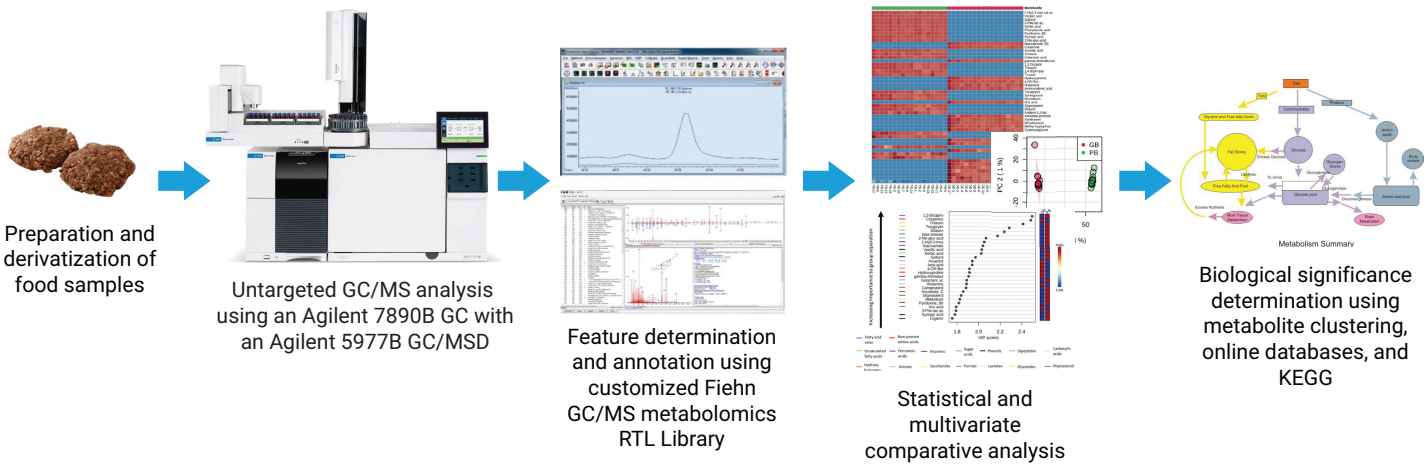

Figure 1. Overview of GC/MS-based metabolomics workflow used to compare the chemical profiles of plant-based and traditional meats.

750 µL of dry methanol and centrifuged for 5 minutes. The crash solvent was spiked with D27-deuterated myristic acid (D27-C14:0) as an internal standard for retention time locking.

For derivatization, the supernatant (700 µL) of each homogenate was transferred to fresh glass vials and dried with toluene as an azeotropic drying agent. Methoxyamine hydrochloride (25 µL) was then added to each sample, followed by sample incubation at 50 °C for 30 minutes for methoximation of certain reactive carbonyl groups. In particular, methoxylation of sugars reduces the number of isomers present, simplifying subsequent data analysis. Compounds were made volatile for GC/MS analysis by replacement of easily exchangeable protons with trimethylsilyl (TMS) groups using N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; 75 µL per sample) at 50 °C for 30 minutes.

GC/MS instrumentation and analysis

GG/MS analysis was carried out using a 7890 GC coupled with a 5977 GC/MSD. Injections were made using an Agilent 7693A Automatic Liquid Sampler (ALS) with an Agilent 7683B GC Injector. The 7890 GC was equipped with an Agilent Multimode Inlet (MMI). Two wall-coated open-tubular (WCOT) Agilent J&W DB-5ms Ultra Inert GC columns (15 m × 25 mm, with 0.25-µm luminal film, part number 122-5512 UI) were connected in series by a purged Ultimate union (PUU). The luminal film is a nonpolar, thermally-stable, phenyl-arylene polymer similar in performance to traditional 5%-phenyl-methylpolysiloxane films. The MMI in combination with a midcolumn PUU enabled hot backflushing of the upstream half of the column at the end of each run to reduce fouling of the GC/MS instrumentation with heavy contaminants and carry over between injections.

The workflow used a modified version of the Fiehn method[4], a dedicated GC/MS analysis method for use with the Fiehn GC/MS Metabolomics RTL Library. Instead of a precolumn, the method used a heat ramp in the MMI to retain nonvolatile compounds in the inlet. Retention indexing with the same nominal column dimensions makes the modification possible. Prior to each daily run (two total), the starting inlet pressure was empirically adjusted so the retention time of the TMS-D27-C14:0 standard was 16.727 minutes. Following distillation in the MMI, the GC oven was ramped from 60 to 325 °C at 10 °C/min. Using these parameters, the derivatized compounds elute from the column at known times within specific tolerance of plus or minus 1 minute.

The 5977 MSD was equipped with an Agilent Extractor EI Source for enhanced response for active compounds and late eluters. The instrument was operated in electron ionization (EI) mode with a scan range of 50 to 600 *m/z*. Data were acquired using Agilent MassHunter software. The GC and MS parameters are provided in Table 1.

Data analysis and visualization

Raw GC/MS data acquired with MassHunter software were imported into the NIST Automatic Mass Spectral Deconvolution and Identification Software (AMDIS version 2.73) for processing including deconvolution, detection of spectral features, and feature annotation. Deconvoluted spectra were annotated using both

Table 1. GC and MS parameters.

Parameter	Value
Gas Chromatograph	
Model	Agilent 7890 GC with an MMI
Columns	Agilent J&W DB-5ms Ultra Inert GC Column, 15 m × 25 mm, 0.25 µm (p/n 122-5512 UI)
Injector Mode	Split, 1:10
Injector Liner	Agilent Inlet liner, Ultra Inert, split, low pressure drop, glass wool, 25/pk (p/n 5190-316)
Injection Volume	1 µL
MMI Temperature Program	Initial 70 °C for 0.02 min, 600 °C/min to 325 °C
Nominal Flow Rate	1 mL/min
Oven Temperature Program	Initial 60 °C for 1 min, 10°C/min to 325 °C
Run Time	31.5 min
Equilibration Time	1.003 min
Mass Spectrometer	
Model	Agilent 5977 GC/MSD
Ion Source	Extractor EI source
Ionization Mode	EI, 70 eV
Tune Method	Etune
Acquisition Mode	Scan, 50 to 600 *m/z*
GC Interface/Transfer Line Temperature	290 °C
Ion Source Temperature	230 °C
Quadrupole Temperature	150 °C

GC retention time (RT), and EI mass spectral fragmentation pattern based on a custom retention-time-locked spectral library of metabolites built on the Fiehn GC/MS Metabolomics RTL Library (part number G1676-90000). The Fiehn GC/MS metabolomics RTL Library is the most comprehensive commercially available GC/MS library of metabolite spectra. It currently contains over 1,400 entries for approximately 800 common metabolites, including spectra corresponding to partial derivatization of metabolites under the conditions described here. Each entry includes the name, CAS, and PubChem numbers of the native molecule for easy compound recognition and subsequent literature, software, and pathway searching. The library and method can easily be expanded with more compounds to meet specific application needs. Additional spectra were added to the library by running pure reagent standards, from the Golm Metabolome Database, and from the Agilent Wiley with NIST MS Library software.

Data processed using AMDIS were manually interrogated to address miscalls and ambiguities in isomeric and other similar species. Compounds were kept for further analysis if detected in ≥80% of samples of either the PB or GB. If a signal for a compound was found in ≥80% of samples of one type but not present in all of the samples of the other type it was assumed absent and given a value close to one prior to log-base-two transformation. After log transformation, the results were tested for normality using Kolmogorov-Smirnov testing ($p < 0.05$). The differences in the abundances of metabolites between the two sample groups were compared using the Wilcoxon rank sum test with Benjamini-Hochberg adjusted p values at 5% (false discovery rate adjusted $p < 0.05$).

Differences in the profiles of the two sample groups and identification of compounds contributing to those differences were visualized using a ranked heat map of the top 50 compounds based on Pearson distance measure and Ward clustering algorithm, and unsupervised principal component analysis (PCA) plots generated using MetaboAnalyst (version 4.0). Partial least square-discriminant analysis (PLS-DA) was applied to determine the variable importance in projection (VIP) of each compound and a VIP plot was generated to rank individual compounds for their ability to discriminate between PB and GB.

Compounds of interest were clustered into metabolite classes according to structural similarity using ChemRICH Chemical Similarity Enrichment Analysis for Metabolomics online software. Bio-activities and health implications of specific compounds were investigated by interrogating the FooDB and PubChem online databases using the Chemical Abstracts Service (CAS) number of the compound of interest. Metabolic pathways were explored using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Results and discussion

GC/MS performance for derivatized compounds

Samples were derivatized to simplify chromatography and make polar metabolites more volatile for GC/MS analysis. Using methoxyamine HCl in pyridine stabilizes reactive carbonyls (C=O) such as alpha-keto (=2-oxo) acids, which are prone to decarboxylation, enolization, and other side reactions that would result in more complex chromatograms. For example, many of the sugars are structural isomers. Methoxyamination of these sugars can reduce isomer formation. Replacement of the exchangeable protons with the trimethylsilyl (TMS) $-Si(CH_3)_3$ [mass = 73] makes polar compounds more volatile.

Despite the complex sample matrix, by applying derivatization the GC/MS method provided adequate separation and detection to facilitate subsequent data processing and analysis.

Comparative metabolomics analysis

Analysis of GC/MS data using false-discovery-rate-adjusted statistical and multivariate methods revealed that 171 out of 190 annotated compounds (90%) were different ($p < 0.05$) between the PB and the GB samples. Many compounds were found exclusively (31) or in greater quantities (67) in PB, while many other compounds were found either exclusively (22) or in greater quantities (51) in the GB compared with the plant-based alternative meat.

A ranked heat map of the 50 compounds that contributed most to the difference between PB and GB enabled easy visualization of the results, providing substantial evidence that the composition of the sample groups was quite different despite their similar NFPs (Figure 2). The score plot (Figure 3) from unsupervised PCA showed a distinct separation in components, with 97.3% of the variance explained by the first principal component (PC1), likewise indicating significant differences between PB and GB. The VIP plot (Figure 4) generated from the PLS-DA models enabled visualization of the ranking of individual compounds that discriminated between the PB and GB.

Individual compounds of interest were clustered into metabolite classes according to structural similarity using ChemRICH. Twenty-four classes with ≥3 structurally similar metabolites were found. Of the 24 metabolite classes, 23 differed significantly (false discovery rates adjusted $p < 0.05$) between the GB and the PB. The metabolite classes that most discriminated between GB and

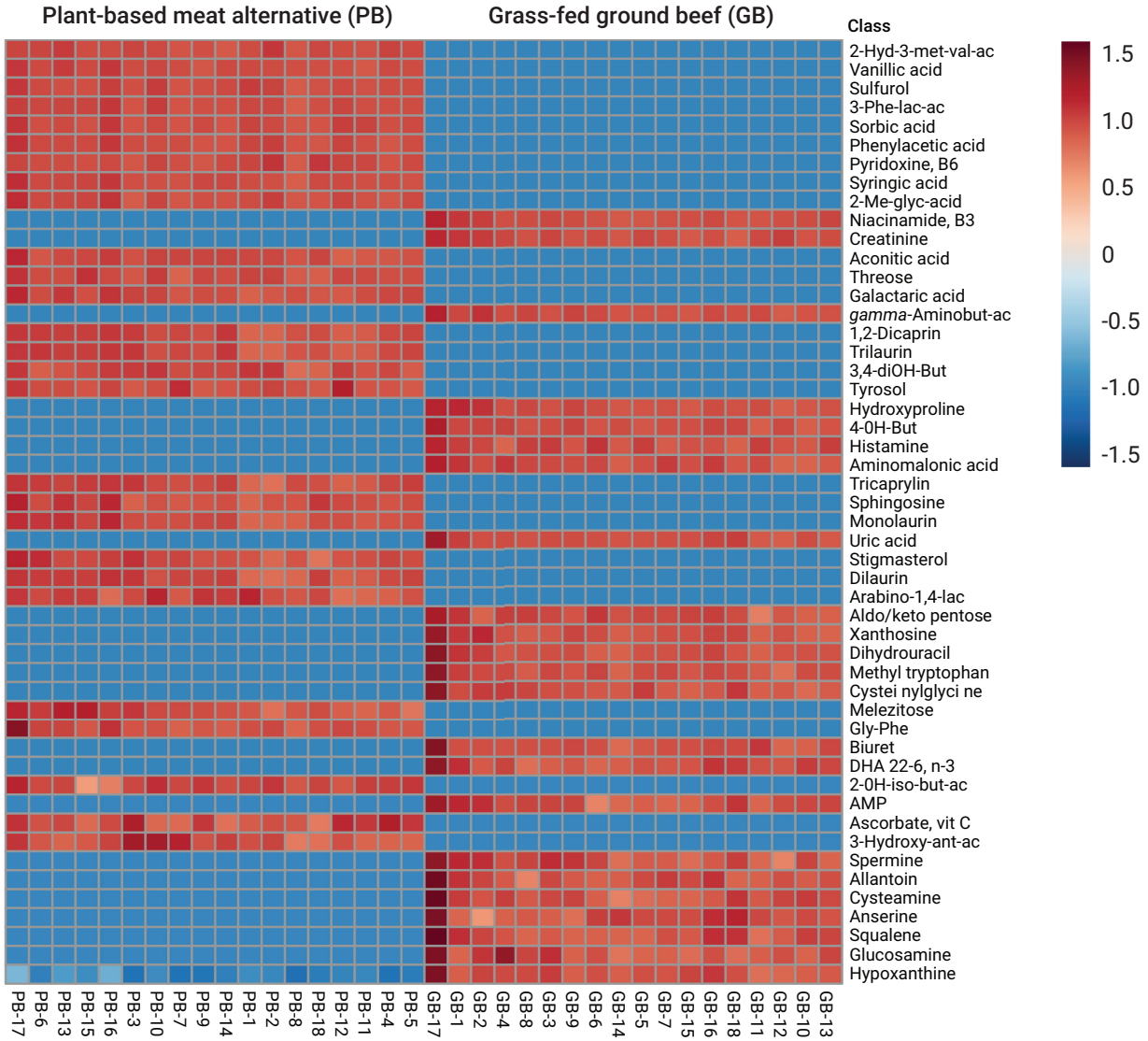

Figure 2. MetaboAnalyst-generated heat map of the top 50 compounds ranked by p values (lowest to highest) that were significantly different ($p < 0.05$) between the GB and the PB. Red (intensity ranges from 0 to 1.5) indicates the higher abundance (upregulation) of a compound, while blue (intensity ranges from −0 to −1.5) indicates the lower abundance (downregulation) of a compound. The coding below the heat map represents the individual samples analyzed. Figure courtesy of Van Vliet, S. *et al*.[3]

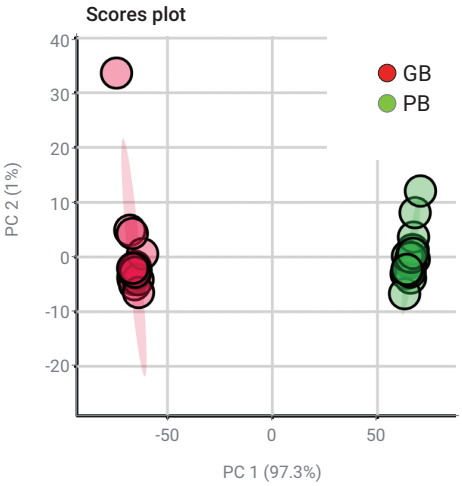

Figure 3. MetaboAnalyst-generated score plot created using unsupervised PCA. Figure courtesy of Van Vliet, S. *et al.*[3]

the PB were amino acids, nonprotein amino acids, saccharides, saturated fatty acids, dicarboxylic acids, phenols, dipeptides, sugar alcohols, vitamins, glycerides, unsaturated fatty acids, and amino alcohols (Figure 4). Metabolites in metabolite classes such as phenols, tocopherols, and phytosterols were found exclusively or in greater abundance in the plant-based meat alternative.

Interrogation of the FooDB and PubChem online databases using CAS number and the KEGG yielded information about the biological significance of the metabolite classes that differentiated GB and PB. For example, the PB contained more tocopherols (α, γ, and δ), which, according to published reports, are compounds with vitamin E activity known for antioxidant properties.[5] The polyunsaturated fatty acids, arachidonic acid (ARA, C20:4, ω-6) and docosahexaenoic acid (DHA, C22:6, ω-3), were found exclusively (ARA) or in greater quantities (DHA) in the GB samples. These fatty acids are major constituents of the brain phospholipid membrane and have important roles in cognition, immunomodulation, platelet function and cell signaling, and their deficiencies are associated with cognitive decline and increased risk of cardiovascular disease.[6]

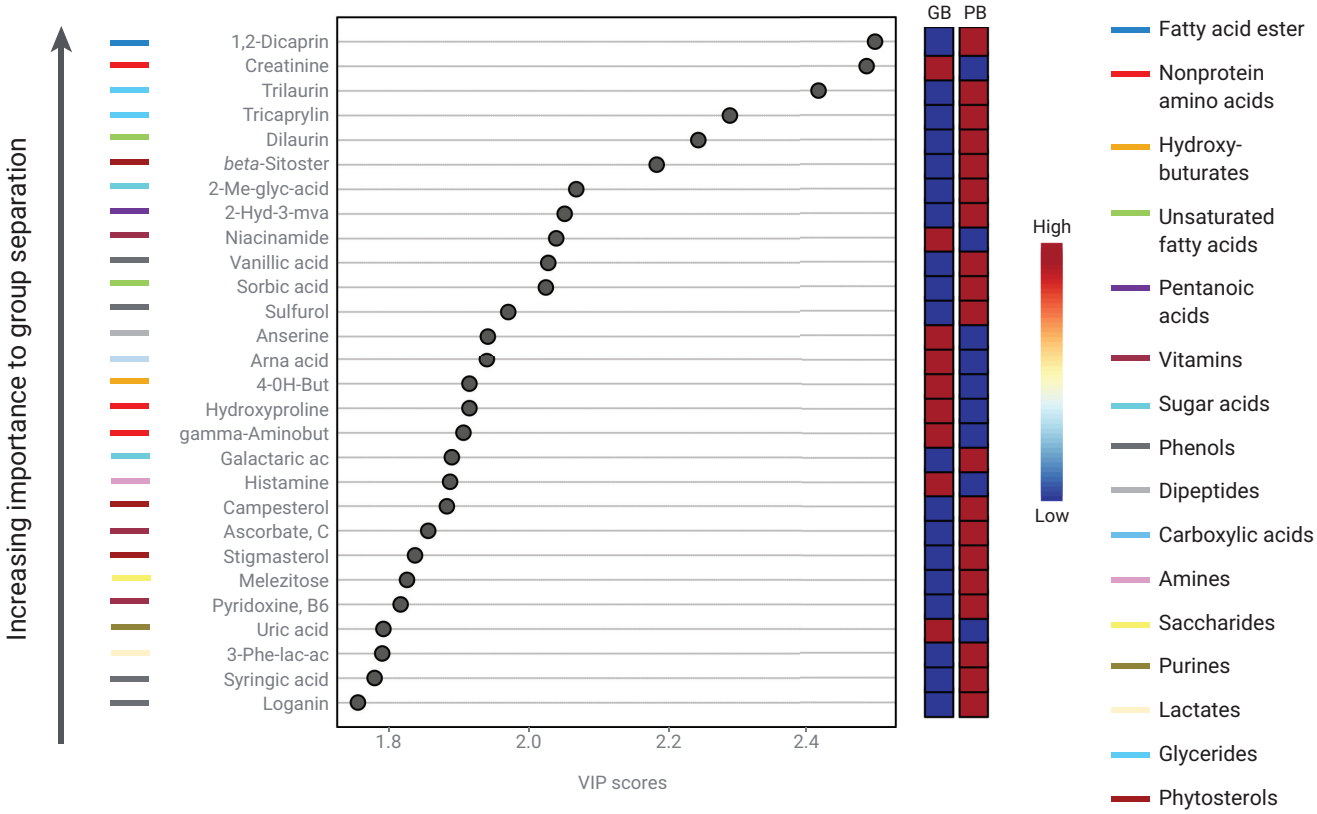

Figure 4. VIP plot generated from the PLS-DA models shows compounds ranked according to their prognostic importance (VIP scores) in separating the chemical profiles of GB and PB. The boxes on the right of the plot show the relative concentrations (blue: low to red: high) of each compound in the GB and PB samples. The colored bars at the left of the ranked compounds list the metabolite class of the ranked compounds that were identified using ChemRICH. Figure courtesy of Van Vliet, S. *et al.*[3]

Taken together, the results suggest that despite nearly identical NFPs, GB and PB are not the same and therefore not nutritionally interchangeable. Though more research is necessary to know for sure, the two different types of meats appear to provide complementary nutritional value.

Method considerations

While GC/MS is a highly robust and relatively inexpensive approach to untargeted sample profiling, it is not well suited to analysis of all metabolites. Other hyphenated techniques, for example LC/MS, can provide additional complementary information about the profiles of the samples analyzed. Table 2 lists the analytes best analyzed by techniques other than GC/MS.

Agilent provides a wide range of robust workflows, including analytical instrumentation and software, for performing global metabolite profiling by GC/MS, LC/MS, CE/MS, and SFC/MS. Though in this application note various custom macros and freeware were used to process and analyze GC/MS MassHunter data, Agilent Mass Profiler Professional (MPP) software is an alternative that provides integrated identification/annotation of compounds and pathway analysis for metabolomics studies. MPP can be applied to any MS-based differential analysis to determine relationships among two or more sample groups and variables. It also offers advanced statistical analysis and visualization tools for GC/MS, LC/MS, CE/MS, and ICP-MS data.

Table 2. Compounds difficult to analyze by GC/MS.[7]

Concern	Example compounds
Compounds that are too light, eluting in solvent front before the MS filament is ignited	Acetic acid, ammonia, hydrogen sulfide
Compounds that are too heavy, have a boiling point that is too high, or that thermally degrade below 325 °C	Heme B, bilirubin, biliverdin, riboflavin (B2), folate (B9), cobalamin (B12)
Nucleotides and other compounds with phosphoanhydride bonds (P-O-P)	Acyl coenzyme As (CoA), nicotinamide adenine dinucleotide (NAD+ ⇔ NADH), ADP, ATP, uridine diphosphate (UDP) glucose
Inherently reactive or otherwise unstable metabolites	α-Aminomalonic acid, adenosine-3',5'-cyclic monophosphate (cAMP) 2-nonenal and 4-hydroxynonenal (4-HNE)
Quaternary amines	Choline, acetylcholine, phosphocholine, arnitine, acetylcarnitine, N6,N6,N6-trimethyllysine, betaine (N,N,N-trimethylglycine), thiamin (B4), trigonelline, trimethylamine-N-oxide (TMAO)
Certain guanidinium compounds	Arginine, arginosuccinate, creatine, phosphocreatine

Conclusion

Given the consumer interest and market growth in plant-based meat alternatives, understanding the differences between alternative and traditional meats beyond what is typically provided in NFPs is essential. With sample derivatization, GC/MS provides an analytical solution that enables measurement of various and numerous compounds with potentially important physiological roles, including amino acids, phenols, vitamins, unsaturated fatty acids, and dipeptides.

In this application note, the 7890 GC with the 5977 GC/MSD provided data well suited to in-depth profiling of the chemical differences between derivatized GB and PB samples. False-discovery-rate-adjusted statistical and multivariate analysis of GC/MS data revealed that that 90% of annotated compounds differed between the PB and the GB samples. Many compounds were found exclusively or in greater quantities in the PB, while many others were found either exclusively or in greater quantities in the GB. Heat maps, PCA score plots, and VIP plots that are commonly used to visualize metabolomics data, as well as clustering of compounds into metabolite classes, provided further insight into the differences between the types of meats. The biological significance of the comparative data was subsequently studied using online databases and pathway analysis tools. The GC/MS-based metabolomics workflow provided substantial evidence that despite nearly identical NFPs, GB and PB are not the same and thus not nutritionally interchangeable. Overall, the workflow presents a robust and relatively inexpensive approach to profiling many types of food samples.

Acknowledgments

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References

1. International Food Council. A Consumer Survey on Plant Alternatives to Animal Meat. **2020**. https://foodinsight.org/wp-content/uploads/2020/01/IFIC-Plant-Alternative-to-Animal-Meat-Survey.pdf. (Accessed April 14, 2022).
2. Barabsi, A. L. *et al.* The Unmapped Chemical Complexity of our Diet. *Nat. Food.* **2020**, *1*, 33–37. https://doi.org/10.1038/s43016-019-0005-1.
3. Van Vliet, S. *et al.* A Metabolomics Comparison of Plant-Based Meat and Grass-Fed Meat Indicates Large Nutritional Differences Despite Comparable Nutrition Facts Panels. *Sci. Rep. 11*, 13828. https://doi.org/10.1038/s41598-021-93100-3.
4. Fiehn, O. *et al.* Identification of Uncommon Plant Metabolites Based on Calculation of Elemental Compositions Using Gas Chromatography and Quadrupole Mass Spectrometry. *Anal. Chem.* **2000**, *72*, 3573-3580. https://doi.org/10.1021/ac991142i.
5. Wu, G. Important Roles of Dietary Taurine, Creatine, Carnosine, Anserine and 4-Hydroxyproline in Human Nutrition and Health. *Amino Acids* **2020**, *20*, 329–360. https://doi.org/10.1007/s00726-020-02823-6.
6. Ruxton, C. H. S. *et al.* The Health Benefits of Omega-3 Polyunsaturated Fatty Acids: a Review of the Evidence. *J. Hum. Nutr. Diet*. **2004**, *17*, 449–459. https://doi.org/10.1111/j.1365-277X.2004.00552.x.
7. Wang, H. M *et al.* Recommendations for Improving Identification and Quantification in Non-Targeted, GC-MS-Based Metabolomic Profiling of Human Plasma. *Metabolites* **2017**, *7(3)*, E45. https://doi.org/10.3390/metabo7030045.

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