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Alternative Proteins Testing

Application Compendium

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Introduction

Alternative Proteins Testing

Alternative proteins are foods, ingredients, or beverages that provide all or a substantial amount of protein from non-animal sources. Whether your lab is testing cell- or plant-based meats, technologies such as ICP-MS, triple quadrupole (QQQ) liquid or gas chromatography mass spectrometry (LC/MS or GC/MS), or high-performance liquid chromatography (HPLC) are recommended by experts for robust testing purposes.

Agilent workflow solutions, designed for the food and beverages industry, are a one-stop shop for testing alternative proteins products to validate their authenticity, nutritional information, and safety for the consumer market.

This application compendium offers a comprehensive introduction to applications, methods, and instruments used in alternative proteins testing. This resource is intended to assist inexperienced scientists working in this field, or experienced analysts who are new to alternative proteins testing.

Extensive expertise in food testing

As a market leader in food testing for over 40 years, Agilent offers full workflows specifically designed to help advance this emerging market to its full potential.

Taste and Flavor

Chapter 1

Application Note

Food Testing

Agilent

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LC/Q-TOF Analysis and Nontargeted Chemometric Profiling of Meats and Plant-Based Alternatives

Food sensory testing using the Agilent 1290 Infinity II LC and Agilent 6546 LC/Q-TOF

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Abstract

Meat-alternative sources of protein, including plant-based and cell-based foods, are gaining popularity globally due to a combination of consumer interest, regulatory changes, and global food systems. For example, as Singapore aims to achieve 30% of its food production levels through self-production by 2030, many established food companies and startups are developing meat-substitute products. The main drivers of Singapore's food production target are around health and environmental concerns. Historically, plant-based meat substitute foods have struggled to achieve the same texture and taste as animal meats. However, recent analogs of plant-based meats are significantly more similar in taste, texture, and composition as traditional meats due to technological advances in production methods. This application note describes a nontargeted profiling method to characterize chemical components of unknown foods, using a high-resolution accurate mass LC-Q/TOF. Also, various statistical tools are presented that translate accurate mass LC/Q-TOF data into more easily understandable information. Principal component analysis (PCA) of the data can be used to identify compounds, abundance distribution of the compounds in different samples, and how the compounds correspond to target taste profiles. Heat maps and hierarchical clustering of raw ingredients show similar distribution of proteins with target taste profiles.

Introduction

Food sensory evaluation is a key method to assess the flavor quality of foods because it measures what consumers perceive. It is, however, subjective. As technology advances, more objective and measurable methods such as liquid chromatography with mass spectrometry (LC/MS) will be used. The five basic tastes (i.e., sweet, salty, sour, bitter, and umami) can now be characterized by LC/MS and the data can be used for the optimization of the overall taste of foods.

Alternative meats are meant to substitute animal-based meat. However, key barriers to consumer adoption have been identified as taste, texture, and nutrition. Testing is critical to ensuring that equivalent health benefits and experience for customers of eating alternative meat foods is achieved. Therefore, there are many studies that compare the difference in nutrition and taste levels between animal-derived meat and meat-alternative products.[1]

Targeted analysis is focused on known groups of nutrients or flavor compounds. The results from targeted analytical methods and sensory evaluation tests may differ as compounds that are not in the targeted list may contribute to the overall taste. In contrast, nontargeted high-resolution accurate mass analysis is not restricted to a specific group of compounds. In an unbiased manner, compounds in the proteins can be profiled, identified, and comparisons made between alternative meat and real meat. As in food sensory analysis testing, nontargeted LC/MS methods do not analyze a particular flavor profile but are unbiased, and focus on total compound profiles, much like taste buds.

Apart from finding the different compounds that contribute to various taste profiles, their abundance in each protein is equally important. Although standards are not often available for quantitative analysis, the relative intensity differences of compounds in the various proteins can be used to tell them apart. A person may only distinguish flavors when there is a drastic abundance difference in some compounds. In this study, quadrupole time of flight (Q-TOF) LC/MS and statistical software were used to identify and differentiate flavor profiles. The method will help the development of equivalent flavor profiles in plant-based protein foods.

Experimental

Solvents

Agilent ultrapure LC/MS grade methanol (part number 5191-4497), acetonitrile (part number 5191-4496), and water (part number 5191-4498) were used. Formic acid for LC/MS (Fluka from Honeywell) and ammonium formate for LC/MS (LiChropur, MerckMillipore) were also used.

Materials

Agilent InfinityLab solvent bottles with cap (part number 9301-6528) were used for the mobile phase. The open-top caps were fitted with an Agilent InfinityLab Stay Safe cap, GL45, one port, one InfinityLab vent valve, 3.2 mm od fitting PTFE insert (part number 5043-1217). The O-ring from the heavy-duty vacuum bottle cap was used to seal the PTFE insert in the bottle. The standard PTFE solvent line was threaded through the PTFE insert. An Agilent stainless steel 12 to 14 µm solvent bottle inlet filter (part number 01018-60025) was then fitted to the solvent line.

Samples

The plant-based meats described in Table 1 were commercially available products. The real meats included minced raw products that were bought from a market.

Table 1. Plant-based alternative meat samples and sample codes.

Sample Code	Description of Food Product
PBC 1	Plant-based chicken
PBC 2	Plant-based chicken
PBB 3	Plant-based beef
PBB 4	Plant-based beef
PBP 5	Plant-based pork
PBP 6	Plant-based pork
PBP 7	Plant-based pork

Sample preparation

All sample collection and preparation steps were done in polyethylene or polypropylene containers. Fifteen and 50 mL high-performance polypropylene centrifuge tubes with plug caps (VWR International Ltd., UK) were used throughout. Agilent 2 mL screw top amber glass autosampler vials (part number 5182-0716) with screw caps (part number 5185-5862) were used. The samples were weighed in a centrifuge tube, 70/30 methanol/water was

then added to the samples at a ratio of 1:2. The samples were vortexed for 10 minutes and centrifuged at 4,000 rpm for 15 minutes. The samples were re-extracted under the same conditions. The extracts were then filtered into the autosampler vials using an Agilent 0.45 µm polyethersulfone (PES) filter (part number 5190-5276).

Instrumentation

An Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II high speed pump (part number G7120A) was used as the HPLC. The system also featured an Agilent 1290 Infinity II multisampler (part number G7167B) fitted with an Agilent InfinityLab sample thermostat and Infinity multiwash option. The LC included an Agilent 1290 Infinity II multicolumn thermostatted column compartment (part number G7116B). An Agilent 6546 Q-TOF MS system (part number G6546A) was used for accurate mass measurements. The mass spectrometer was run in "Data Independent All Ions Fragmentation" scan acquisition mode where all ions passed through the Q-TOF collision cell operating under positive ion polarity.

Data analysis was done using Agilent MassHunter Qualitative Analysis 10.0, Profinder 10.0, and Mass Profiler Professional 15.1 software.

Table 2. Agilent 6546 LC/Q-TOF LC/MS system (G6546A) operating conditions.

HPLC Conditions	
Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
Injection Volume	5 µL
Mobile Phase	A) 10 mM NH_4F + 0.1% FA in DIW B) Acetonitrile
Initial	A) 98 % 10 mM NH_4F + 0.1% FA in DIW B) 2 % acetonitrile
Gradient	Time (min) %A %B 0.30 98.0 2.0 7.27 20.0 80.0 10.27 1.0 99.0 12.00 1.0 99.0 12.10 98.0 2.0 15.00 98.0 2.0
Flow	0.4 mL/min
MS Conditions	
ESI	Positive
Source Parameters	
Gas Temperature	300 °C
Gas Flow	11 L/min
Nebulizer	35 psi
Sheath Gas Heater	350 °C
Sheath Gas Flow	11 L/min
Capillary	3,500 V
V Charging	1,000

Results and discussion

The LC/Q-TOF data were acquired using an All Ions full scan from *m/z* 100 to 1,700 Da and fragmentation spectrum at three different collision energies (10, 20, and 40 V). For compound identification, the accurate mass data were searched against a custom MS fragmentation library consisting of compounds that may impact taste. These compounds included amino acids, short peptides, nucleotides, fatty acids, and various vitamins.

As shown in Figure 1, an overall view of the raw data shows some slight differences between the spectra of the actual meats and substitute meats. Also, it would be time-consuming to screen through the spectral library to identify an individual compound via a library match. Therefore, statistical analysis tools become useful in converting the raw data from the nontargeted analysis into more useable information.

For principal component analysis (PCA), three injections of each extract were performed to check the repeatability of data by observing the clustering of samples. Generally, it was observed that the replicates for each food sample were tightly clustered, indicating a high degree of repeatability in the method (Figure 2). Under the score plot view in Figure 2, each dot represents an injection of a sample. Protein samples were assigned distinct colored data points by target flavor profiles and individual products were assigned different shapes to differentiate them. This score plot view in Figure 2 shows which trends in the sample set contribute to the differences between flavor profiles and particular products. Also, the plot can show if different samples are similar by sharing the same general region in the PCA scores plot. The alternative protein foods were found to cluster well in their targeted flavor profile and there were significant differences between flavor profiles of each food-type, as expected. In contrast, in the PCA loading plot view (Figure 3B), each dot represents a compound. This plot provides information on which compounds impact the scores plot in the PCA. Compounds with the highest loadings (indicated by their symbols) on a principal component correlate with higher abundances of those compounds in the samples.

Figure 2 shows an overview of the distinct types of meat and their plant-based alternatives. From the two-dimensional (2D) PCA plot of nonvolatile compounds, each meat (e.g., chicken) and its plant-based equivalent (e.g., PBC 1 and PBC 2) are more similar to each other compared to the other meats (e.g., beef or pork).

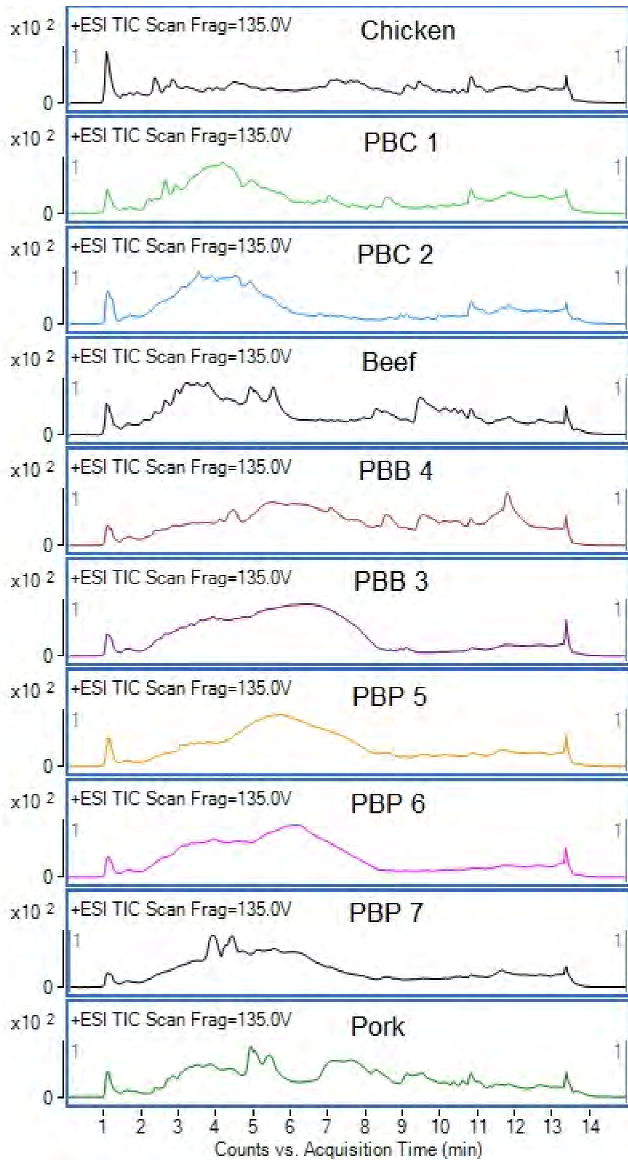

Figure 1. TIC overview of actual meats and plant-based meat equivalents.

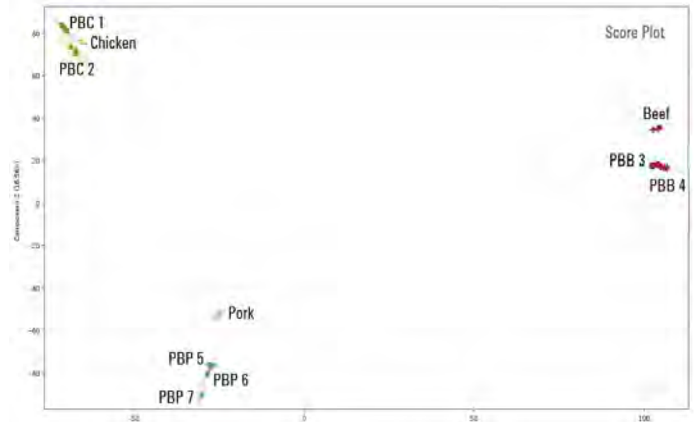

Figure 2. 2D PCA score plot of pork, beef, and chicken and their plant-based equivalents.

Viewing the PCA score plot and loading plot side by side for one meat-type makes it easy to correlate the compounds associated with the group of nutrients or flavor compounds. Figure 3A shows a 3D score plot for chicken, with different groups separated along each axis. The loading plot (Figure 3B) provides information on the compounds that cause the differences in the score plot.

A heat map is a data visualization technique that shows the abundance of a compound on a color scale, with red representing high abundance and blue low abundance. Heat maps allow users to quickly see compound abundance differences of a particular set of flavor profiles, as shown in Figure 4.

Free amino acids that form on the surface of meat at typical cooking temperatures provide the "grilled-meat" flavors that consumers like.[1] It is important, therefore, that manufacturers of plant-based beef foods control the abundance of various amino acids in their products. Figure 4 shows that plant-based beef products, PBB 3 and 4, contain some of the bitter amino acids in higher abundance than real beef. These amino acids may affect the final taste of these products. Profile data of amino acids in foods can be used to select base ingredients that provide a similar abundance of a class of flavor compounds to the desired one.

The plant-based chicken, PBC 2, has more short peptides, while sample PBC 1 has more nucleotide flavor enhancer, which may come from soy or bean-based ingredients. Real chicken meat is known to contain fatty acids, amino acids, and acetyl carnitine, as shown in Figure 3. Chicken is at the top right of the plot, between Y and Z-axis. PBC 1 is near the origin of the three axes, and PBC 2 is at the bottom end of the X-axis. Compounds such as glutamine-glutamine (glu-glu), adenosine diphosphate (ADP), inosine-5-diphosphate (IDP), and valine-glutamine (val-glu) would provide plant-based meats with more of the umami flavor of real chicken.[2]

Higher levels of these flavors in PBC 1 and PBC 2 would be shown by a shift in the compound data points up the Y-axis, closer to the region of chicken.

The nucleotide hypoxanthine, which is a naturally occurring purine derivative, plays a critical role in the umami flavor of chicken. However, with its low purine (guanine) content, PBC 2 may be a healthier choice for reducing the formation of uric acid, which can lead to gout.[3]

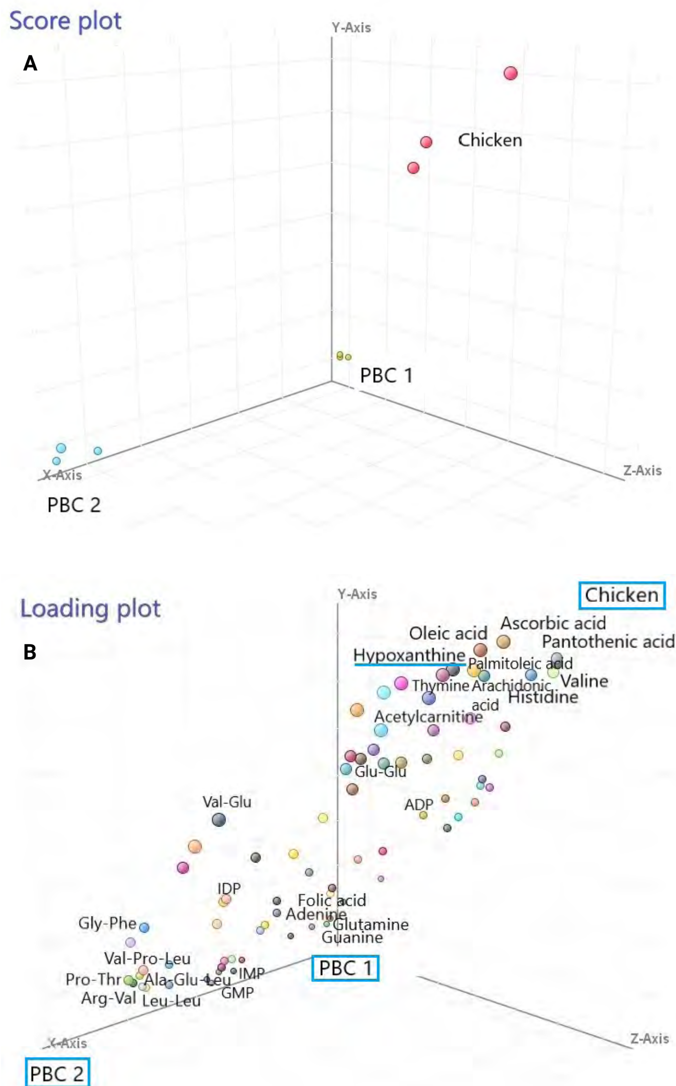

Figure 3. 3D PCA score plot (A) and loading plot (B) of chicken and its plant-based alternatives, PBC 1 and PCB 2.

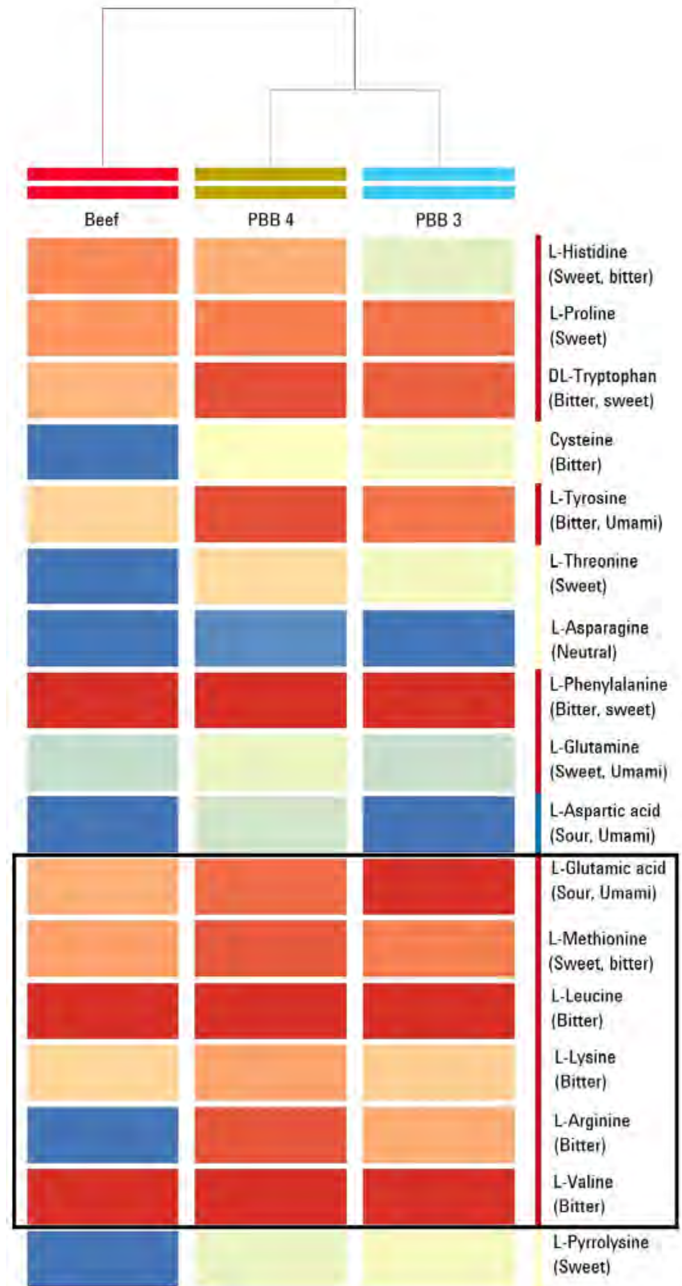

Figure 4. Heat map of amino acids in beef and its plant-based equivalents, PBB 3 and PBB 4.

Conclusion

The flavor, texture, and nutritional value of meat-alternative protein sources are critical to consumer perception, acceptability, and assessment of value.

A nontargeted, data-independent, All Ions workflow using a high-resolution Agilent 6546A Q-TOF LC/MS system successfully profiled and identified many flavor compounds in chicken, beef, pork, and their plant-based alternatives. Agilent Mass Profiler Professional (MPP) software was used to determine relationships among the real meat and alternative plant-based meats using advanced statistical analysis and visualization tools. PCA score and loading plots are useful for comparing compounds in food products. Heat maps are also useful tools for visualizing the profiling of compounds, such as amino acids, in meat and commercially available plant-based meat substitute foods.

The comprehensive LC/MS data acquisition and statistical workflow provides manufacturers of alternate protein foods with critical molecular insights of their products. The profile data would help manufactures to fine-tune a product's ingredients to better replicate the taste of animal-derived meats.

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Application Note

Food Testing & Agriculture

Automation of Sample Derivatization Using the Agilent 1260 Infinity II Prime LC System for Amino Acid Analysis

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Abstract

Amino acid analysis plays an important role in research, industrial processes, and the assessment of food quality. This application note describes the use of an Agilent 1260 Infinity II Prime LC System for amino acid analysis in different beverages. Using the Agilent 1260 Infinity II Multisampler, amino acids were automatically derivatized by an injector program enabling a fast reaction time and high reproducibility. By application of the Agilent 1260 Infinity II Flexible Pump combined with an Agilent AdvanceBio Amino Acid Analysis (AAA) column, 23 analytes could be separated in a run time of 9 minutes showing a retention time precision of less than 0.1% RSD for all analytes. The multi-emission feature of the Agilent 1260 Infinity II Fluorescence Detector enabled sensitive detection of all amino acid derivatives in a single run showing LODs down to 0.225 pmol/µL. To show its potential for several application areas, the developed method is used for analysis of amino acids in a soft drink and red wine sample.

Introduction

Amino acids are small organic molecules containing an amino and carboxyl group that are relevant for formation of peptides and building of proteins. They are involved in several other biological functions as key precursors for a variety of nitrogenous compounds and hormones playing a role in chemical messaging and energy metabolism. As many amino acids are essential nutrients and present in a variety of food and beverages, reliable determination of amino acids for assessment of food quality is indispensable.

Amino acid analysis can be performed using a variety of analytical methods (e.g., CE/MS, GC/MS, or LC/MS). Automated derivatization of amino acids before analysis via reversed-phase chromatography in combination with fluorescence or diode array detection has proven value and eliminates the need of MS detection. In-loop derivatization with *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid for primary as well as 9-fluorenylmethylchloroformate (FMOC) for secondary amino acids provides a rapid and easy approach to overcome the insufficient analyte retention on reversed-phase columns as well as weak fluorescence and ultraviolet absorbance.[1]

This application note demonstrates the use of a 1260 Infinity II Prime LC System with a 1260 Infinity II Fluorescence Detector for sensitive and precise analysis of amino acids in different beverages. Thereby, the 1260 Infinity II Multisampler is used for automated, precolumn derivatization of amino acids, enabling fast and reproducible masking of amine functionalities with protective groups without any manual labor.

Experimental

Instruments

- Agilent 1260 Infinity II Flexible Pump (G7104C), no mixer equipped
- Agilent 1260 Infinity II Multisampler (G7167A), 100 µL loop (G4267-60311), 0.12 mm seat assembly (G4267-87012)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A), standard heat exchanger (G7116-60015)
- Agilent 1260 Infinity II Fluorescence Detector Spectra (G7121B), 8 µL FLD cell (G1321-60005)
- 0.12 mm id system capillaries

Software

Agilent OpenLab CDS (Version 2.4)

Analytical standards/samples

- Amino Acid Supplement (part number 5062-2478) containing: L-asparagine, L-glutamine, L-tryptophan, L-4-hydroxyproline, L-norvaline, and sarcosine (1 g each)
- AA standard, 1 nmol/µL (part number 5061-3330)
- AA standard, 250 pmol/µL (part number 5061-3331)
- AA standard, 100 pmol/µL (part number 5061-3332)
- AA standard, 25 pmol/µL (part number 5061-3333)
- AA standard, 10 nmol/µL (part number 5061-3334)

Extended amino acid (EAA) stock solution (1.8 nmol/µL) and internal standard (IS) stock solution (1 nmol/µL) were prepared in 0.1 M HCl in water. EAA stock solution includes L-asparagine, L-glutamine, L-tryptophan, and L-4-hydroxyproline. IS stock solution consists of L-norvaline and sarcosine. To avoid freeze-thaw cycles, it is recommended to distribute stock solutions (e.g., in 1 mL aliquots) and store them at –20 °C. The EAA and IS stock solutions were mixed 1:1 to get the desired volume of the EAA-IS stock solution. Afterwards, the EAA-IS stock solution can be diluted 1:10 with, for example, 100 pmol/µL AA standard solution to reach the final concentration of 90 pmol/µL for each amino acid and 50 pmol/µL for internal standards.

Samples (soft drink and red wine) were obtained from a local store and were filtered using 15 mm Agilent Captiva premium syringe filters with 0.2 µm regenerated cellulose membrane (part number 5190-5108)

All samples and standard mixtures were transferred into amber vials (part number 5182-0716) with glass inserts with polymer feet (part number 5181-1270) and screw caps (part number 5190-7024)

Columns

- Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm (part number 695975-322)
- Agilent AdvanceBio AAA guard columns, 3.0 × 5 mm, 2.7 µm, 3/pk (part number 823750-946)

Solvents

- **Mobile phase A:** Weigh in 2.8 g of sodium phosphate dibasic (Na_2HPO_4) and 7.6 g of disodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$), add 1.9 L of water and 3.5 mL of fuming hydrochloric acid (37%), mix until homogeneous, fill up to the total volume of 2 L with water. It is recommended to use an amber 2 L solvent bottle (part number 9301-6341) to avoid algae growth.

- **Mobile phase B:** acetonitrile/methanol/water 45/45/10 (v/v/v)
- Fresh, ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point of use cartridge (Millipak).
- Other mobile phase ingredients were obtained from Merck, Germany.

Reagents

- **Borate buffer:** 0.4 M in water, pH 10.2, 100 mL (part number 5061-3339)
- **FMOC reagent:** 2.5 mg/mL in ACN, 10 × 1 mL ampoules (part number 5061-3337)
- **OPA reagent:** 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 × 1 mL ampoules (part number 5061-3335)
- **Injection diluent:** 5 mL mobile phase A + 100 µL *ortho*-phosphoric acid (85%) from Merck, Germany

After opening an OPA or FMOC ampoule, the reagents should be distributed to amber vials (part number 5182-0716) with inserts (part number 5181-1270) and screw caps (part number 5190-7024) and stored for no longer than a week. Borate buffer and injection diluent can be transferred to vials without inserts. All reagents should be stored at 4 °C and should be exchanged daily.

Injector program

1. Draw 5.00 µL from location 1 (borate buffer) with the default speed using the default offset.
2. Wash the needle as defined in the method.
3. Draw 1.00 µL from the sample with the default speed using the default offset.
4. Wash the needle as defined in the method.
5. Draw 1.00 µL from location 2 (OPA reagent) with the default speed using the default offset.
6. Wash the needle as defined in the method.
7. Mix 7.00 µL from air with the default speed 10 times.
8. Draw 0.40 µL from location 3 (FMOC reagent) with the default speed using the default offset.
9. Wash the needle as defined in the method.
10. Mix 7.40 µL from air with the default speed 10 times.
11. Draw 32.00 µL from location 4 (injection diluent) with the maximum speed using the default offset.
12. Wash the needle as defined in the method.
13. Mix 20.00 µL from air with the maximum speed 5 times.
14. Inject.

Calibration/limit of detection

Calibration was conducted using 0.5 to 90 pmol/µL of analytical standards diluted in 0.1 M HCl in water. Limit of detection (LOD) values were determined using a signal-to-noise ratio (S/N) of at least 3.

Method parameters

Parameter	Value
Flow	1.2 mL/min
Timetable	0 min: 2% B, 0.2 to 6.8 min: 2 to 57% B, 7.0 to 7.4 min: 100% B, 7.5 min: 2% B
Stop Time	9 min
Needle Wash	5 s in flush port, wash solvent: 0.1 M HCl in water/acetonitrile 1/1 (v/v)
Column Compartment Temperature	40 °C
Advanced Multisampler Parameters	
Draw Speed	100 µL/min
Eject Speed	400 µL/min
Wait Time After Draw	1.2 s
Offset	0 mm
Vial/Well Bottom Sensing	Off
Advanced Pump Parameters	
Minimum Stroke/Primary Channel	Automatic
Flow Ramp Up/Down	50 mL/min²

FLD parameters

Parameter	Value
Multi-Emission	A) 455 nm B) 315 nm
Excitation	0 min: 345 nm 5.68 min: 265 nm
PMT Gain	10
Peak Width	>0.013 min (0.25 s resp. time) (37.04 Hz)

Results and discussion

To enable chromatographic separation by a reversed-phase column and detection via FLD, derivatization of the primary and secondary amine functionalities of amino acids was conducted using the 1260 Infinity II Multisampler. Therefore, in-loop derivatization with OPA and FMOC was executed by an injector program resulting in high reproducibility without any manual work. During the derivatization program, samples were alkalized with borate buffer, derivatized with OPA/FMOC, and quenched with injection diluent in around 3.5 minutes. After each draw of the sample or reagent, a 5-second needle wash step using a 1:1 mixture of sample solvent and acetonitrile was included to minimize carryover. By automation of these processes, a peak area precision (n = 10) of less than 1% relative standard deviation (RSD) could be achieved for the majority of the compounds (Table 1).

Due to the pH sensitivity of the reaction, derivatization of samples with an overly acidic pH value can result in a strongly decreased yield and consequently lower signal intensity. Thus, samples with a pH value below the pH value of the sample solvent (0.1 M HCl has a pH value of 1) might be neutralized before derivatization. For example, after a classical hydrolysis method for cleavage of proteins into amino acids using 6 M hydrochloric acid,[2] sample neutralization might be a necessary step before derivatization. For samples containing too many matrix components, a more selective method might be considered to avoid coelution of amino acids with matrix components.

Application of hydrophilic interaction chromatography with low-pH solvents and positive ion mode in MS detection using multiple reaction monitoring has shown to be a suitable approach for amino acid analysis.[3]

Table 1. Method validation showing calibration linearity, detector sensitivity, and repeatability (RSD calculations are based on 10 consecutive injections using the standard mixture with a final concentration of 4.5 pmol/µL for amino acids and 2.5 pmol/µL for internal standards).

Peak No.	Compound	Calibration Range (pmol/µL)	LOD (pmol/µL)	R^2	RSD RT (%)	RSD Area (%)
1	L-Aspartic acid	0.9 to 90	0.225	0.9999	0.08	0.95
2	L-Glutamic acid	0.9 to 90	0.225	1.0000	0.10	0.90
3	L-Asparagine	0.9 to 90	0.225	0.9996	0.04	0.90
4	L-Serine	0.9 to 90	0.225	0.9997	0.04	0.92
5	L-Glutamine	0.5 to 45	0.225	0.9985	0.03	0.83
6	L-Histidine	0.9 to 90	0.225	0.9998	0.02	1.62
7	Glycine	0.9 to 90	0.225	0.9997	0.02	0.56
8	L-Threonine	0.9 to 90	0.225	0.9998	0.02	0.80
9	L-Arginine	0.9 to 90	0.225	0.9997	0.04	0.90
10	L-Alanine	0.9 to 90	0.225	0.9998	0.02	0.95
11	L-Tyrosine	0.9 to 90	0.225	0.9998	0.01	0.95
12	L-Cystine	5 to 90	2.25	0.9990	0.05	2.61
13	L-Valine	0.9 to 90	0.225	0.9997	0.04	0.90
14	L-Methionine	0.9 to 90	0.225	0.9998	0.04	0.74
15	L-Norvaline	1.25 to 50	0.25	0.9998	0.03	0.80
16	L-Tryptophan	0.9 to 90	0.225	0.9998	0.03	0.84
17	L-Phenylalanine	0.9 to 90	0.225	0.9998	0.03	0.91
18	L-Isoleucine	0.9 to 90	0.225	0.9999	0.03	0.97
19	L-Leucine	0.9 to 90	0.225	0.9998	0.03	0.81
20	L-Lysine	4.5 to 90	0.9	0.9990	0.05	1.26
21	L-4-Hydroxyproline	4.5 to 90	0.9	0.9996	0.03	1.84
22	Sarcosine	5 to 50	1.25	0.9995	0.01	1.88
23	L-Proline	4.5 to 90	0.9	0.9995	0.02	4.15

An AdvanceBio AAA LC column and the corresponding guard column enabled separation of 23 target substances in a run time of 9 minutes (Figure 1). The superficially porous particle technology of the Agilent InfinityLab Poroshell column resulted in good chromatographic separation at a moderate backpressure of up to 510 bar.

OPA- and FMOC-derivatized amino acids can be detected via FLD using an emission wavelength of 455 and 315 nm, respectively. To detect both derivatives in a single run, the multi-emission functionality of the 1260 Infinity II Infinity Fluorescence Detector was used. Additionally, the excitation wavelength needs to be switched from 345 to 265 nm after the elution of L-leucine (Peak 19) to detect the FMOC-derivatized L-4-hydroxyproline (Peak 21), L-proline (Peak 22), and sarcosine (Peak 23). If FMOC-derivatized amino acids are not of interest, it is recommended to keep the excitation and emission wavelength at 345 and 455 nm, respectively, over the entire run time.

Calibration was performed using individual concentrations from 0.5 to 90 pmol/µL and showed an excellent linearity with R^2 values of around 0.99 for all amino acids (Table 1). LODs showed an S/N of at least 3 and ranged from 0.225 to 2.25 pmol/µL, showing the high sensitivity of the 1260 Infinity II Infinity Fluorescence Detector. The use of the 1260 Infinity II Prime LC system resulted in excellent retention time precision (n = 10), showing values lower than 0.1% RSD for all compounds (Table 1).

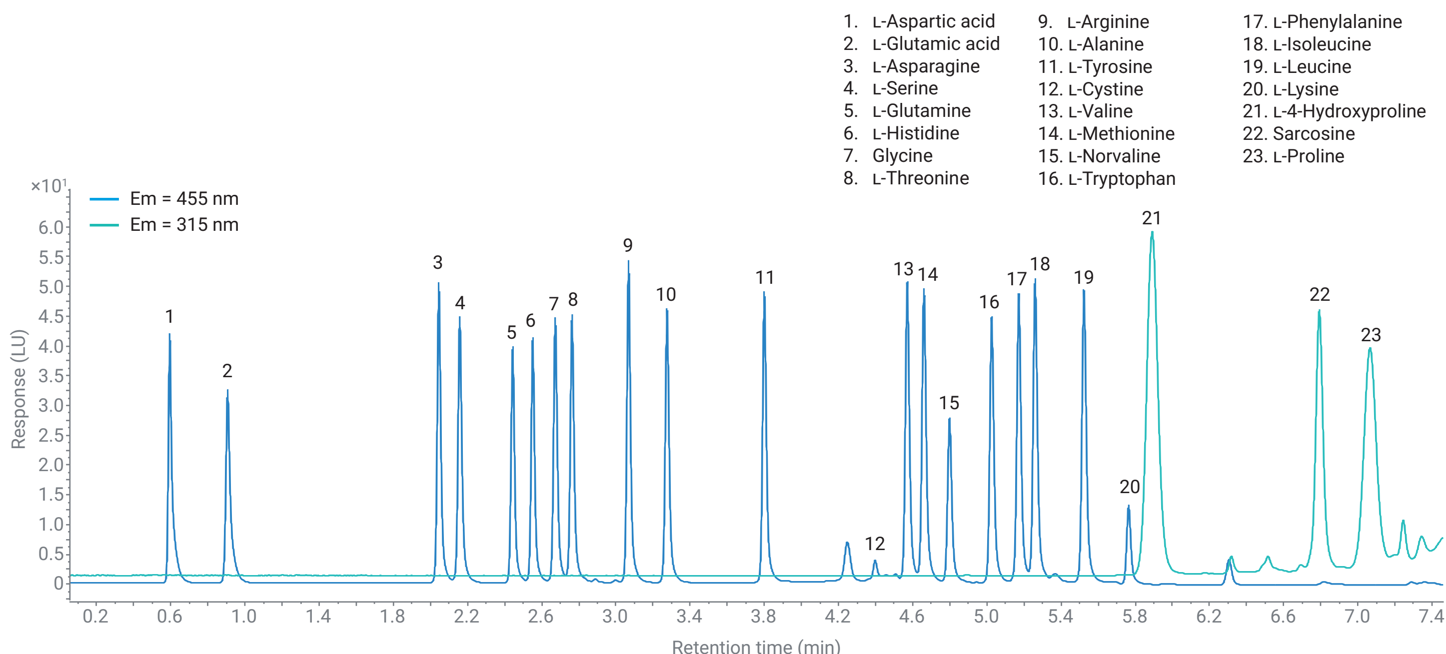

Figure 1. Analysis of 22.5 pmol/µL amino acid standard mixture showing an overlay of FLD signals at an emission wavelength of 455 and 315 nm.

To demonstrate the utility of this method, a commercially available soft drink and a red wine sample were analyzed showing different amino acid profiles (Figure 2). Samples were filtered using a 0.2 µm regenerated cellulose membrane, and the red wine sample was diluted 1:10 with water before injection. Due to high concentrations of certain amino acids, the photomultiplier tube (PMT) gain was adapted to the samples' individual concentration range to avoid oversaturated signals. Each step of the PMT gain approximately doubles the signal; signal-to-noise ratio is decreased with lower values for PMT gain. For the soft drink sample, the PMT gain was decreased from 10 to 9 over the entire run, and for the diluted red wine sample, the PMT gain was switched from 10 to 8 after 6.5 minutes to get a sufficient peak height for proline, which is usually the most abundant amino acid in red wine.[4] Consequently, recalibration should be performed daily and in accordance with the required PMT gain settings.

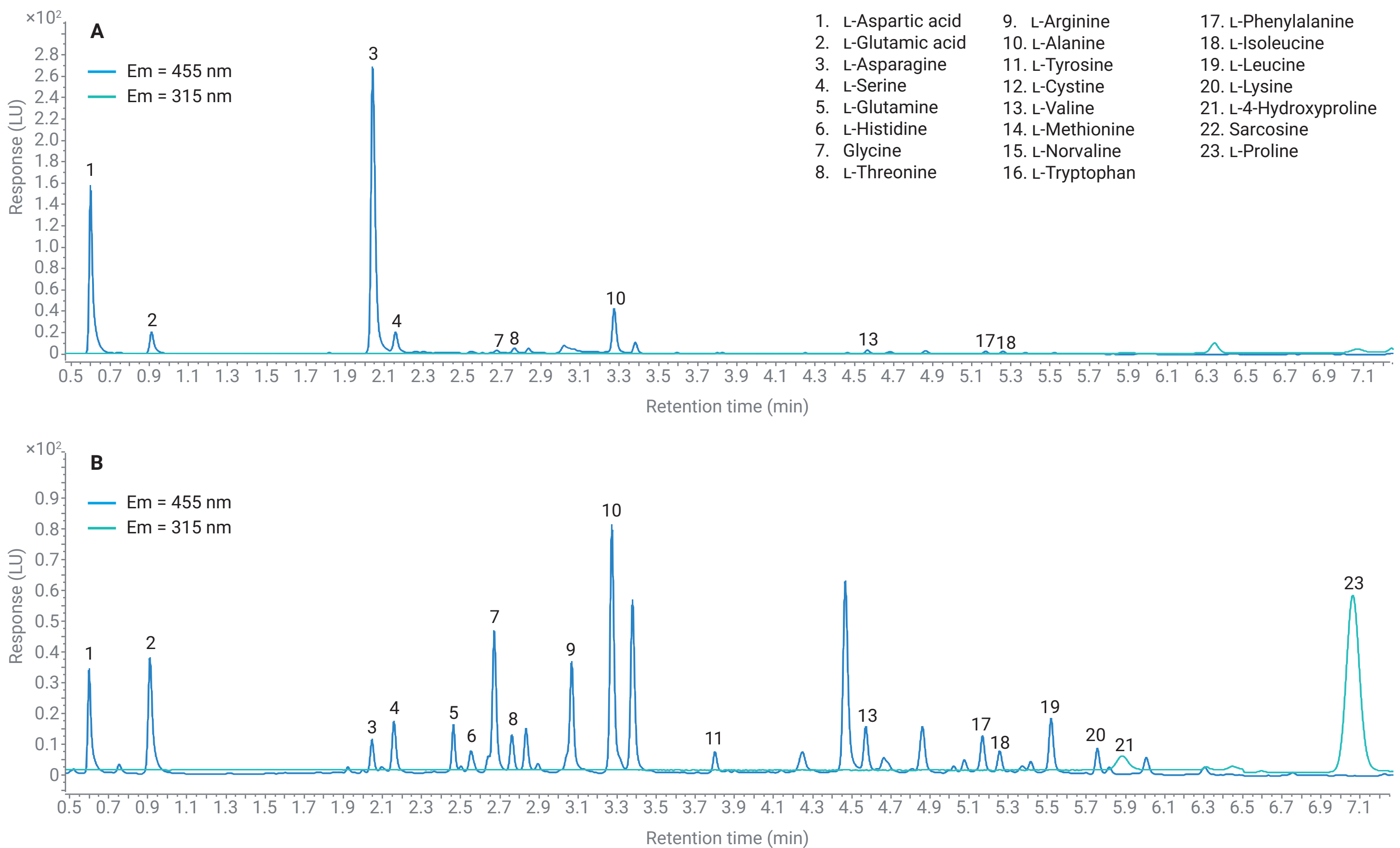

Figure 2. Analysis of amino acids in (A) soft drink and (B) red wine showing an overlay of FLD signals at an emission wavelength of 445 and 315 nm.

Conclusion

This application note demonstrates the use of a 1260 Infinity II Prime LC System for efficient and reliable analysis of amino acids. The 1260 Infinity II Multisampler was used for automated in-loop derivatization of amino acids with OPA and FMOC without the need of any manual work. Derivatization could be achieved in approximately 3.5 minutes and showed high reproducibility, with a peak area precision of less than 1% RSD for most of the compounds. Using the 1260 Infinity II Flexible Pump resulted in excellent chromatographic separation of 23 analytes in a run time of 9 minutes, showing a retention time precision of less than 0.1% RSD. Application of the 1260 Infinity II Infinity Fluorescence Detector enabled simultaneous detection of OPA and FMOC derivatives and showed a high sensitivity with LODs down to 0.225 pmol/µL for most of the compounds. Application of the method for the analysis of a red wine and soft drink sample demonstrates its potential for use in several application areas.

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Nutrition

Chapter 2

Application Note

Food Testing

Agilent

Trusted Answers

Simultaneous Detection and Quantitation of 14 Water-Soluble Vitamins in a Supplement by Triple Quadrupole LC/MS/MS

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Abstract

This application note describes a method for the simultaneous determination of 14 water-soluble B vitamins in a dietary supplement using an Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS system with Agilent MassHunter Workstation software. The method was used to quantify the water-soluble B vitamins in a highly complex multivitamin tablet matrix. All tested water-soluble vitamins met the manufacturer claimed concentrations. It was concluded that the method can be used for quality control and establishment of nutrition labels for water-soluble vitamin-containing supplement products.

Introduction

Vitamins are essential nutrients for human health. Vitamin supplements are often consumed to ensure adequate vitamin intake and are available in various forms such as tablets, capsules, gummies, softgels, and drinks.

In particular, B vitamins are essential for proper nervous system function and for converting food into cellular energy.[1] The water-soluble B vitamins include B1 (thiamine), B2 (riboflavin), B3 (nicotinic acid and nicotinamide), B5 (pantothenic acid), B6 (pyridoxine and pyridoxal-5-phosphate), B7 (biotin), B9 (folic acid and 5-methyltetrahydrofolate), and B12 (cyanocobalamin, adenosylcobalamin, methylcobalamin and hydroxocobalamin). The structures of these vitamins are given in Figure 1.

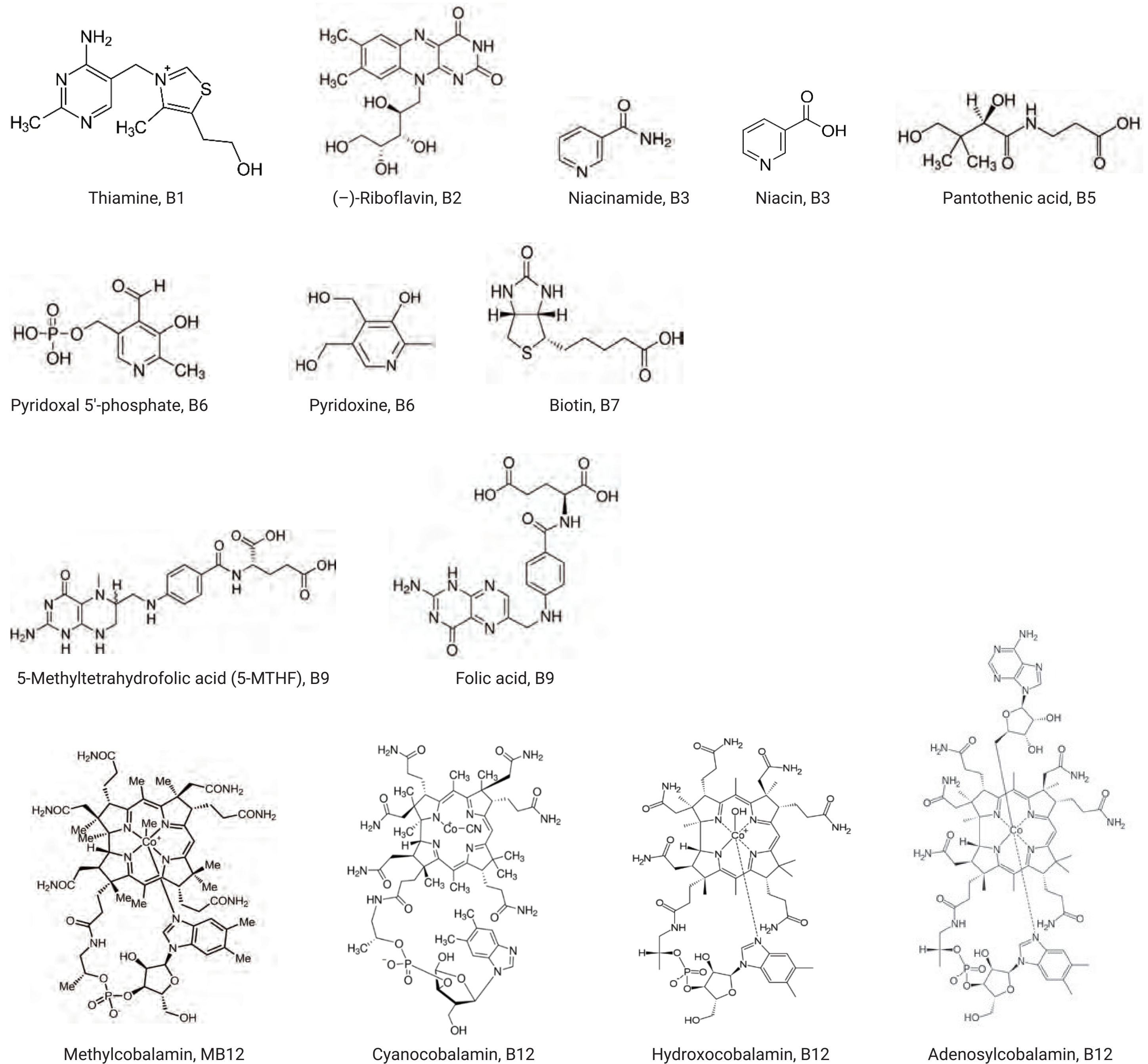

Figure 1. Structure of water-soluble B vitamins.

Accurate quantitative measurements for B vitamins are required to ensure product quality and regulatory compliance. Traditional measurements include microbiological methods, which have significant shortcomings for accuracy, specificity and throughput; and LC-UV methods, which have poor sensitivity for low-level vitamins and poor selectivity within complex matrices. These techniques often involve multiple assays to quantify all B vitamins. Instead, LC/MS/MS has become increasingly popular for vitamin detection due to the high sensitivity, selectivity, and accuracy it provides.

In this study, a fast and sensitive LC/MS/MS method was developed and evaluated to provide identification and accurate quantification of B vitamins in a complex multivitamin tablet matrix. More efficiently, all 14 B vitamins were analyzed simultaneously in one run. The postextraction matrix-matched standard and standard additions were included to compensate for matrix effects. Method criteria for data acceptance were also established.

Experimental

Equipment

All experiments in this study were performed using an Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II multisampler (G7167B), an Agilent 1290 Infinity II high speed pump (G7120A), and an Agilent 1290 Infinity II multicolumn thermostat (G7116B) coupled to an Agilent 6470 triple quadrupole LC/MS system (G6470A). The system was controlled by Agilent MassHunter Acquisition software version 10.1. Data processing was performed with MassHunter quantitative analysis software version 10.1 and MassHunter qualitative analysis software version 10.0.

Sample and standards

The study matrix was Nature's Way Alive! Men's Max Potency Daily Multivitamin (Nature's Way, WI, USA). Standards of thiamine, riboflavin, niacin, niacinamide, pantothenic acid, pyridoxine, pyridoxal-5-phosphate, biotin, folic acid, cyanocobalamin, adenosylcobalamin, methylcobalamin, hydroxocobalamin, riboflavin-$^{13}C_4$, ^{15}N solution, and niacinamide-$^{13}C_6$ solution were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA); 5-methyltetrahydrofolate was obtained from Cayman Chemical (Ann Arbor, MI, USA); pyridoxine-$^{13}C_4$ and biotin-D_2 were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA).

Individual analyte and internal standard stock solutions were made at concentrations of 100 µg/mL in 0.1% H_3PO_4 + 5% ACN in water and took purity, water content, and counter-ions into account. The sample and standards were stored at 5 °C or –20 °C accordingly.

Chromatographic conditions

Parameter	Setting
Analytical Column	Agilent InfinityLab 120 Poroshell 120 Phenyl-Hexyl, 3.0 × 100 mm, 2.7 µm (p/n 695975-312)
Column Oven	30 ±2 °C
Injection Volume	1 µL
Run Time	9 min
Autosampler	15 ±2 °C
Mobile Phase A	*5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	0.1% formic acid in methanol
Needle Wash	50/50 MeOH/H_2O
Gradient	Time (min) / Flow (mL/min) / %A / %B 0 / 0. 5 / 97 / 3 1.0 / 0. 5 / 94 / 6 4.5 / 0. 5 / 55 / 45 5.5 / 0. 5 / 10 / 90 6.5 / 0. 5 / 10 / 90 6.6 / 0. 5 / 97 / 3 9.0 / 0. 5 / 97 / 3

* To achieve the best peak shape, the column needs a relatively long time to equilibrate, or up to 20 mM ammonium formate can be used.

MS parameters

Parameter	Setting
MS Acquisition	Dynamic MRM (dMRM)
Ion Source Type	Agilent Jet Stream electrospray ionization (AJS ESI)
Drying Gas Temperature	270 °C
Drying Gas Flow	13 L/min
Nebulizer	40 psi
Sheath Gas Heater	375 °C
Sheath Gas Flow	11 L/min
Capillary	2,500 V
Nozzle Voltage	0 V
Precursor Ion and Production Ion Resolution	Unit
Compound-Specific Conditions	See Table 1

Method

Below is the detailed description of the optimized sample preparation protocol followed in this study. Table 1 gives the analyte-specific LC/MS/MS conditions.

1. Determine the average weight of the tablet using an analytical balance. Grind the sample into a fine powder to form a homogeneous mixture.
2. Weigh out a 5 g sample into a 100 mL amber volumetric flask.
3. Add approximately 70 mL extraction solvent (0.1% H_3PO_4 + 5% ACN + 0.5% EDTA + 0.5% vitamin C in water) to the flask (Vitamin C was added to the solution to prevent oxidation of the target analytes).
4. Shake for 20 minutes.
5. Fill the flask to the 100 mL volume mark and mix well.
6. Draw approximately 1.5 mL of sample extract to a 2 mL microcentrifuge tube.
7. Heat the flask to 90 to 95 °C in a water bath for 25 minutes for releasing vitamin B2.
8. Cool down to room temperature.
9. Draw approximately 1.5 mL of sample extract to a 2 mL microcentrifuge tube.
10. Centrifuge the sample extract from step 5 and step 8 for 5 minutes at 13,000 rpm.
11. Dilute the supernatants with 0.1% H_3PO_4 + 5% ACN in water as needed.
12. Prepare the postspiked samples along with the diluted samples.
13. Inject samples to LC/MS/MS using positive ESI mode for analysis.

Table 1. Analyte-specific LC/MS conditions: precursor to product ion transitions, fragmentor, collision energies (CE), cell accelerator voltage (CAV), and retention times (RT).

Compound Group	Compound Name	Type	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Delta RT (min)	Fragmentor (V)	CE (V)	CAV (V)
B1	Thiamine	Target	265.1	144.0	1.14	1	97	8	4
B1	Thiamine	Target	265.1	121.9	1.14	1	97	8	4
B1	Thiamine	Target	265.1	80.9	1.14	1	97	28	4
B2	Riboflavin	Target	377.1	243.0	6.31	1	167	16	4
B2	Riboflavin	Target	377.1	198.1	6.31	1	167	44	4
B2	Riboflavin	Target	377.1	172.0	6.31	1	167	16	4
B2	Riboflavin-$^{13}C_4$, $^{15}N_2$	IS	383.1	249.0	6.31	1	167	16	4
B3	Niacin	Target	124.0	80.1	1.41	1	107	20	5
B3	Niacin	Target	124.0	78.1	1.41	1	107	24	5
B3	Niacin	Target	124.0	53.0	1.41	1	107	32	5
B3	Niacinamide	Target	123.1	80.0	1.82	1	112	20	4
B3	Niacinamide	Target	123.1	53.0	1.82	1	112	36	4
B3	Niacinamide-$^{13}C_6$	IS	129.0	86.0	1.81	1	112	20	5
B5	Pantothenic acid	Target	220.1	202.1	3.52	1	107	4	4
B5	Pantothenic acid	Target	220.1	90.0	3.52	1	107	8	4
B5	Pantothenic acid	Target	220.1	71.9	3.52	1	107	16	4
B6	Pyridoxal 5'-phosphate	Target	247.9	150.0	2.06	1.5	142	12	4
B6	Pyridoxal 5'-phosphate	Target	247.9	94.1	2.06	1.5	142	28	4
B6	Pyridoxine	Target	170.1	152.1	2.09	1	92	12	4
B6	Pyridoxine	Target	170.1	134.0	2.09	1	92	24	4
B6	Pyridoxine	Target	170.1	77.0	2.09	1	92	40	4
B6	Pyridoxine-$^{13}C_4$	IS	174.1	138.0	2.09	1	92	24	4
B7	Biotin	Target	245.1	227.1	5.95	1	102	8	4
B7	Biotin	Target	245.1	123.0	5.95	1	102	28	4
B7	Biotin	Target	245.1	97.1	5.95	1	102	32	4
B7	Biotin-D_2	IS	247.1	125.0	5.94	1	102	28	4

B9	Folic acid	Target	442.1	295.0	5.43	1	102	20	4
B9	Folic acid	Target	442.1	176.0	5.43	1	102	44	4
B9	Folic acid	Target	442.1	120.0	5.43	1	102	44	4
B9	5-Methyltetrahydrofolic acid (5-MTHF)	Target	460.1	313.0	4.40	1	102	16	4
B9	5-Methyltetrahydrofolic acid (5-MTHF)	Target	460.1	180.1	4.40	1	102	48	4
B12	Adenosylcobalamin	Target	790.1	359.1	6.28	1	152	28	4
B12	Adenosylcobalamin	Target	790.1	147.1	6.28	1	152	32	4
B12	Cyanocobalamine	Target	678.1	456.9	5.68	1	152	30	4
B12	Cyanocobalamine	Target	678.1	359.0	5.68	1	152	25	4
B12	Cyanocobalamine	Target	678.1	341.0	5.68	1	152	25	4
B12	Methylcobalamine	Target	673.0	665.1	6.42	1	132	14	4
B12	Methylcobalamine	Target	673.0	359.1	6.42	1	132	20	4
B12	Methylcobalamine	Target	673.0	147.1	6.42	1	132	48	4
B12	OH-cobalamine	Target	665.1	359.1	4.93	2	152	20	4
B12	OH-cobalamine	Target	665.1	147.1	4.93	2	152	40	4

Evaluation procedure

Method performance was evaluated by analyzing a complex multivitamin tablet sample. The product label for water-soluble B vitamins is listed in Table 2.

Quantitation was performed using isotopically labeled internal calibration curves with 1/x weighting, or external calibration curves with 1/x weighting and single point postmatrix spike correction, or postmatrix standard addition curve.

Evaluation criteria

Specificity:

- The relative error (RE %) of retention time of each analyte peak to the average of standard peaks is less than 5%
- The ion ratio is within the tolerance of 30%

Linearity and range:

- Calibration curve has R^2 >0.99
- Calculated working standard values should be within ±30% of the theoretical value
- The calibration standards should bracket the analyte concentration level

Accuracy:

- The tested result for each water-soluble B vitamin meets ≥100% claimed on the product label (see Table 2)
- Postspike recovery is within 70% to 130% (due to the variations in method and instrument performance, the criteria should be determined by each individual lab)

Table 2. Supplement facts (serving size: 3 tablets/serving).

Compound Name	Vitamins	Compound Form in Tablet	Claim (mg/serving)
Thiamin	B1	As thiamin mononitrate	40
Riboflavin	B2	As free riboflavin	40
Niacin	B3	As niacinamide	80
Pantothenic Acid	B5	As calcium pantothenate	62.5
Vitamin B6	B6	As total of pyridoxine HCl and pyridoxal 5'-phosphate	42.5
Biotin	B7	As free biotin	0.033
Folate	B9	As 5-methyltetrahydrofolate glucosamine salt	0.42
Vitamin B12	B12	As methylcobalamin	0.2

Results and discussion

Column selection

In this study, two columns were evaluated with the same LC conditions, Agilent InfinityLab Poroshell 120 Phenyl-Hexyl (100 × 3.0 mm, 2.7 µm) and Agilent InfinityLab Poroshell 120 StableBond-Aqueous (100 × 2.1 mm, 2.7 µm). Better selectivity was achieved using the phenyl-hexyl column with a balanced mixture of retention mechanisms, including hydrophobic interactions, the π-π interactions of the phenyl group and the hexyl linkage of the stationary phase. Methanol is a good choice when paired with phenyl columns.[2] See Figure 2 for a selectivity comparison between the two columns. The phenyl-hexyl column provided an enhanced molecular interaction and selectivity to improve the chromatographic separation and peak shape.

Specificity

A dynamic multiple reaction monitoring (dMRM) acquisition method was used for vitamin quantitation. Monitoring MS/MS transitions with evaluation of the ratio of their relative intensities and RT of analyte peaks allows the target analyte to be distinguished from potential interferences in quantitative analysis. Figure 3 shows an example of an extracted ion chromatogram of a 10 ng/mL working standard in 0.1% H_3PO_4 + 5% ACN in water. Figure 4 shows that a reagent blank is free of analytes in 0.1% H_3PO_4 + 5% ACN in water.

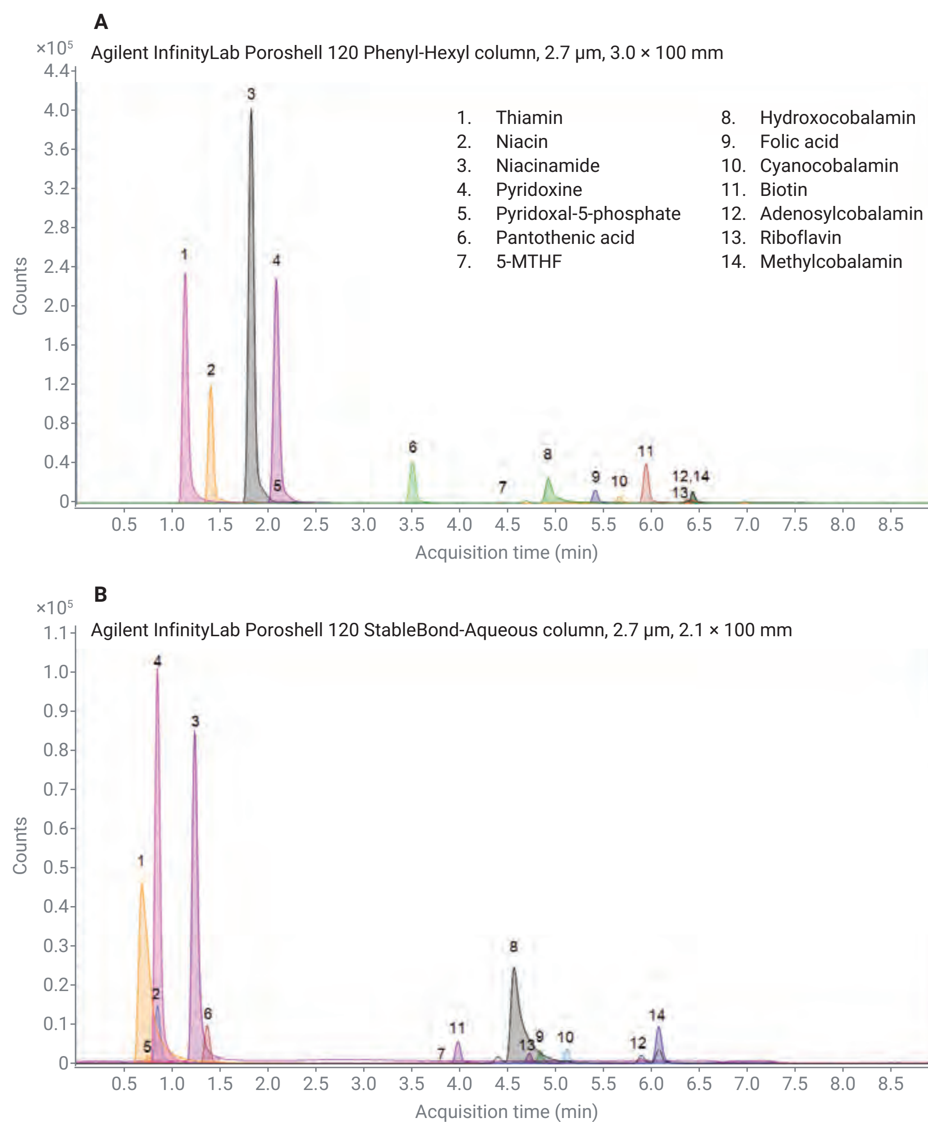

Figure 2. Comparison of the selectivity between Phenyl-Hexyl and StableBond-Aqueous columns.

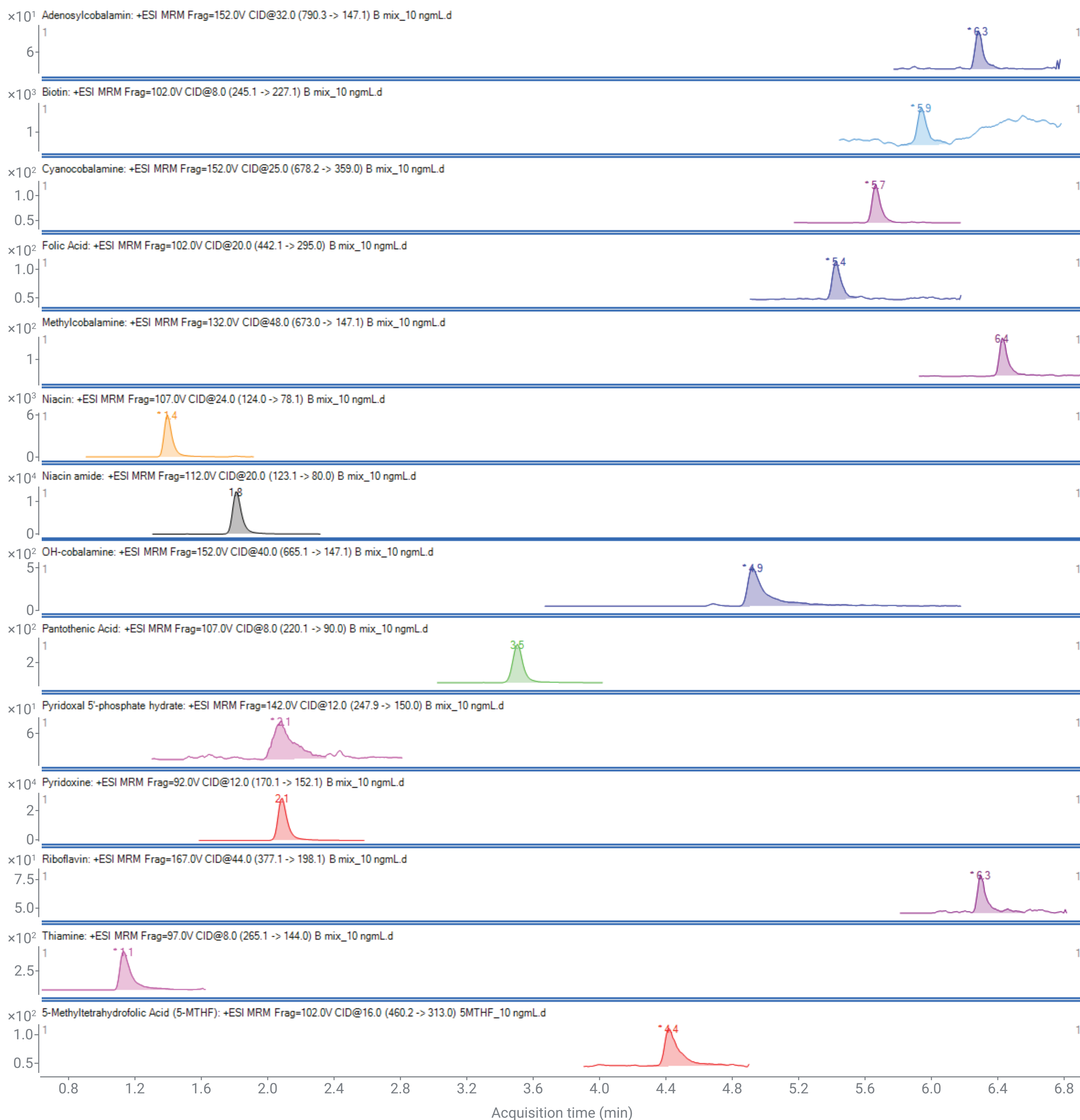

Figure 3. Extracted ion chromatogram of vitamin composite working standards at 10 ng/mL in 0.1% H_3PO_4 + 5% ACN in water, 1 µL injection volume.

Range and linearity

The method was evaluated over the range of 0.5 to 500 ng/mL. To evaluate response linearity of the method, eight working standard (WS) solutions of B vitamins were made at 0.5, 1, 2, 5, 10, 50, 100, and 500 ng/mL. The calibration curve residuals were ≤30% for WS1 to WS8. The linearity was determined by using a linear calibration with a 1/x weighting factor. The coefficient of determination (R^2) values were >0.99. Table 3 lists the statistical data of the calibration curve residuals, linear range and coefficients of determination.

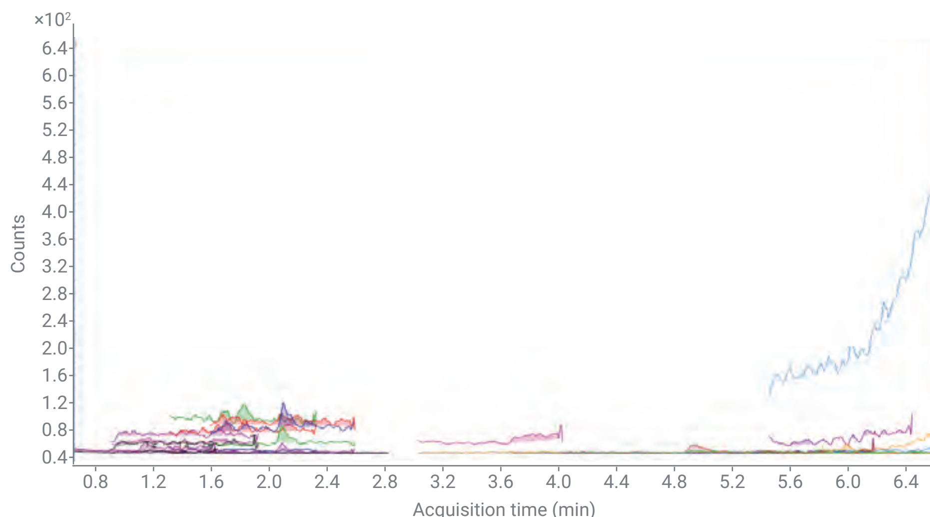

Figure 4. Extracted ion chromatogram of a solvent blank 0.1% H_3PO_4 + 5% ACN in water.

Table 3. Calibration curve statistical data.

Compound Name	Calibration Curve Residual (%)								Range (ng/mL)	R^2
	WS1 0.5	WS2 1	WS3 2	WS4 5	WS5 10	WS6 50	WS7 100	WS8 500		
	ng/mL									
Thiamine	28	17	7	−3	−20	−3	−3	2	0.5 to 500	0.9980
Riboflavin	18	9	12	6	−10	3	−8	3	0.5 to 500	0.9970
Niacin	7	2	−14	−7	−2	−3	3	0	0.5 to 500	0.9995
Niacinamide	23	−10	−4	3	−11	5	7	−2	0.5 to 500	0.9985
Pantothenic Acid	–	17	10	−9	−13	−5	0	2	1 to 500	0.9980
Pyridoxine	27	2	−1	1	−15	0	4	0	0.5 to 500	0.9991
Pyridoxal 5'-phosphate	–	–	–	−13	−17	−3	−2	2	5 to 500	0.9992
Biotin	–	–	–	28	−12	−5	−3	2	5 to 500	0.9978
5-Methyltetrahydrofolic Acid (5-MTHF)	–	–	–	–	25	−12	−8	−10	25 to 500	0.9913
Folic Acid	–	–	–	12	−1	−4	−4	1	5 to 500	0.9993
Methylcobalamine	–	25	3	−13	−4	−14	2	1	1 to 500	0.9981
Cyanocobalamine	−23	8	21	−6	1	0	0	9	0.5 to 500	0.9991
OH-cobalamine	–	30	−7	−2	−13	−6	−4	2	1 to 500	0.9987
Adenosylcobalamin	–	–	–	−23	12	−12	−11	4	5 to 500	0.9936

Approaches for accurate quantitation

Interfering substances in the matrix may be observed and can affect the electrospray ionization process, causing ion suppression or enhancement. Currently, there is no guideline for matrix effects in vitamins due to the variations in method and instrument performance. However, matrix effects need to be addressed for proper quantitation. A postspiked matrix-matched standard or standard addition can address the matrix effect or any other matrix interactions when the internal standard is not available or not easy to obtain.[3]

Postspike recovery was determined by fortifying samples after extraction with an analyte composite standard solution. The results were corrected using postspike recovery if it was within 70 to 130%. If the postspike recovery was outside the range of 70 to 130%, a standard addition curve was generated.

Sample tests

An increasing number of methylcobalamin B12 based supplements have entered the market due to its natural form and higher bio-availability. Naturally occurring folate (5-methyltetrahydrofolate) is a biologically

active form of folic acid and is rapidly gaining popularity as the preferred supplemental form. Nature's Way Alive! Men's Max Potency Daily Multivitamin incorporates high potency B-vitamins, including the active forms of B12 and folate, and food-based blends including Daily Greens, Orchard Fruits and Garden Veggies, Cardio, Digestive Enzyme, and more blends.[4] The complexity of the product formulation served as an excellent matrix to demonstrate unambiguous identification, accurate quantitation, and high sensitivity for a variety of B vitamins by LC/MS/MS.

The high sensitivity of LC/MS/MS allows a large dilution after sample extraction. The postspike recoveries for B1, B2, B3, B5, B6, B7, and B12 fell into the accepted range and the results were corrected. Given that significant ion enhancement for vitamin B9 (folate) was observed, a standard addition curve was generated for quantitation. See Figure 5 for this curve generated from the MassHunter quantitative analysis software. The corrected results for all B vitamins met ≥100% claims on the product label. Sample results and postspike recoveries for each analyte are shown in Table 4. The results of riboflavin, niacinamide, pyridoxine and biotin were also corrected using internal standards to compare with those from post matrix-matched correction. The results from both techniques were found to be in good agreement with each other (see Table 4).

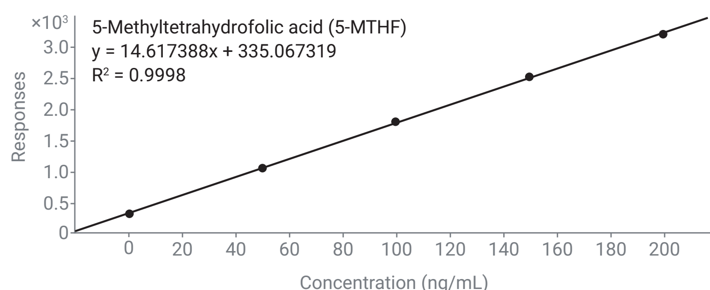

Figure 5. Standard addition curve for 5-MTHF.

Table 4. Sample results for Nature's Way Alive! Men's Max Potency Daily Multivitamin.

Compound Name	Vitamins	Serving Size (g) (3 tablets)	Claim on Product Label (mg/serving)	*Corrected Results by IS mg/serving	*Corrected Results by postspike Recovery (mg/serving)	Postspike Recovery (%)
Thiamin	B1	5.2	40		41	102
Riboflavin	B2	5.2	40	57	57	97.1
Niacinamide	B3	5.2	80	89	87	108
Pantothenic Acid	B5	5.2	62.5		75.9	97.0
Total of Pyridoxine and Pyridoxal 5'-Phosphate	B6	5.2	42.5	47.1	43.0	111
Biotin	B7	5.2	0.033	0.047	0.050	94.7
5-Methyltetrahydrofolate Glucosamine Salt	B9	5.2	0.42		0.44	By standard addition
Methylcobalamin	B12	5.2	0.20		0.21	70.8

Conclusion

A rapid, sensitive, and accurate UHPLC/MS/MS method for the identification and quantitation of water-soluble B vitamins in a complex supplement matrix was presented. The method used an Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS system. All 14 B vitamins were detected simultaneously in one run for high efficiency, throughput, and cost reduction when compared to the traditional involvement of multiple assays. This method demonstrated linearity over three orders of magnitude for all analytes tested with an R^2 value of 0.99. All water-soluble B vitamins in the supplement product met the specifications with the quantitation approaches of matrix-matched standard or standard addition. The evaluation demonstrated that the method can achieve the necessary specificity, linearity, and accuracy required for water-soluble vitamin B analysis.

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Application Note

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Analysis of Water-Soluble Vitamins and Their Metabolites

Performance gains in hydrophilic interaction chromatography (HILIC) with LC/MS/MS

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Abstract

Water-soluble vitamins and their metabolites were analyzed by LC/MS/MS with Agilent InfinityLab Poroshell 120 HILIC-Z columns. The effect of varying mobile phase additives with different pH values was explored. The best overall performance was achieved with a mid-pH additive using 10 mM ammonium acetate and acetonitrile as the mobile phase. Performance gains for phosphorylated compounds could be obtained using peek-lined column hardware and InfinityLab deactivator additive.

Introduction

There are increased demands for comprehensive analysis of water-soluble vitamins and their metabolites. Most analytes are small, highly polar compounds that are not well retained on a reversed-phase column. An InfinityLab Poroshell 120 HILIC-Z column with superficially porous particle technology is ideal for the retention of this class of compounds. This Application Note separated 24 vitamins and their metabolites using the InfinityLab Poroshell 120 HILIC-Z column with an Agilent 6460 triple quadrupole LC/MS system. The influence of different mobile phase additives was explored when using the Poroshell 120 HILIC-Z column. In addition, the effect of peek-lined hardware in combination with the InfinityLab deactivator additive, a mobile phase additive to reduce interactions with active metal surfaces, was examined. These effects were compared with standard phosphoric acid wash protocols and conventional chromatography that does not use any passivation strategy.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher. HPLC grade acetonitrile was purchased from J. T. Baker (Center Valley, PA, USA.). Water was purified using an ELGA PURELAB Chorus system (High Wycombe, UK). Formic acid, acetic acid, ammonium acetate, and ammonium hydroxide were purchased from J&K Scientific (Beijing, China). All vitamins and the metabolite standards (Table 1) were obtained through Anpel (Shanghai, China). The standards stock solutions were prepared with concentrations and solvent listed in Table 1.

The mixture solution was prepared by mixing the individual stock solutions and diluted with acetonitrile. Table 1 shows the individual concentration of all the components.

Equipment and materials

- Column inlet: Agilent InfinityLab Quick Connect LC fitting (p/n 5067-5965)
- Column outlet: Agilent InfinityLab Quick Turn LC fitting (p/n 5067-5966)
- Agilent Captiva Econofilter, PTFE membrane, 13 mm diameter, 0.2 µm pore size (p/n 5190-5265)
- Agilent vial, screw top, amber, write on spot, certified, 2 mL (p/n 5182-0716)
- Agilent bonded screw cap, bonded blue, PTFE/red silicone septa (p/n 5190-7024)
- Agilent InfinityLab solvent bottle, amber, 1,000 mL (p/n 9301-6526)
- Agilent InfinityLab Stay Safe cap, GL45, three ports, one vent valve (p/n 5043-1219)
- Eppendorf pipettes and repeater
- Sonicator (VWR, Radnor, PA, USA)

Table 1. Stock solutions for all the analytes.

Compound Name	Concentration (mg/mL)	Solvent	Concentration in Mixture (µg/mL)
Thiamine	1.0	ACN:water (9:1)	0.16
Thiamin diphosphate	1.0	ACN:water (1:1)	3.4
Thiamin monophosphate	1.0	Water	3.4
Riboflavin	0.125	Ethanol:1% NH_4OH in water (9:1)	0.43
Riboflavin phosphate	1.0	Water	3.4
Flavin adenine dinucleotide (FAD)	1.0	Water	3.4
Nicotinamide	1.0	ACN:water (9:1)	0.04
Niacin	1.0	ACN:water (9:1)	3.4
NAD	1.0	ACN:water (7:3)	1.7
NADH	1.0	ACN:water (7:3)	6.9
NADP	1.4	ACN:water (7:3)	10.8
NADPH	1.0	ACN:water (7:3)	6.9
D-Pantothenic acid	1.0	ACN:water (7:3)	3.4
Pyridoxine	1.0	ACN:water (9:1)	0.04
Pyridoxal	1.0	ACN:water (9:1)	0.02
Pyridoxamine	1.0	ACN:water (9:1)	0.12
Pyridoxal 5'-phosphate	1.0	Water	3.4
Biotin	0.5	ACN:water (1:1)	1.7
Folic acid (FA)	0.5	ACN:1% NH_4OH in water (1:1)	1.7
Folinic acid/Folinate	0.5	ACN:water (1:1)	1.7
5-Methytetrahydrofolate	0.5	ACN:water (1:1)	1.7
Tetrahydrofolate (THFA)	0.5	ACN:water (1:1)	8.6
Dihydrofolate (DHFA)	0.5	ACN/0.1% NH_4OH in water (1:1)	1.7
Cyanocobalamin (VB12)	0.5	ACN:water (1:1)	5.1

Instrumentation

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6460 triple quadrupole LC/MS (G6460A)

Software

- Agilent MassHunter software for LC/MS data acquisition, version B.08.02
- Agilent MassHunter workstation for qualitative analysis software, version 10.0

Table 2. LC/MS method parameters.

HPLC Conditions	
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm (p/n 685775-924) InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100, 2.7 µm, PEEK-lined (p/n 675775-924)
Stock solution	A) 100 mM ammonium formate in water adjusted pH to 3.0 with formic acid B) 100 mM ammonium acetate in water C) 100 mM ammonium acetate in water adjusted pH to 9.0 with ammonium hydroxide
Mobile Phase 1	A) 100 mL of stock solution a with 900 mL of water B) 100 mL of stock solution a with 900 mL of acetonitrile
Mobile Phase 2	A) 100 mL of stock solution b with 900 mL of water B) 100 mL of stock solution b with 900 mL of acetonitrile
Mobile Phase 3	A) 100 mL of stock solution c with 900 mL of water B) 100 mL of stock solution c with 900 mL of acetonitrile
Mobile Phase 4	A) 100 mL of stock solution c with 900 mL of water, added with 1.0 mL deactivator additives B) 100 mL of stock solution c with 900 mL of acetonitrile, added with 1.0 mL deactivator additives
Gradient	0 to 1 minute, 100% B; 1 to 8 minutes, 100 to 50% B; Stop time: 10 minutes
Flow Rate	0.30 mL/min
Column Temperature	40 °C
Injection Volume	0.5 µL
MS Conditions	
Ion Mode	ESI/Jet Stream ESI, Positive/ Negative
Drying Gas Temperature	250 °C
Drying Gas Flow	6 L/min
Nebulizer Pressure	35 psi
Sheath Gas Temperature	325 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	Positive 3,500 V; Negative 2,500 V
Nozzle Voltage	Positive 500 V; Negative 1,000 V
ΔEMV	0 V

Results and discussion

Three kinds of mobile phase additives with different pH values were explored. With an increase in mobile phase pH from pH 3.0 to pH 7.0, most compounds were retained on the InfinityLab Poroshell 120 HILIC-Z column, and achieved better resolution using mid-pH mobile phase. When comparing pH 7.0 and pH 9.0 mobile phases, retention of most compounds changed very little. However, the mid pH mobile phase provided higher signals in basic conditions under positive mode. Therefore, the mid-pH mobile phase addition provides the best overall separation of all compounds shown in Figure 1.

During the investigation, the phosphorylated molecules including pyridoxal 5'-phosphate, riboflavin phosphate, thiamine diphosphate, and thiamine monophosphate had poor peak shape. When these compounds interact with the steel pathway, including the pump and steel tubing, they often cause peak tailing tail at high concentrations and disappear completely at low concentrations (Figure 2A).

A simple solution was to deactivate the metal sites on the steel surface. This deactivation was done using a mild phosphoric acid wash (0.5% phosphoric acid in 90:10 acetonitrile:water).[1] The phosphoric acid strongly bonds to the active sites on the system, enabling satisfactory analysis of sticky compounds. The peak shapes of four phosphorylated molecules were all improved after washing with 0.5% phosphoric acid as shown in Figures 2A and 2B.

Table 3. Masses monitored by multiple-reaction monitoring.

Compound	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (V)	Polarity
Nicotinamide	123.1	80.1	100	21	Positive
Pyridoxal	168.1	150	75	9	Positive
Pyridoxine	170.1	152	80	13	Positive
Riboflavin	377.2	243	135	25	Positive
Pyridoxamine	169.1	152	170	25	Positive
Niacin	124	80.1	110	21	Positive
Thiamine	265.1	122	70	13	Positive
Biotin	245.1	227	105	13	Positive
D-Pantothenic acid	220.1	90.1	80	13	Positive
D-Pantothenic acid	218.1	87.9	145	9	Negative
VB12	678.3	147.1	165	40	Positive
FAD	786.2	348	180	21	Positive
Riboflavin phosphate	457.1	439	140	13	Positive
Pyridoxal 5'-phosphate	248	150	135	13	Positive
NADH	666.1	136.4	180	60	Positive
NAD	664.1	136.3	175	60	Positive
Folinate	474.2	327	110	17	Positive
5M-TFH	460.2	313	120	17	Positive
Thiamine monophosphate	345.1	122	85	17	Positive
FA	442.2	295	95	9	Positive
DHFA	444.2	178	110	9	Positive
Thiamine diphosphate	425.1	122	95	21	Positive
THFA	446.2	299	115	17	Positive
NADP	744	136.4	180	60	Positive
NADPH	746	136.4	180	60	Positive

Another solution is to add deactivator additive (1 mL of activator, part number 5191-3940 or 5191-4506 in 1 L mobile phase),[2] which chelates free metals and covers exposed active metal sites in the sample flowpath. This additive reduces metal-analyte interaction. Before adding deactivator additive to the mobile phase, perform the same phosphoric acid wash of the system. The results demonstrate a continued improved peak shape shown in the third chromatograms of Figure 2C. As an alternative solution to improve peak shape of these compounds, use the PEEK-lined column with activator additives added. The peak shapes of some compounds were also improved in the chromatograms of Figure 2D.

Figure 1. Selected product ions chromatograms for the analytes.

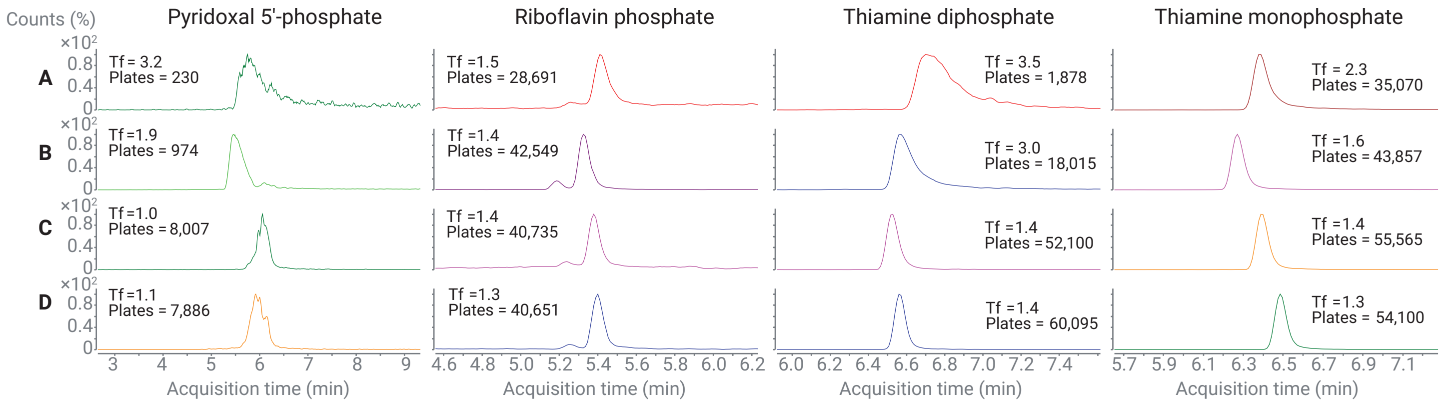

Figure 2. Interactions of phosphorylated metabolites with steel: before and after wash. A) Before system wash; B) After system wash, HILIC-Z column; C) After system wash, HILIC-Z column, with deactivator; D) After system wash, HILIC-Z PEEK-lined column, with deactivator.

Conclusion

The best overall performance of these water-soluble vitamins and their metabolites analysis was achieved using an ammonium acetate-supplemented mobile phase at mid pH with the Agilent InfintyLab Poroshell 120 HILIC-Z column. The peak shape of some phosphorylated metabolites could be significantly improved by flushing the instrument with 0.5% phosphoric acid in 90:10 acetonitrile:water and InfinityLab deactivator additives. The peek-lined InfintyLab Poroshell 120 HILIC-Z column further improved the peak shape of phosphorylated metabolites. The method described in this Application Note is well suited for the analysis of water-soluble vitamins and their metabolites.

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Application Note

Food Testing & Agriculture

Determination of Isoflavones in Soybean by LC/MS/MS

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Abstract

This application note describes a sensitive and reliable method for the determination of seven isoflavones in soybean samples by liquid chromatography/tandem mass spectrometry (LC/MS/MS). The best separation of daidzein, glycitein, daidzin, genistin, genistein, and rutin was obtained on a reversed-phase C18 column (Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100 mm, 1.8 µm, p/n 959758-902) under gradient elution. The proposed method is simple, fast, and presented a linear calibration with correlation coefficients greater than 0.998. The limits of detection (LODs) and limits of quantification (LOQs), based on the signal-to-noise ratio (S/N), were in the range of 0.7 to 6.7 and 2.3 to 22.5 ppb, respectively. The method was successfully used to determine isoflavones in soybean samples.

Introduction

Soybean (*Glycine max*) is a complex food matrix containing low starch, approximately 20 % oil, and 40 % high-quality protein, in addition to several important bioactive compounds. Soybean products are consumed worldwide as both foods and food additives. They can be cooked and eaten, or used to make other products such as tofu, soy milk, and soy sauce. Soybean is also used as an additive in processed foods to enhance texture, flavor, or nutritional content. It is commonly used as a vegetarian alternative to conventional products to produce soy infant formula, soy yogurt, veggie burgers, and so forth. In addition to high protein and nutrient content, soybean also contains isoflavones, compounds similar to the female hormone estrogen. Isoflavones may be present in soybean foods and supplements as aglycones (daidzein, genistein, and glycitein), glycosides (daidzin, genistin, and genistein), or malonyl- and acetyl-glycosides. The potential impact of phytoestrogens on humans and animals has fueled an ever-increasing interest in the study of these compounds in foods, especially in soybean. These compounds also have an influence on a plants insect resistance and can therefore in certain situations allow crop yields to be increased, ensuring greater food security.

Analytical methods for the determination of phytoestrogens in edible plants, plant products, and biological matrices include gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). These can be coupled with various techniques such as ultraviolet absorption (UV), electrochemical detection (ED), fluorescence detection, mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. In addition, immunoassays are adopted to analyze isoflavones in food products and biological samples. Each method has its own advantages and limitations. This study develops and validates a method for the determination of seven isoflavones (daidzin, glycitin, rutin, genistin, daidzein, glycitein, and genistein) in soybean samples using LC/MS/MS. Figure 1 shows the molecular structures of isoflavones analyzed in this work.

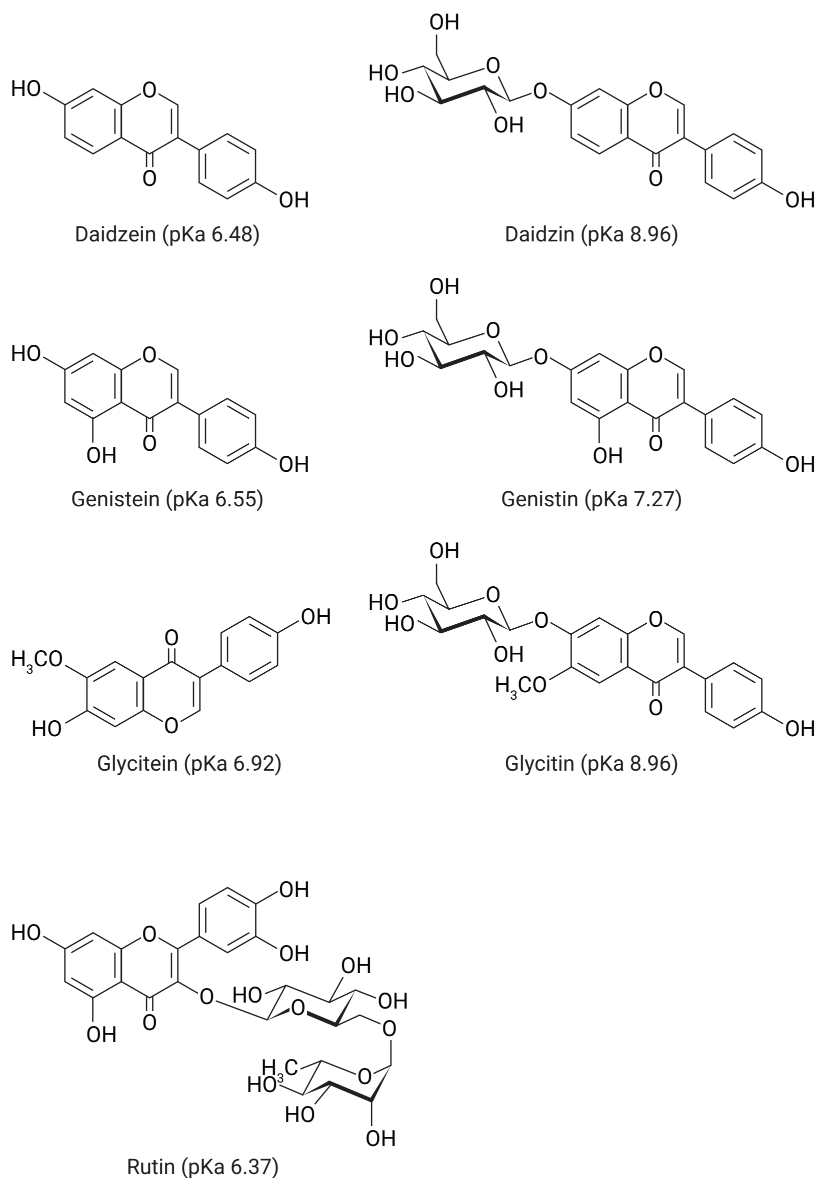

Figure 1. Molecular structure of isoflavones analyzed with predicted pKa values (calculated at www.chemicalize.org (accessed July, 2018).

Experimental

The mass spectrometer was operated in positive multiple reaction monitoring (MRM) mode using two specific transitions for each isoflavone. The most intense transition was used for quantification, and the other was used as a qualifying ion. Table 1 lists the retention time (RT), monitored ions, and other MS/MS acquisition parameters used for the identification and quantification of isoflavones in soybean.

Sample preparation

Samples of soybean seeds from two different cultivars, Dowling (resistant to sucking insects) and Silvania (susceptible to sucking insects), were obtained from the Embrapa Cerrados Research Center (Brasília, DF, Brazil). The samples were homogenized using liquid nitrogen in a porcelain mortar and pestle to obtain a fine flour. An aliquot of 2.0 g was weighed and added to a glass vial (15-mL) containing 10 mL of methanol and 2 mL of aqueous 0.1 M HCl. The samples were sonicated for 20 minutes at room temperature. Then, the supernatant was separated and filtered through a filter paper, followed by a second filtration step using a syringe filter with a 0.45 µm PTFE membrane. The solvent was removed under low pressure using a rotary evaporator, and the final volume was adjusted to 2 mL of methanol.

LC conditions

Instrument	Agilent 1290 Infinity II LC
Column	ZORBAX Eclipse Plus C18 2.1 mm × 100 mm, 1.8 µm (p/n 959758-902)
Column temperature	40 °C
Injection volume	1 µL
Mobile phase	A) Water with 0.1 % formic acid B) Acetonitrile
Gradient	Time (min) %A %B 0.0 90 10 0.5 90 10 6.0 50 50 7.0 10 90 8.0 10 90 8.01 90 10
Stop time	9 minutes
Flow rate	0.300 mL/min

MS conditions

Instrument	Agilent 6470 triple quadrupole LC/MS
Ion mode	AJS-ESI, positive ionization
Capillary Voltage	4,000 V
Sheath gas heater	300 °C
Sheath gas flow	10 L/min
Drying gas flow (N_2)	10 L/min
Drying gas temperature	300 °C
Nebulizer pressure	20 psi
VCharging	500 V

Table 1. RT and MS/MS acquisition parameters used for the identification and quantification of isoflavones in soybean.

Compound	RT (min)	Q1[a] (*m/z*)	Q3[b] (*m/z*)	CE[c] (V)	FE[d] (V)
Daidzin	3.54	417.1	255.0* 199.0	20 52	101
Glycitin	3.64	447.2	285.0* 270.0	12 52	101
Rutin	3.88	611.2	303.0* 85.1	24 56	101
Genistin	4.23	433.1	271.0* 153.0	20 60	96
Daidzein	5.47	255.1	199.0* 91.1	28 44	125
Glycitein	5.62	285.1	270.0* 118.0	28 52	125
Genistein	6.43	271.1	153.0* 91.1	32 44	135

a = precursor ion (Q1); b = fragment ion (Q3); c = collision energy; d = fragmentor energy.

Results and discussion

Isoflavones have acidic-basic characteristics, with pKa values ranging from 6.37 to 8.96 (see Figure 1). Accordingly, the mobile phase was acidified with 0.1 % aqueous formic acid to prevent the deprotonation of analytes, and to improve the shape of the chromatographic peaks. The mobile phase composition, gradient composition, and injected volume were optimized for separation efficiency and sensitivity. Figure 2 shows a representative dMRM chromatogram obtained for isoflavone standards under optimized conditions using the Agilent MassHunter Qualitative software (B.08.00).

The developed method has the potential to quantify isoflavones in soybean by standard addition, since it is not possible to find a blank for soybean. To check the linearity of the isoflavones standards, calibration curves were constructed with at least nine distinct levels of concentration with each measured in triplicate. Calibration curves were constructed with standard solutions with at least nine distinct levels of concentration in triplicate. The correlation coefficients of calibration curves were greater than 0.998, with relative standard deviations (RSDs) ranging from 0.2 to 3.6 % for run-to-run precision. The LODs and LOQs were determined with reference to the corresponding concentration to three and ten times, respectively, the baseline noise, in a time close to the RT measured around each isoflavone in matrix.

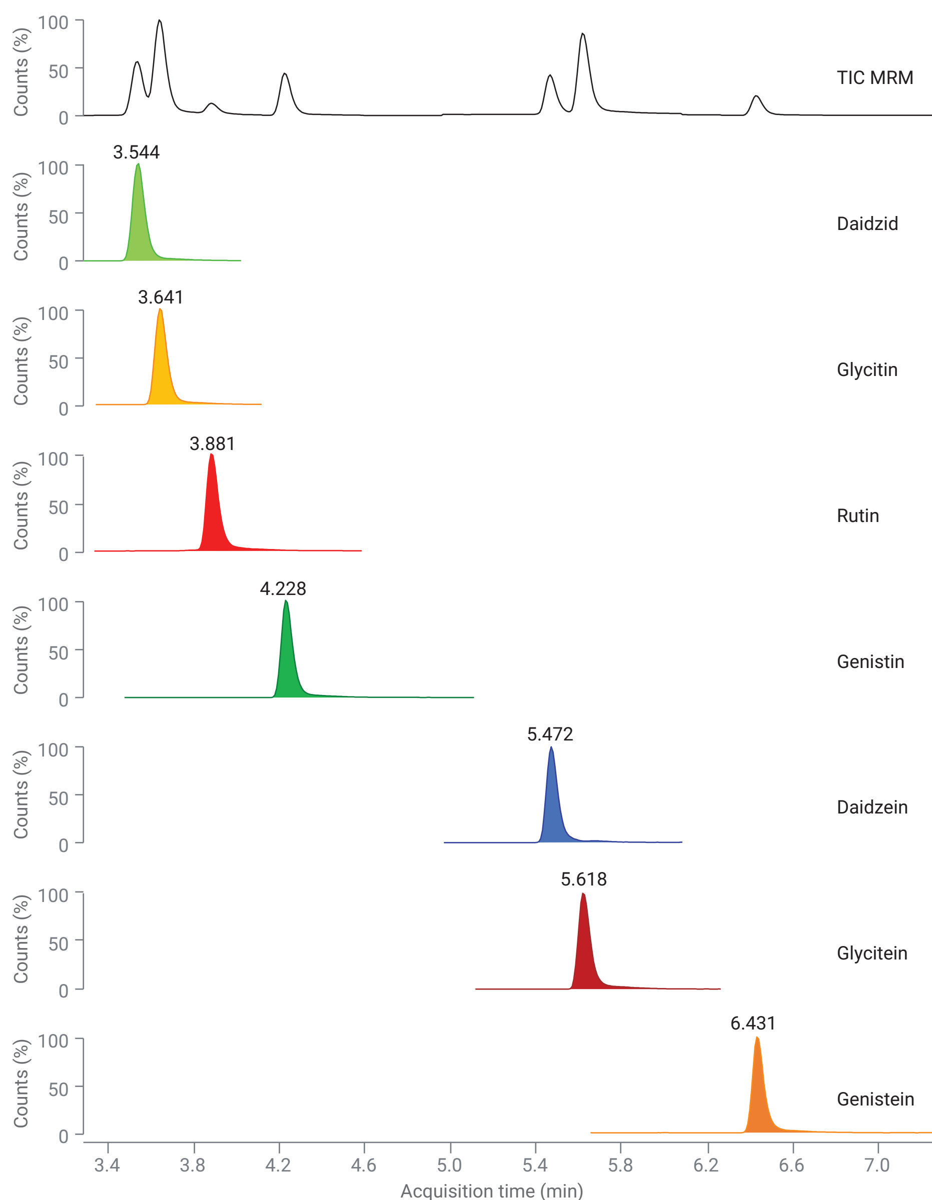

Figure 2. Normalized dMRM chromatogram at optimum conditions for the isoflavone standards at 40 ppb each, using the MassHunter qualitative software (B.08.00).

Table 2 shows the regression equations and other characteristic parameters for the developed method, while Figure 3 shows the calibration curve for genistin, using the MassHunter quantitative software (B.08.00).

Table 2. Figures of interest from the method developed for the determination of isoflavones in soybean by LC/MS/MS.

Compound	Linear range (ppm)	y = ax + b	R^2	LOD (ppb)	LOQ (ppb)
Daidzin	0.01 – 2.5	y = 664.2x – 5,044.9	0.998	0.7	2.4
Glycitin	0.01 – 2.5	y = 265.6x – 4,880.2	0.998	0.7	2.3
Rutin	0.04 – 5.0	y = 230.9x – 2,3876.8	0.999	6.7	22.5
Genistin	0.01 – 4.0	y = 155.5x – 173.3	0.998	0.7	2.3
Daidzein	0.01 – 1.3	y = 380.9x + 8215.7	0.998	1.3	4.4
Glycitein	0.01 – 2.5	y = 51.6x + 524.9	0.998	1.5	5.0
Genistein	0.01 – 1.3	y = 314.7x + 1,600.8	0.998	1.6	5.4

a = slope; b = intercept; R^2 = determination coefficient

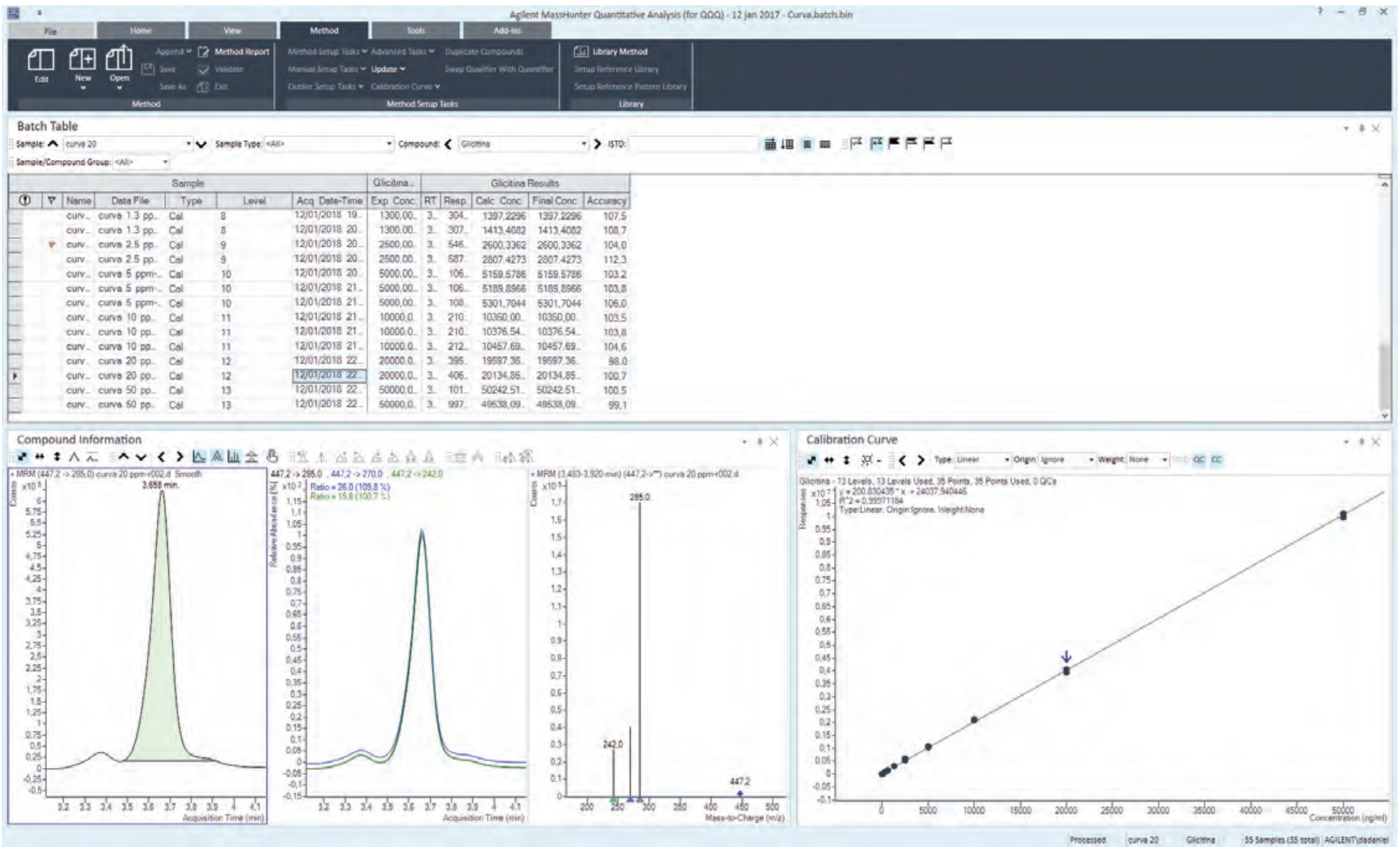

Figure 3. Calibration curve for genistin using MassHunter quantitative software (B.08.00).

To determine isoflavones in four soybean samples obtained from Embrapa Cerrados Research Center (Brasília, DF, Brazil), the consistency of the proposed method was evaluated by applying it to real samples, using standard addition methodology to avoid matrix effects. The RSD was lower than 10.2 %. Table 3 summarizes these results. The values obtained also confirmed previously published results, which showed that the Dowling cultivar produces a lower amount of the isoflavones identified here compared to Silvania soybean cultivar.

Table 3. Concentration (µg/g of soybean) of isoflavone in soybean samples (n = 3) as well the RSD (%) values.

Compound	Silvania 1	Silvania 2	Dowling 1	Dowling 2
Daidzin	3.8 ± 0.3 (7.9 %)	2.8 ± 0.2 (7.1 %)	1.6 ± 0.1 (6.2 %)	1.6 ± 0.1 (6.2 %)
Glycitin	3.6 ± 0.2 (5.5 %)	2.2 ± 0.2 (9.1 %)	1.3 ± 0.1 (7.6 %)	1.4 ± 0.1 (7.1 %)
Rutin	0.025 ± 0.002 (8.0 %)	0.036 ± 0.002 (5.5 %)	0.036 ± 0.002 (5.5 %)	0.029 ± 0.002 (6.9 %)
Genistin	20.1 ± 1.5 (7.4 %)	16.9 ± 1.3 (7.7 %)	6.9 ± 0.5 (7.2 %)	11.8 ± 1.2 (10.2 %)
Daidzein	0.080 ± 0.003 (3.7 %)	0.081 ± 0.004 (4.9 %)	0.031 ± 0.003 (9.7 %)	0.072 ± 0.004 (5.5 %)
Glycitein	0.108 ± 0.005 (4.6 %)	0.260 + 0.004 (1.5 %)	0.055 ± 0.002 (3.6 %)	0.079 ± 0.003 (3.8 %)
Genistein	0.036 ± 0.002 (5.5 %)	0.035 ± 0.001 (2.8 %)	0.025 ± 0.002 (8.0 %)	0.046 ± 0.003 (6.5 %)

Conclusion

We have shown that LC/MS/MS is well suited to determine isoflavones in soybean samples. The proposed method presented a linear response with excellent precision data for replicate injections and LODs lower than 7 ppb. In addition, the method is simple, fast, and lasts less than nine minutes per sample. It presents excellent potential for application in food analysis laboratories, not only for the analysis of soybean isoflavones, but also for other types of matrices.

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Application Note

Food & Beverage Testing

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Trusted Answers

Determine Sugars and Artificial Sweeteners in a Single Run

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Abstract

The determination of sugars in food and beverages is an established procedure in many analytical laboratories. With an industry and consumer trend towards products with reduced sugar content and added low-calorie sweeteners, analytical laboratories need to run a second method to analyze the sweeteners. This application note presents a method enabling simultaneous determination of sugars and sweeteners in a single run, reducing the effort and time spent on the analysis.

Introduction

The excessive consumption of sugars as part of a widespread poor diet has been identified by the World Health Organization (WHO) as a major risk factor for premature deaths.[1] The WHO therefore strongly recommends reducing the intake of free sugars. To limit the addition of sugars to food and beverages, many countries have installed excise duties on added sugar in food and beverages. For this reason, the food and beverage industry seeks to reduce added sugar in products. To maintain sensory identity and quality of a product, however, omitted sugar is frequently replaced with low-calorie sweeteners. This trend is reflected in decreasing sales or consumption of added sugars and increase of sweeteners (e.g., in Canada[2], the United States[3,4], and the European Union[5]). The increasing number of products containing both sugars and sweeteners drives the need for analytical methods enabling simultaneous analysis of both analyte classes. Whereas most artificial sweeteners exhibit a chromophore, making them amenable to UV detection, sugars lack a chromophore and thus cannot be reliably and sensitively detected by a UV detector. A refractive index detector (RID), on the other hand, can detect any compound distinct from the mobile phase, which makes it an ideal detector for sugars.

This application note presents a method for simultaneous separation and quantitation of four commonly used sweeteners and five mono- and disaccharides found in natural ingredients as well as food additives.

Experimental

Instrumentation

The Agilent 1260 Infinity II LC consisted of the following modules:

- Agilent 1260 Infinity II Isocratic Pump (G7110B)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Refractive Index Detector (G7162A)

Column

Agilent ZORBAX Carbohydrate Analysis, 4.6 × 250 mm, 5 µm (part number 840300-908)

Software

Agilent OpenLab CDS, version 2.6, or later versions

Solvents

HPLC gradient-grade acetonitrile (ACN) was purchased from VWR (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

Chemicals and samples

Acesulfame, ammonium acetate, aspartame, cyclamate, fructose, glucose, lactose monohydrate, maltose monohydrate, saccharine, and sucrose were obtained from Merck (Darmstadt, Germany).

A calibration stock solution of all standards was made up in 20 mM aqueous ammonium acetate/acetonitrile, 1:1 (by volume). Pure stock solution was used for the highest calibration point; other calibration points were created by diluting the stock solution with mobile phase. Calibration curves were constructed in a range from 31 to 1,000 µg/mL for sweeteners, and from 156 to 5,000 µg/mL for sugars. Each point was measured in triplicate.

Soda samples were sourced from a local grocery store. Before injection, the sample was centrifuged for 5 minutes at 14,100 × g, filtered using an Agilent Captiva premium syringe filter (0.45 µm, regenerated cellulose, part number 5190-5107), and diluted with pure ACN or mobile phase.

Method settings

Table 1. Chromatographic conditions.

Parameter	Value
Mobile Phase	20 mM ammonium acetate in acetonitrile/water 75:25 (v:v)
Flow Rate	1.5 mL/min
Injection Volume	5 µL
Sample Temperature	Ambient
Column Temperature	35 °C
RI Detector	35 °C Peak width >0.025 min (0.5 s response time, 18.5 Hz) Signal polarity: positive (+)

Results and discussion

Figure 1 shows the chromatogram of the separation of the highest calibration point. Four artificial sweeteners and five sugars were successfully separated. Resolution between all analytes was typically larger than 2; the only exception was a resolution ranging from 1.6 to 1.7 between aspartame and fructose, which is just acceptable for quantitation. Between fructose and glucose (peaks 5 and 6), each calibration point exhibited a negative peak. The negative signal was largest in the highest calibration sample and decreased with increasing dilution of the calibrant; a blank injection of pure mobile phase did not show any negative peaks. It is therefore hypothesized that the different buffer concentration between calibration solvent and mobile phase was detected by the RID. The lower the buffer concentration in the less diluted calibration samples, the larger the negative peak area. To integrate peak 6 in a reproducible way, the intercept of the baseline after peak 5 and 6 was treated as the start of peak 6.

Calibration curves for all analytes were constructed measuring six points in triplicate. Concentration levels were 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL for the sweeteners, and 156.25, 312.5, 625, 1,250, 2,500, and 5,000 µg/mL for the sugars. Excellent correlation (R^2) and standard deviations of the procedure (s_{x0}) were found for all analytes, as shown in Table 2. Limits of detection and quantitation were calculated for each analyte based on the signal-to-noise ratio, with the noise determined according to the ASTM method E 685-93.

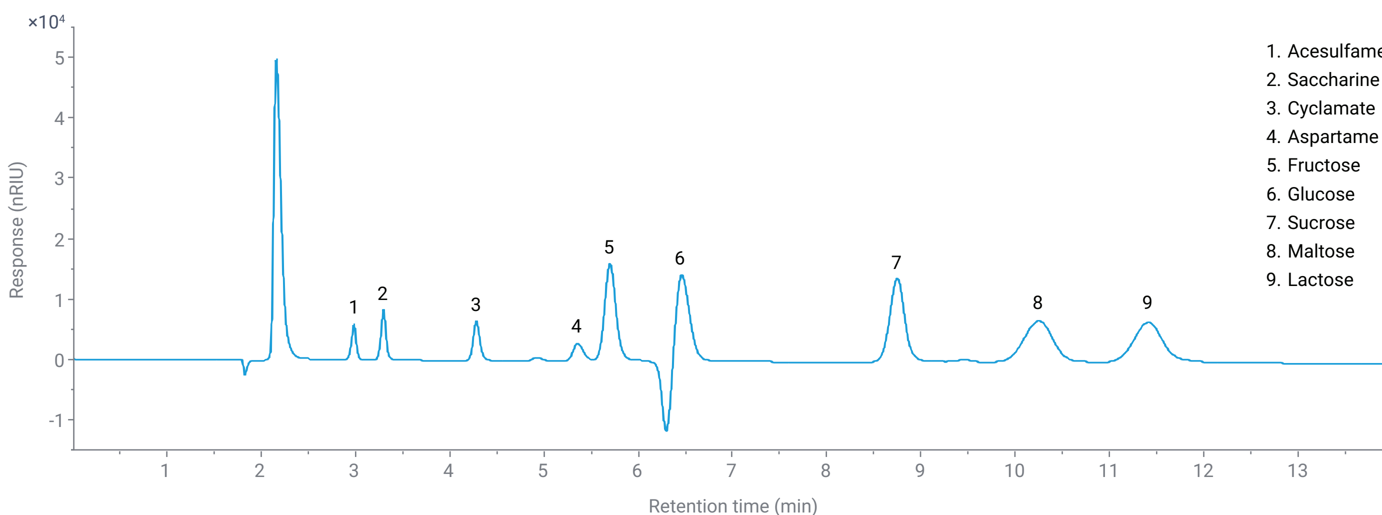


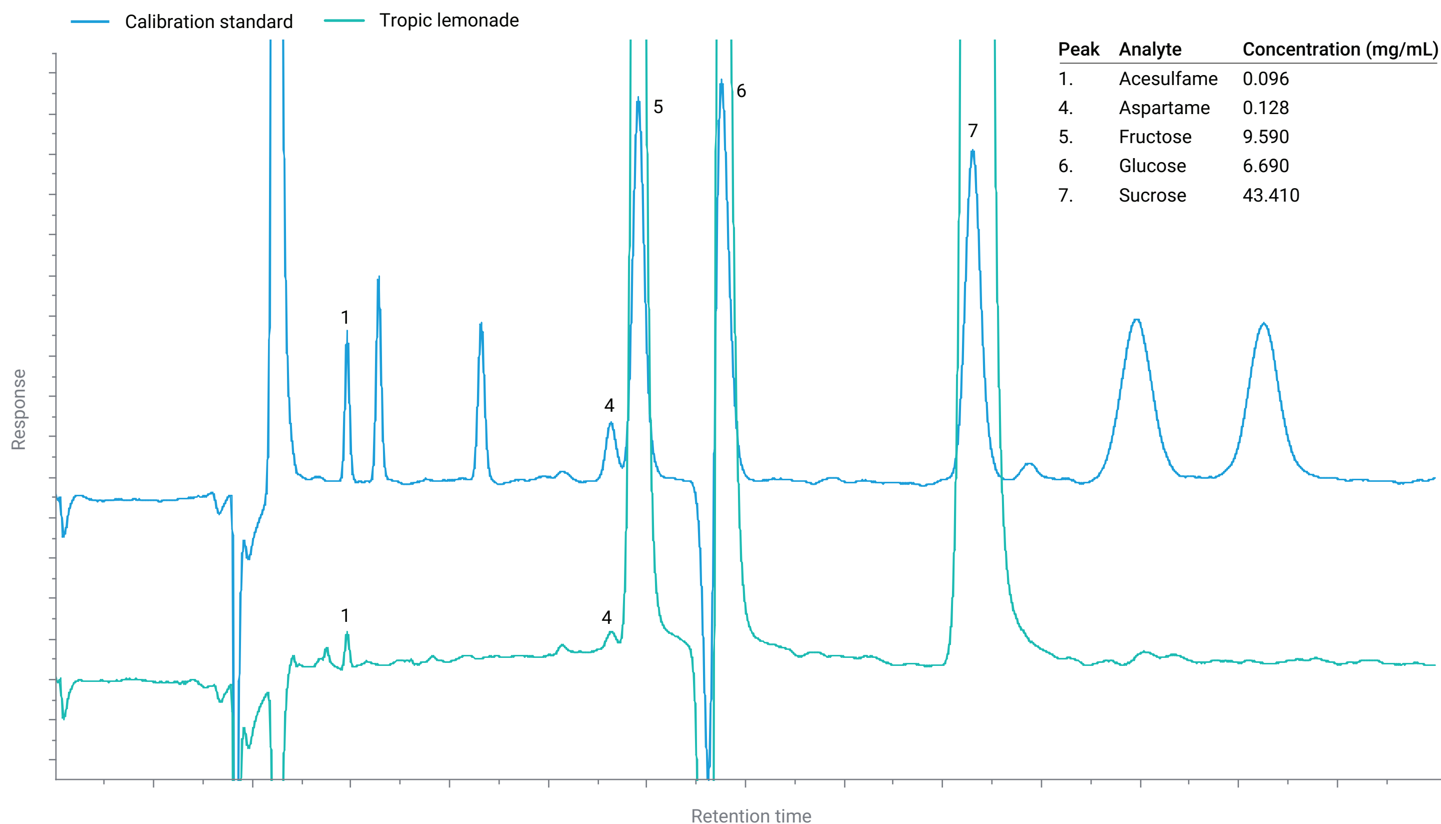
Figure 1. Separation of the calibration sample.

Table 2. Statistics of the calibration.

Analyte	Calibrated Range (µg/mL)	Resolution	R^2	LOQ (mg/mL)	LOD (mg/mL)	s_{x0} (mg/mL)
Acesulfame	31.25–1,000	–	0.99998	0.006	0.002	0.002
Saccharine	31.25–1,000	3.4–3.6	0.99994	0.003	0.001	0.003
Cyclamate	31.25–1,000	8.8–9.0	0.99999	0.004	0.001	0.001
Aspartame	31.25–1,000	6.6–6.8	0.99947	0.011	0.003	0.010
Fructose	156.25–5,000	1.6–1.7	0.99998	0.010	0.003	0.010
Glucose	156.25–5,000	3.3–3.4	0.99999	0.010	0.003	0.005
Sucrose	156.25–5,000	8.5–8.9	0.99999	0.010	0.003	0.006
Maltose	156.25–5,000	3.5–3.6	0.99999	0.019	0.006	0.005
Lactose	156.25–5,000	2.0–2.1	0.99999	0.020	0.006	0.008

The different calibration ranges for sweeteners and sugars were chosen to account for the expected concentrations of these two analyte classes in real samples. The range between sweeteners and sugars in the selected samples was in fact so high that it required dilution to accurately quantify the sugar amount. To avoid sample breakthrough, aliquots of the samples were diluted with the threefold volume of pure ACN, mimicking the composition of the mobile phase. If the sugar concentration was still out of the calibration range, another aliquot of the sample was diluted tenfold with pure mobile phase. Two lemonades were analyzed: one with reduced sugar content and added sweeteners ("tropic lemonade"), and one diet lemonade based on whey, with added sweeteners but without added sugar ("whey lemonade").

Figure 2 shows the analysis of the tropic lemonade, diluted 1:4 with ACN. Two sweeteners and three sugars were detected. To quantify the amount of sugar, a tenfold dilution of the same sample needed to be analyzed (not shown). Acesulfame and aspartame were quantified at 96 and 128 µg/mL, respectively. Fructose, glucose, and sucrose were found in larger amounts, namely 9.59, 6.69, and 43.41 mg/mL. The sum of these three sugars, 59.69 mg/mL, matches the amount given on the nutrition label (60 mg/mL). The amount of added sweeteners was not provided on the label, but the measured concentration is in good agreement with the amounts that can be found in the literature: 126 ±72 µg/mL for acesulfame, and 162 ±120 µg/mL for aspartame, based on a survey of 57 different drinks.[6]



Peak	Analyte	Concentration (mg/mL)
1.	Acesulfame	0.096
4.	Aspartame	0.128
5.	Fructose	9.590
6.	Glucose	6.690
7.	Sucrose	43.410

Figure 2. Chromatogram overlay of the tropic lemonade sample (diluted 1:4 with ACN) with a calibration standard.

The analysis of the whey lemonade is shown in Figure 3. Again, two sweeteners could be detected, this time acesulfame at 168 µg/mL and cyclamate at 244 µg/mL. Since this lemonade is based on whey, a significant amount of lactose was expected, and lactose was quantified at 14.05 mg/mL. The label of the lemonade only declared the total sugars, which were given at 15 mg/mL. This number, however, includes the amount of caramelized sugar that is added for color. In response to a direct inquiry, the manufacturer reported a lactose concentration of 14 mg/mL. Neither of the sweeteners was given with a quantity on the label, but again, the numbers found are in accordance with the literature[6]: 126 ±72 µg/mL for acesulfame, 207 ±47 µg/mL for cyclamate.

The sum of sweeteners found in the whey lemonade (412 µg/mL) is higher than in the tropic lemonade (224 µg/mL), which can be explained in two ways: First, the whey lemonade only contains a quarter of the amount of sugar found in the tropic lemonade. To meet customer expectations of lemonade sweetness, more added sweetener might be required. Second, cyclamate, found in the whey lemonade but not in the tropic lemonade, has a sweetening power six times lower than aspartame and acesulfame[7], which were added to the tropic lemonade. With this factor calculated out of the cyclamate concentration, the sum of sweeteners is about the same in both lemonades (224 compared to 209 µg/mL).

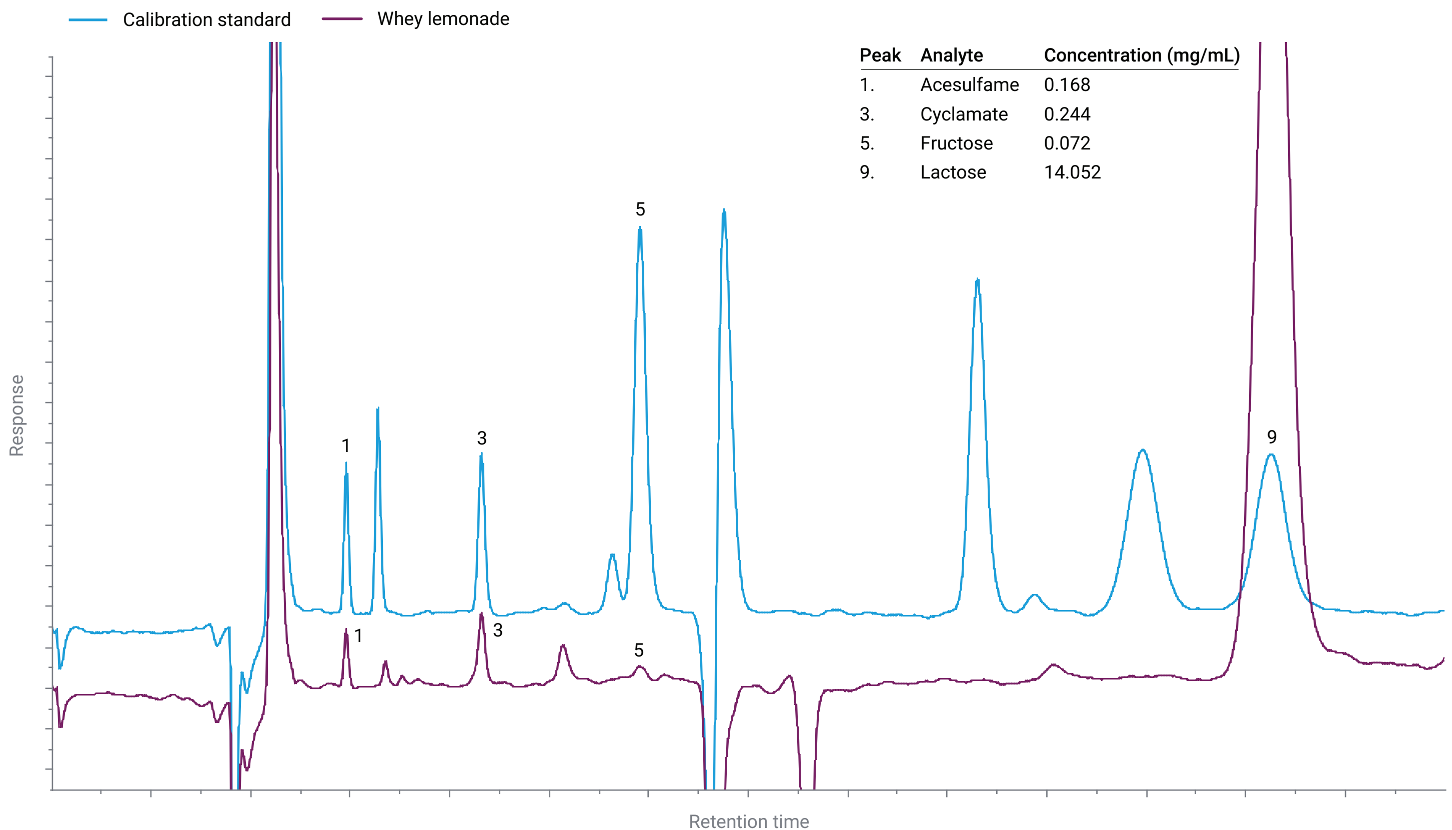


Figure 3. Chromatogram overlay of the whey lemonade sample (diluted 1:4 with ACN) with a calibration standard.

Conclusion

This application note presents an isocratic method capable of simultaneously analyzing four sweeteners and five mono- and disaccharides found in food and beverages. Within 14 minutes, the nine analytes were separated and quantified. For all compounds except maltose and lactose, LOQs were at 11 µg/mL or lower, equaling 55 ng on column. The simultaneous determination of sweeteners and sugars in a single run can significantly reduce the analysis time of samples containing both analyte classes, which makes the analysis faster and more cost-efficient.

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Application Note

Food Testing

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Trusted Answers

A Comparison Study of the Analysis of Volatile Organic Acids and Fatty Acids

Using J&W DB-FATWAX Ultra Inert and other WAX GC columns

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Abstract

This application note evaluates the performance of the Agilent J&W DB-FATWAX Ultra Inert GC column for the analysis of aqueous C2–C7 free fatty acids, C2–C18 organic acids, and Agilent WAX UI test mixtures. The DB-FATWAX Ultra Inert GC column has excellent stability to repeated injections of aqueous matrices. Compared with acid-modified wax columns such as the Agilent J&W DB-FFAP GC column, DB-FATWAX UI columns provide comparable or better peak shape for short-chain volatile organic acids. The results indicate that DB-FATWAX UI provides superior inertness, thermal stability, and retention time reproducibility compared to other commercially available WAX columns for the analysis of underivatized volatile organic acids and free fatty acids.

Introduction

Monitoring types of volatile organic acids and fatty acids is a common analysis required in food, flavors, alcoholic beverages, and other industries. In life science research, laboratories routinely analyze the profiles of the fatty acids extracted from bacterial culture media to identify the bacteria[1]. Short-chain organic acids (C2 to C7) are also regularly monitored for anaerobic or aerobic digestion in fermentations. It can be done using a combination of headspace/GC or liquid-liquid extraction followed by GC analysis. The most common method is by direct injection of the acids in water[2]. Underivatized volatile organic acids are difficult to quantify by GC because these highly polar compounds interact strongly with any active sites on the column, resulting in tailing or poorly resolved peaks that can make quantitation difficult at low levels. For some acids, adsorption can become irreversible. The direct injection method requires the use of GC column stationary phases, which do not decompose in strong acids and water.

Normally, fatty acids are derivatized to methyl esters for analysis. To identify and quantify consumer products in the food industry, fatty acid esters are routinely analyzed by determining the ratio of various saturated and unsaturated fatty acids in fat and oil[3]. These pretreatment procedures are tedious and time-consuming. They carry the potential for incomplete conversions of acids to esters and the loss of short-chain fatty acid esters in the extraction process. Therefore, to eliminate the problems associated with derivatization, extraction, and cleanup procedures, analysis of underivatized organic acids and free fatty acids is recommended. These fatty acids are typically analyzed in their free form using two types of GC columns: one is acid-modified WAX columns, such as the FFAP columns; another is ultra-inert WAX columns.

Previous articles have detailed the GC analysis of FAMEs using DB-FATWAX UI columns[4,5]. This Application Note discusses the analysis of free fatty acids using DB-FATWAX UI, DB-FFAP, and other WAX columns.

Experimental

Chemicals and standards

All standard compounds and reagents in the test mixtures were purchased from ANPEL Scientific Instrument Co. Ltd (Shanghai, China). The purity of each standard compound and solvent is more than 98 %. The standard solution mixtures were prepared from individual pure compounds. The WAX ultra-inert test mixture[6] consisted of the 12 compounds listed in Table 1, which were analyzed using the chromatographic conditions in Table 4.

Table 1. WAX UI test mixture, in dichloromethane.

Peak no.	Compound	Amount on-column (ng)
1	2-Nonanone	3.3
2	Decanal	3.3
3	Propionic acid	3.3
4	Ethylene glycol	3.3
5	Heptadecane	1.65
6	Aniline	3.3
7	Methyl dodecanoate	3.3
8	2-chlorophenol	3.3
9	1-Undecanol	3.3
10	Nonadecane	1.65
11	2-Ethylhexanoic acid	6.6
12	Ethyl maltol	6.6

Instrumentation

The analyses were performed using an Agilent 7890B GC equipped with a flame ionization detector (FID). Sample introduction was achieved using an Agilent 7683B automatic liquid sampler with a 5 µL syringe (p/n G4513-80213), and a split/splitless injection port. Tables 2–4 summarize the instrumental configuration and analytical conditions. Table 5 lists the other supplies used in this study.

Table 2. Method 1 experimental conditions (C2–C7 free fatty acids in water).

Parameter	Value
GC system	7890B/FID
Column	J&W DB-FATWAX Ultra Inert, 30 m, 0.25 mm, 0.25 µm (p/n G3903-63008) J&W DB-FFAP, 30 m, 0.25 mm, 0.25 µm (p/n 122-3232) Commercially available wax columns from suppliers 1 and 2, 30 m, 0.25 mm, 0.25 µm
Carrier gas	Helium, 42 cm/s, constant flow mode
Inlet	Split/splitless, 250 °C, split ratio 30:1
Oven	162 °C isothermal
FID	250 °C, Hydrogen: 40 mL/min; Air: 400 mL/min; make-up gas: 25 mL/min
Sample	0.06–0.13 % each acid in water
Injection	0.1 µL

Table 3. Method 2 experimental conditions (C2–C18 organic acids).

Parameter	Value
GC system	7890B/FID
Column	J&W DB-FATWAX Ultra Inert, 30 m, 0.25 mm, 0.25 µm (p/n G3903-63008) J&W DB-FFAP, 30 m, 0.25 mm, 0.25 µm (p/n 122-3232) Wax columns from suppliers 1 and 2, 30 m, 0.25 mm, 0.25 µm
Carrier gas	Helium, 42 cm/s, constant flow mode
Inlet	Split/splitless, 280 °C, split ratio 50:1
Oven	120 °C (2 minutes), 5 °C/min to 140 °C (3 minutes); 20 °C/min to 250 °C (10 minutes)
FID	280 °C, Hydrogen: 40 mL/min; Air: 400 mL/min; make-up gas: 25 mL/min
Sample	0.05–0.1 % each component in dichloromethane
Injection	1 µL

Table 4. Method 3 experimental conditions (WAX UI test mixture).

Parameter	Value
GC system	7890B/FID
Column	J&W DB-FATWAX Ultra Inert, 30 m, 0.25 mm, 0.25 µm (p/n G3903-63008) J&W DB-FFAP, 30 m, 0.25 mm, 0.25 µm (p/n 122-3232) Wax columns from suppliers 1 and 2, 30 m, 0.25 mm, 0.25 µm
Carrier gas	Helium, 40 cm/s, constant flow mode
Inlet	Split/splitless, 250 °C, split ratio 75:1
Oven	130 °C isothermal
FID	250 °C, Hydrogen: 40 mL/min; Air: 400 mL/min; make-up gas: 25 mL/min
Injection	0.5 µL

Table 5. Flowpath supplies.

Supply	Description
Vials	Amber, write-on spot, certified, 2 mL, screw top vial packs (p/n 5182-0554)
Septa	Nonstick BTO septa (p/n 5183-4757)
Column nut	Self Tightening, inlet/detector (p/n 5190-6194)
Ferrules	15 % graphite: 85 % Vespel, short, 0.4 mm id, for 0.1 to 0.25 mm columns (10/pk, p/n 5181-3323)
Liner	Agilent Ultra Inert split liner with glass wool (p/n 5190-2295)
Inlet seal	Ultra Inert, gold-plated, with washer (p/n 5190-6144)

Results and discussion

Aqueous C2–C7 free fatty acid test mixture

Direct injection of free fatty acids dissolved in water for GC analysis is quite challenging. Due to the presence of strong acids and water, conventional WAX-type phases have been unstable and active, resulting in poor peak shapes and reproducibility, as well as decreased lifetime. To prevent vapor volume overloading of the liner, the injection volume for aqueous samples should be less than 1 µL.

Figure 1 shows a GC/FID chromatogram of a mixture of C2–C7 free fatty acids in water on a J&W DB-FATWAX Ultra Inert (UI) column. Table 2 lists the analysis conditions. Due to the Ultra Inert performance of the DB-FATWAX UI GC column, all acids were well resolved with sharp and symmetrical peaks.

Repeatability of the analysis and column performance stability were tested by 15 injections of aqueous C2–C7 free fatty acids sample. Figure 2 shows that there is no retention time stability drift resulting from repeated injections of aqueous samples. Peak shapes were also maintained over the course of this study. Relative standard deviations (RSDs) for retention times were less than 0.03 %, and absolute peak areas were within 2 % for all free fatty acids.

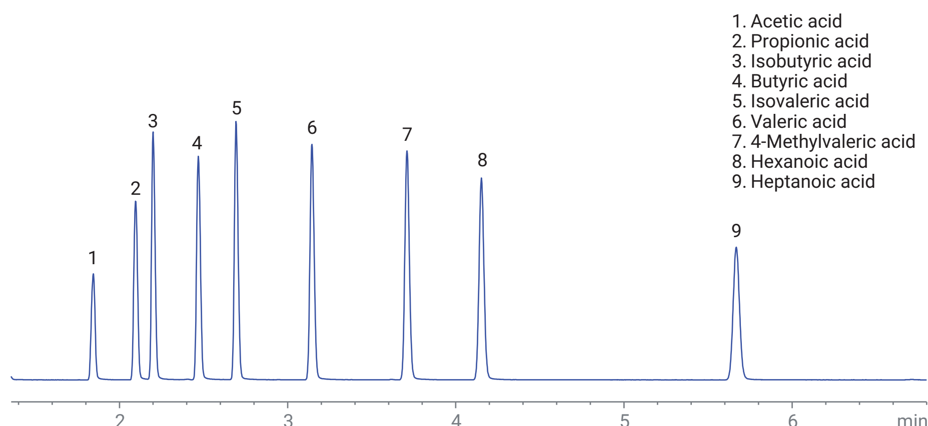

Figure 1. GC/FID chromatogram of a mixture of C2–C7 free fatty acids in water on a 30 m × 0.25 mm id, 0.25 µm J&W DB-FATWAX Ultra Inert column (conditions listed in Table 2).

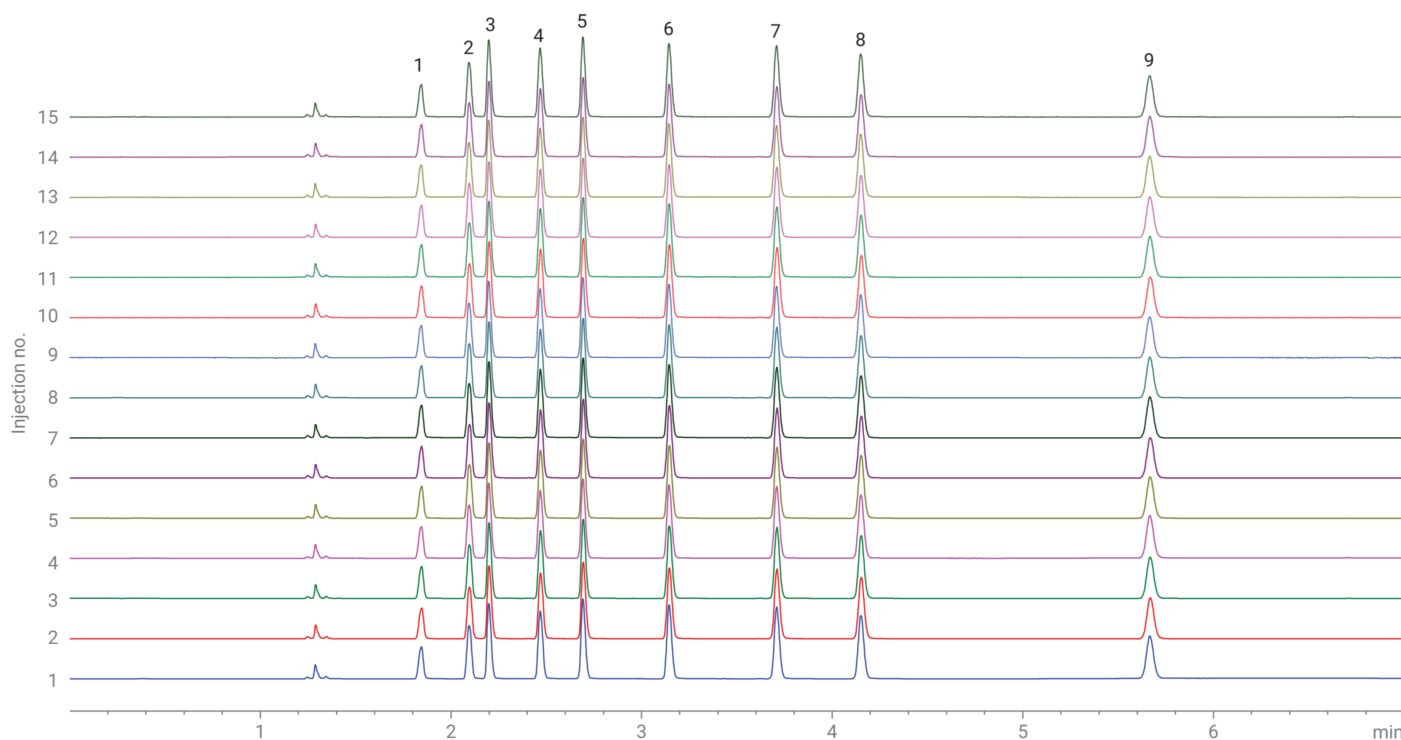

Figure 2. Overlaid GC/FID chromatograms of repeat injections of the same aqueous C2–C7 free fatty acids sample shown in Figure 1.

DB-FFAP, an acid-modified WAX column, is designed primarily for the analysis of organic acids, free fatty acids, or samples that require quantitation of acidic impurities. Figure 3 shows the chromatograms of the aqueous C2–C7 free fatty acids test mix on both a DB-FFAP column and a DB-FATWAX UI column. The analysis was completed in approximately five minutes on the DB-FFAP column, and six minutes on the DB-FATWAX UI column. Both columns provide excellent peak shape and good resolution for all these acids. Table 6 shows peak asymmetry at 10 % peak height (As.10 %) of each peak in this chromatogram; As.10 % for DB-FATWAX UI is between 0.98–1.16, and DB-FFAP is between 0.91–1.20. DB-FATWAX UI provides more symmetrical peaks of acetic acid (peak 1) and isobutyric acid (peak 3).

Two other commercially available WAX-type columns from different suppliers were used to do the same tests under the same conditions. Peak tailing was observed for the WAX columns from other suppliers using the aqueous C2–C7 free fatty acids mix (Figure 4 and Table 6). The column activity and unstable phase, especially Supplier 1's WAX column, also lead to retention time drifting, poorly resolved peaks, and loss of responses of critical analytes of interest, such as acetic acid (peak 1), propionic acid (peak 2), and isobutyric acid (peak 3). The same results are shown in Figure 6.

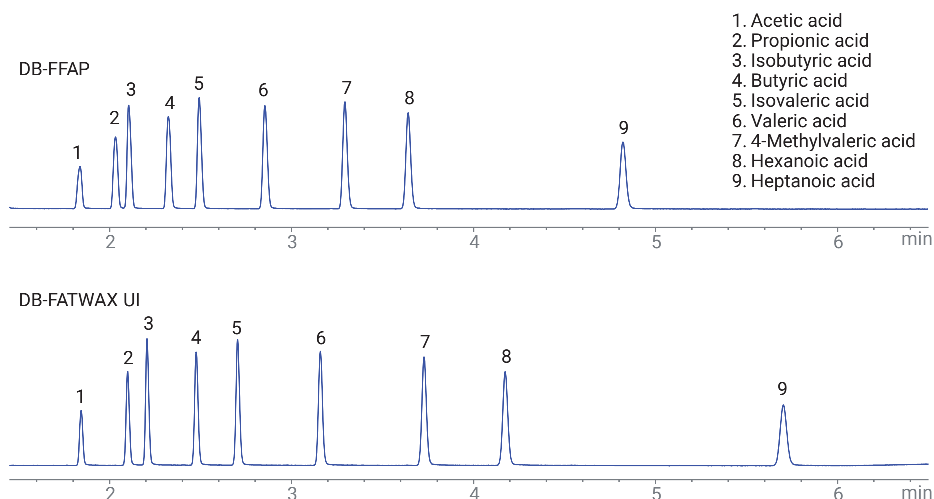

Figure 3. GC/FID chromatograms of the aqueous C2–C7 free fatty acids sample on a 30 m × 0.25 mm id, 0.25 µm J&W DB-FFAP column and a DB-FATWAX Ultra Inert column using Method 1 (see Table 2).

Table 6. Peak asymmetry at 10 % peak height (As. 10 %).

Peak no.	As. 10 %								
	1	2	3	4	5	6	7	8	9
DB-FATWAX UI	0.98	1.04	1.15	1.16	1.14	1.08	1.06	1.07	1.04
DB-FFAP	0.91	1.05	1.20	1.16	1.16	1.15	1.09	1.05	1.06
Supplier 1 WAX	1.56	coelution	coelution	1.97	1.65	2.04	1.96	1.96	1.87
Supplier 1 WAX	0.97	1.08	1.32	1.22	1.28	1.23	1.27	1.26	1.23

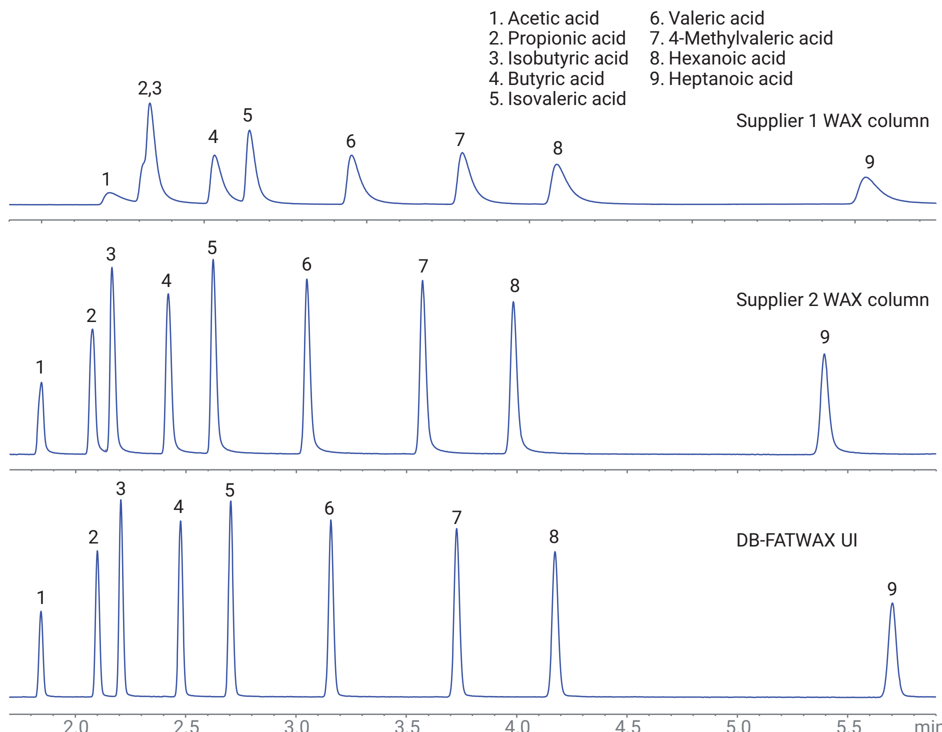

Figure 4. GC/FID chromatograms of the aqueous C2–C7 free fatty acids sample on a J&W DB-FATWAX UI column and WAX columns from Suppliers 1 and 2 using Method 1 (see Table 2).

C2–C18 organic acids test mixture

Figures 5–7 show the example GC/FID chromatograms of the C2–C18 organic acids test mixture on J&W DB-FFAP and DB-FATWAX UI columns as well as WAX columns from different suppliers. The testing was performed after the columns were conditioned for 50 hours at 250 °C. As shown in Figure 5, DB-FFAP demonstrated relatively lower column bleed at the high temperature (250 °C) and shorter analysis time to elute the higher molecular weight organic acids such as C18 fatty acids compared with DB-FATWAX UI. However, the DB-FATWAX UI column provided comparable or even better peak shapes for C2–C12 volatile organic acids than DB-FFAP according to Figure 6, the enlarged section of Figure 5. These volatile acids significantly affect the flavor and quality of food[7], the content of these active volatile acids is an index for quality assurance in some foods. Figure 7 shows that inertness performance of WAX columns from different suppliers deteriorated differently after conditioning the column for 50 hours. The WAX column from Supplier 2 was reported as an inert wax phase column. On this column, all of the compounds, including isomers, could be separated, but there was noticeable tailing of C2–C7 free organic acids in the chromatogram. The WAX column from Supplier 1 is a typical conventional wax phase GC column. The lack of column inertness and thermal stability for the WAX column from Supplier 1 lead to severe peak tailing, and negatively affected sensitivity, resolution, and selectivity especially for active C2–C7 free fatty acids and levulinic acid.

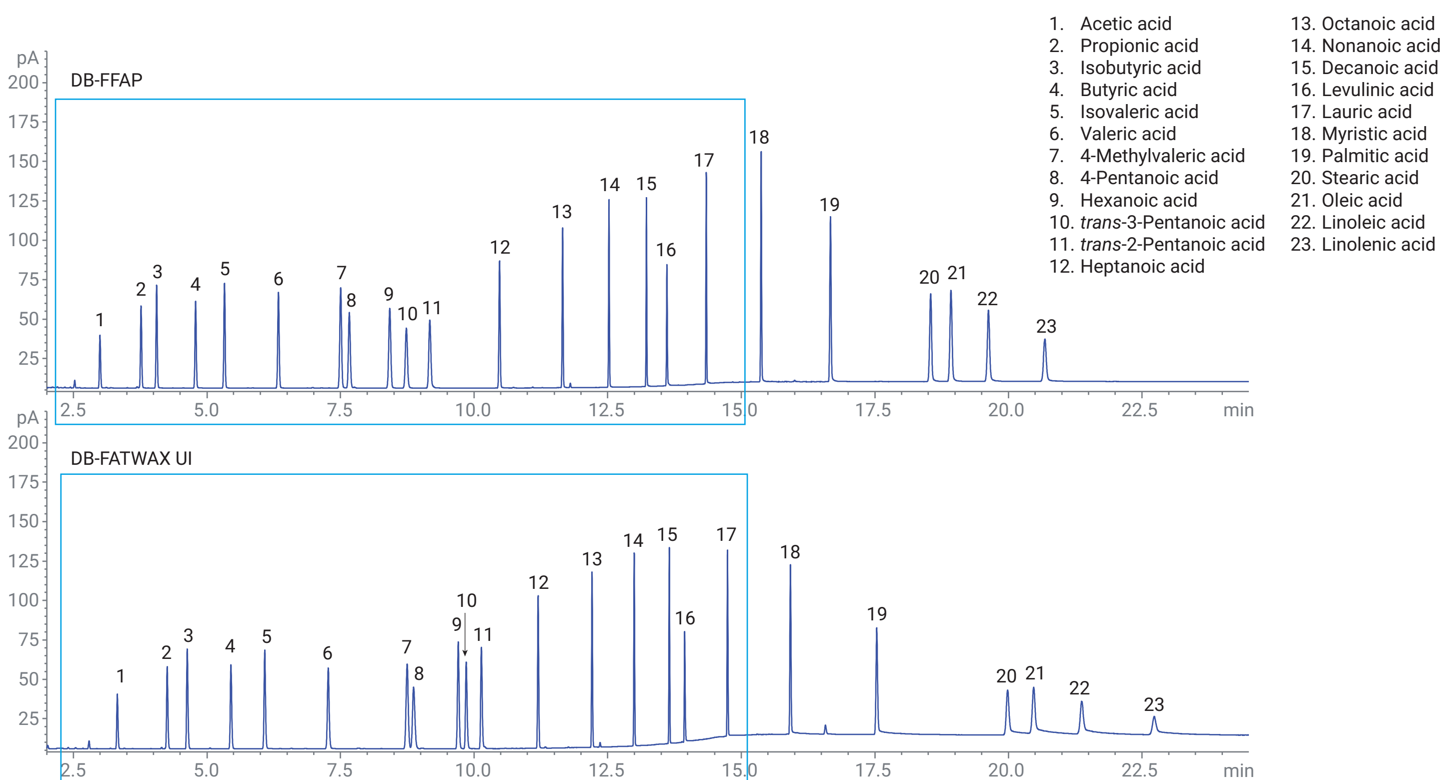

Figure 5. Example FID chromatograms of the organic acids (C2–C18) test mix on J&W DB-FFAP and DB-FATWAX UI GC columns using Method 2 (see Table 3).

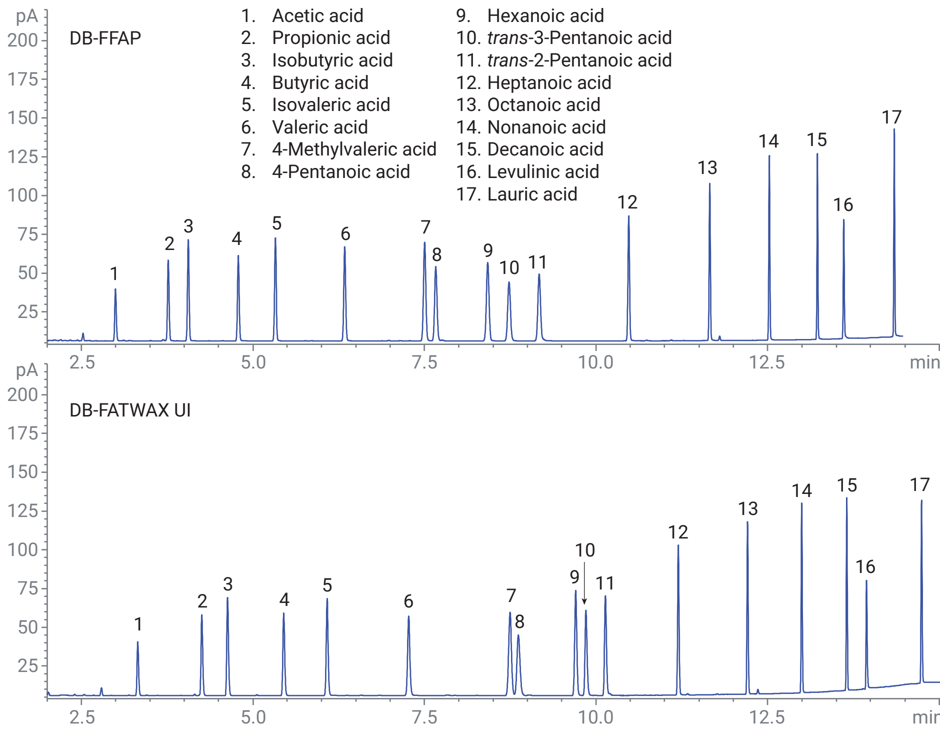

Figure 6. Enlarged section of the GC/FID chromatogram of the organic acids (C2–C18) test mix separated on J&W DB-FFAP and DB-FATWAX UI GC columns (the elution order is the same as Figure 5).

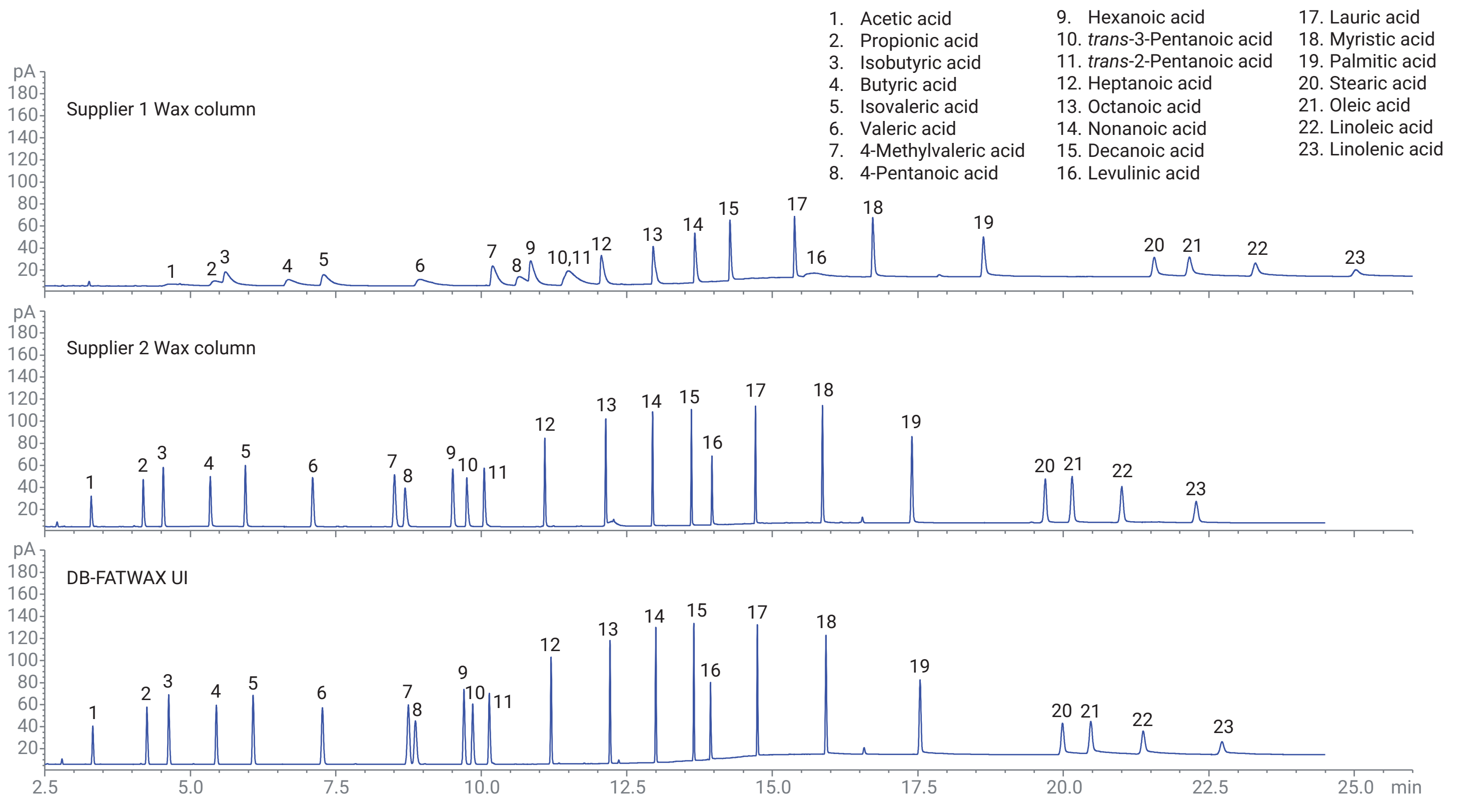

Figure 7. Example GC/FID chromatograms of the organic acids (C2–C18) test mix on a J&W DB-FATWAX UI and other WAX columns from different suppliers (the elution order was the same as Figure 5).

Agilent WAX UI test mixture

DB-FATWAX UI GC columns are part of the J&W Ultra Inert GC column family. To ensure inertness performance for these active polar compounds, every column is tested with the industry's most demanding test probe mixture[6]. The WAX UI test mixture includes propionic acid, ethyl hexanoic acid, and other active compounds. Figures 8 and 9 show the chromatograms of the WAX UI test mixture separated on DB-FATWAX UI, DB-FFAP, and WAX columns from Suppliers 1 and 2. Excellent peak shapes were obtained for the compounds of interest with the DB-FATWAX UI column. DB-FFAP and DB-FATWAX UI share a similar inertness performance for propionic acid and 2-ethylhexanoic acid, but Figure 8 shows that there is noticeable tailing with the peak of ethylene glycol in the chromatograms for the DB-FFAP column. The As. 10 % for ethylene glycol was 0.37 on DB-FFAP, and 0.91 on DB-FATWAX UI. In addition, reduced response is evident in the chromatogram for ethylene glycol on DB-FFAP. The WAX column from Supplier 2 exhibits tailing peaks of decanal, propionic acid, and ethylene glycol, with peak asymmetry values of 1.28, 1.32, and 1.26, respectively. The column activity of the WAX column from Supplier 1 was characterized by tailing peaks and loss of response of critical analytes of interest, such as decanal, propionic acid, ethylene glycol, 2-ethylhexanoic acid, and ethyl maltol (Figure 9).

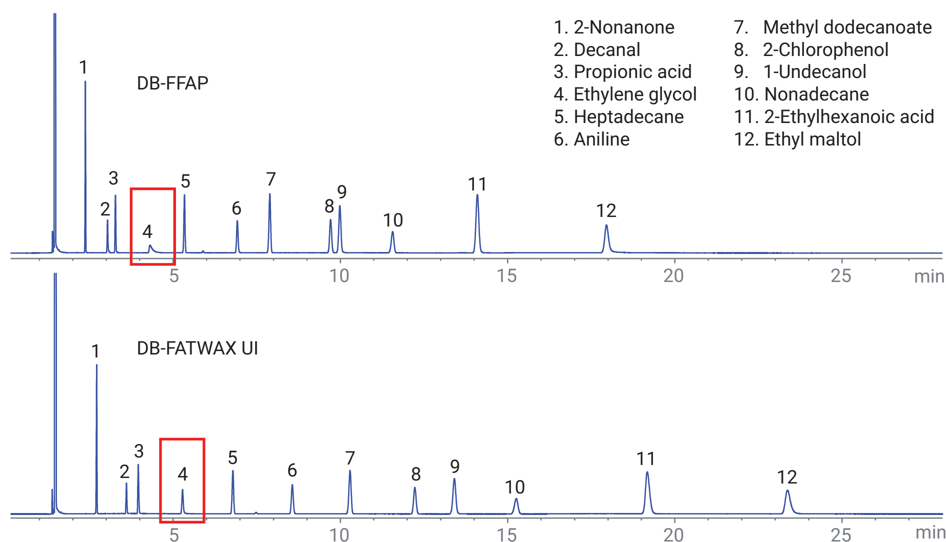

Figure 8. Example GC/FID chromatograms of the WAX UI test mix on J&W DB-FFAP and DB-FATWAX UI GC columns using Method 3 (see Table 4).

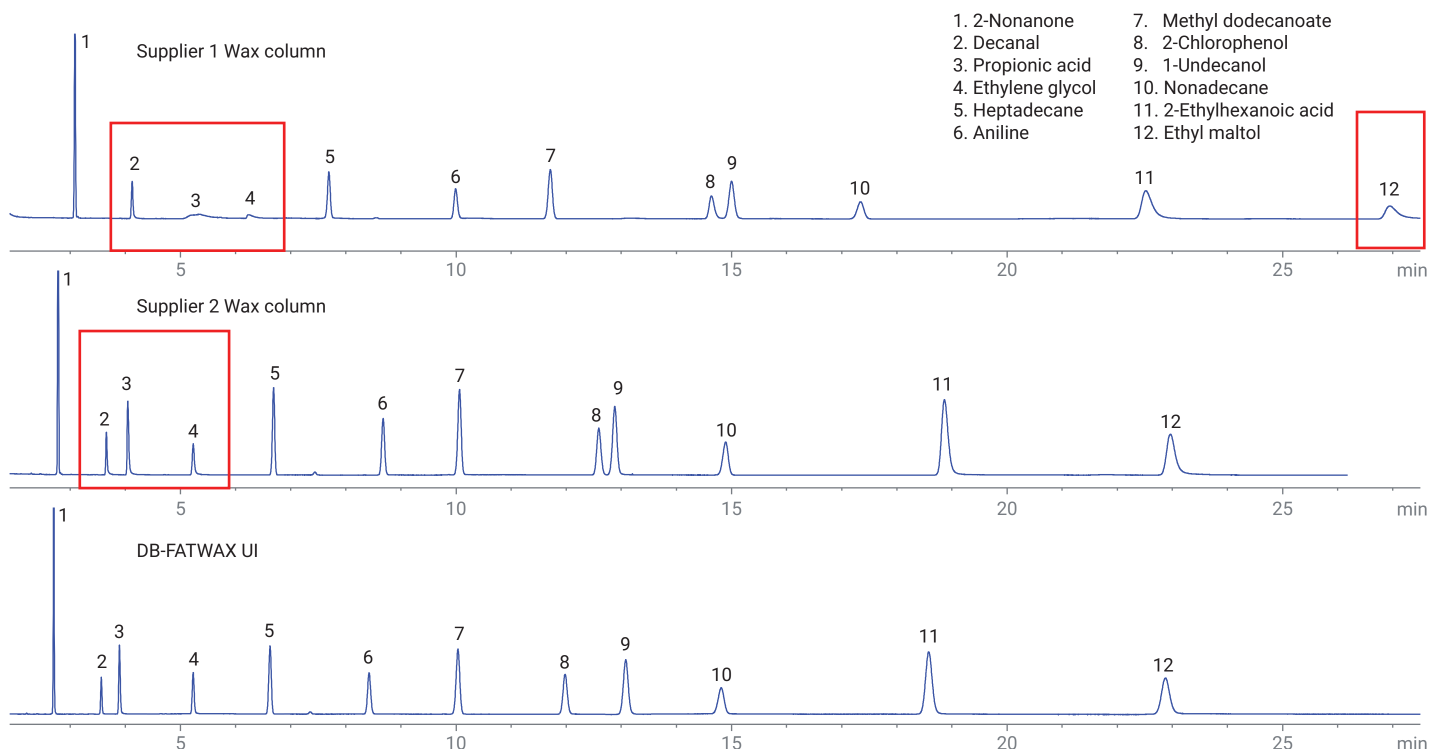

Figure 9. Example GC/FID chromatograms of the WAX UI test mix on a J&W DB-FATWAX UI and WAX GC columns from other suppliers using Method 3 (see Table 4).

Conclusion

A J&W DB-FATWAX Ultra Inert GC column was evaluated by analyzing organic acids and WAX UI test mixtures using GC/FID. High inertness and the improved thermal stability of the DB-FATWAX UI provided better peak shapes and more consistent analytical results than the other suppliers' WAX columns evaluated in this report. This was especially true in the presence of strong short-chain volatile organic acids and aqueous samples. Volatile organic acids and free fatty acids are well resolved, with sharp and symmetrical peaks on both DB-FATWAX UI and DB-FFAP GC columns. The better column for C18 fatty acids analysis is the DB-FFAP column due to reduced column bleed at high temperatures (250 °C) and shorter analysis times. The best column for complex samples including alcohols, diols, glycols, and organic acids is the DB-FATWAX Ultra Inert because it produces sharper peaks and higher responses especially for these most active compounds, enhancing the sensitivity and reproducibility for these challenging analytes. The excellent phase stability of DB-FATWAX UI for aqueous injections was demonstrated by the reproducible analysis of C2–C7 free fatty acids in water.

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Quality

Chapter 3

Application Note

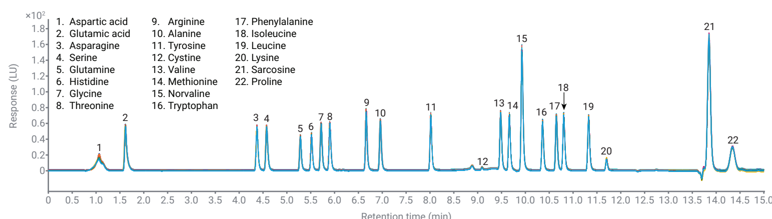
Food & Beverage Testing

Agilent

Trusted Answers

Comparison of Plant-Based Meat Alternatives and Meat

Analysis of amino acid profiles using an Agilent 1260 Infinity II LC



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Abstract

Increasing numbers of plant-based meat alternatives are being developed in response to consumer demand for sustainable food supplies and a healthy diet. The comparison of the amino acid profile of proteins from meat and plant-based meat alternatives is of interest from a nutritional quality perspective.

This application note shows the determination of the amino acid composition of beef burger patties and different plant-based burger patties following acidic hydrolysis of sample proteins. Amino acids are analyzed with an Agilent 1260 Infinity II LC using reversed-phase LC with fluorescence detection (FLD) and automated precolumn derivatization using the injector program available with the Agilent 1260 Infinity II Multisampler.

Introduction

In recent years, there has been increasing consumer interest in meat alternatives. Reasons for this trend include negative impressions of the health impact of meat, environmental stress associated with animal meat production, and animal welfare.[1] Meat alternatives include plant-based, cell-based (*in vitro* or cultured meat), and fermentation-based (mycoproteins) products. Plant-based meat alternatives represent a primary sector of this industry, and their market has grown exponentially in recent years.[1] The transition towards a more plant-based diet is considered to reduce a person's environmental footprint compared with the consumption of animal-based foods. This transition appears to be supported by plant-based products that directly mimic meat, and thus do not lead to a fundamental change in dietary habits.[2] Most plant-based meat alternatives in development are protein-based, and considering their availability, cost, and processing functionality, soy and pea proteins as well as wheat gluten are most widely used.[1] From a nutritional quality perspective, the comparison of the amino acid profile of meat and plant-based meat alternatives is of interest.

The amino acid profile of a protein is typically analyzed following hydrolysis with 6 M HCl at 110 °C for 24 hours.[3] Analysis of amino acids can be performed using various analytical methods, such as LC with fluorescence or UV detection following derivatization, LC/MS, or CE/MS.[3,4] Precolumn derivatization with o-phthalaldehyde (OPA) and 3-mercaptopropionic acid for primary as well as 9-fluorenylmethylchloroformate (FMOC) for secondary amino acids overcomes the insufficient analyte retention on reversed-phase columns and the weak fluorescence and ultraviolet absorbance of amino acids.[4] Furthermore, derivatization of amino acids followed by LC with FLD increases selectivity of the analysis.

This application note demonstrates the analysis of the amino acid profile of beef burger patties and different plant-based burger patties using a 1260 Infinity II LC with FLD. The injector program available with the 1260 Infinity II Multisampler enables automated precolumn derivatization of amino acids[4], avoiding manual liquid handling steps and saving time and cost generated by manual work. Possible errors resulting from manual work are also prevented.

Experimental

Equipment

The Agilent 1260 Infinity II LC System comprised the following modules:

- Agilent 1260 Infinity II Binary Pump (G7112B)
- Agilent 1260 Infinity II Multisampler (G7167A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Fluorescence Detector Spectra (G7121B) with flow cell, 8 µL, 20 bar (G1321-60005)

Software

Agilent OpenLab CDS version 2.6, or later versions

Columns

Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm (part number 695975-322) with Agilent AdvanceBio AAA guard column, 3.0 × 5 mm, 2.7 µm (part number 823750-946)

Chemicals

All solvents were LC grade. Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, EMD Millipore, Billerica, MA, USA). Sodium phosphate dibasic, disodium tetraborate decahydrate, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid, 37%, and phosphoric acid, 85%, were obtained from Merck (Darmstadt, Germany), and hydrochloric acid, 6 N, was obtained from Fluka (Steinheim, Germany).

Amino acid standards and derivatization reagents were obtained from Agilent:

- Amino acid supplement (part number 5062-2478) containing: L-asparagine, L-glutamine, L-tryptophan, L-4-hydroxyproline, L-norvaline, and sarcosine (1 g each)
- Amino acid standard, 100 pmol/µL (part number 5061-3332)
- Amino acid standard, 25 pmol/µL (part number 5061-3333)
- Amino acid standard, 10 pmol/µL (part number 5061-3334)
- Borate buffer 0.4 N in water, pH 10.2, 100 mL (part number 5061-3339)
- FMOC reagent, 2.5 mg/mL 9-fluorenylmethylchloroformate in acetonitrile, 10 × 1 mL (part number 5061-3337)

– OPA reagent, 10 mg/mL each of o-phthalaldehyde and 3-mercaptopropionic acid in 0.4 M borate buffer, 6 × 1 mL (part number 5061-3335)

Samples

Beef burger patties and different plant-based burger patties based on pea, soy, and wheat protein were obtained from a local supermarket.

Preparation of solvents and derivatization reagents

– **Mobile phase A:** Weigh 2.8 g of sodium phosphate dibasic (Na_2HPO_4) and 7.6 g of disodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$), add 1.9 L of water and 1.5 mL of fuming hydrochloric acid (37%), mix until homogeneous, fill up to the total volume of 2 L with water and adjust the pH with fuming hydrochloric acid to pH 8.2. It is recommended to use an amber 2 L solvent bottle (part number 9301-6341) to avoid algae growth.

– **Mobile phase B:** Acetonitrile:methanol:water 45:45:10 (v:v:v)

– **Injection diluent:** 10 mL of mobile phase A + 200 µL of phosphoric acid (85%)

– After opening an OPA or FMOC ampoule, the reagents are distributed to amber vials (part number 5182-0716) with inserts (part number 5181-1270) and screw caps (part number 5190-7024) and stored for no longer than a week. Borate buffer and injection diluent are transferred to vials without inserts. All reagents should be stored at 4 °C and reagents in the autosampler should be exchanged daily.

Preparation of amino acid standard solutions

– An extended amino acid (EAA) stock solution containing 1.8 nmol/µL each of asparagine, glutamine, and tryptophan was prepared in 0.1 M HCl in water. The EAA stock solution was diluted to 0.9 nmol/µL, 0.45 nmol/µL, 0.18 nmol/µL, 90 pmol/µL, 45 pmol/µL, 18 pmol/µL, and 9 pmol/µL with 0.1 M HCl in water.

– An internal standard (IS) stock solution containing 1.0 nmol/µL each of norvaline and sarcosine was prepared in 0.1 M HCl in water.

– The EAA solutions were combined 1:1 with the IS stock solution to obtain amino acid concentrations of 4.5 to 900 pmol/µL and IS concentrations of 500 pmol/µL.

– Amino acid calibration solutions were prepared at 0.45, 0.90, 2.25, 4.5, 9.0, 22.5, 45, and 90 pmol/µL of amino acids and 50 pmol/µL of internal standards by combination of the EAA-IS solutions with amino acid standards and 0.1 M HCl in water.

Sample preparation

For determination of the amino acid profile, proteins contained in the samples were hydrolyzed using 6 N HCl following a procedure described by Dai *et al.*[3] Note that acidic hydrolysis leads to the conversion of asparagine, glutamine, and cysteine to aspartic acid, glutamic acid, and cystine, respectively. Tryptophan is decomposed during acidic hydrolysis.[3]

To investigate method suitability, amino acid recoveries were determined in triplicate from BSA. For this purpose, approximately 50 mg of BSA (equivalent to 0.75 µmol of protein) was weighed into a 15 mL Kimax glass tube, 10 mL of 6 N HCl was added, and the tube was gassed with nitrogen and capped.

For determination of the amino acid profiles of the samples, approximately 2 g of sample was accurately weighed and homogenized in 8 mL water using a laboratory homogenizer. Approximately 1.2 g of the resulting suspension was weighed into a 15 mL Kimax glass tube and appropriate amounts of water and fuming hydrochloric acid (37%) were added to result in 10 mL 6 M HCl. The tube was gassed with nitrogen and capped.

The tubes were placed in an oven with an inside temperature of 110 °C for 24 hours. After 2 hours, the tubes were gently shaken to ensure that the sample was completely covered by the solution. After the 24-hour period, the tubes were allowed to cool to room temperature, and the whole solution was transferred to a 100 mL flask and made up to the final volume with water. One hundred microliters of the resulting hydrolysate was combined with 50 µL of the IS stock solution and 850 µL water and filtered using a 1 mL plastic syringe with Agilent Captiva premium syringe filters, regenerated cellulose, 15 mm, 0.2 µm (part number 5190-5108).

Table 1. Method for analysis of derivatized amino acids.

Parameter	Value
Column	Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm with Agilent AdvanceBio AAA guard column, 3.0 × 5 mm, 2.7 µm
Solvent	A) 10 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$, pH 8.2 B) Acetonitrile:methanol:water (45:45:10, v:v:v)
Gradient	0.00 min – 2% B 0.40 min – 2% B 13.60 min – 57% B 14.00 min – 100% B Stop time: 17 min Post time: 3 min
Flow Rate	0.600 mL/min
Temperature	40 °C
Detection	Excitation: 345 nm; emission: 455 nm 13.00 min: change excitation: 265 nm; change emission: 315 nm PMT gain: 10 Peak width: >0.025 min (18.52 Hz)
Injection	Use sample preparation method (injector program) shown in Table 2 for derivatization of amino acids Injection volume: 1 µL Needle wash: 5 s in acetonitrile:0.1 M HCl in water (50:50; v:v) Draw speed: 100 µL/min Eject speed: 400 µL/min Wait time after draw: 1.2 s Use vial/well bottom sensing

Table 2. Sample preparation method (injector program) for derivatization of amino acids.

Function	Parameter
Draw	Draw 5.00 µL from location "Borate Buffer" with default speed using default offset
Wash	Wash needle as defined in method
Draw	Draw 1.00 µL from sample with default speed using default offset
Wash	Wash needle as defined in method
Draw	Draw 1.00 µL from location "OPA reagent" with default speed using default offset
Wash	Wash needle as defined in method
Mix	Mix 7.00 µL from air with default speed 10 times
Draw	Draw 0.40 µL from location "FMOC reagent" with default speed using default offset
Wash	Wash needle as defined in method
Mix	Mix 7.40 µL from air with default speed 10 times
Draw	Draw 32.00 µL from location "Injection Diluent" with maximum speed using default offset
Wash	Wash needle as defined in method
Mix	Mix 20.00 µL from air with maximum speed five times
Inject	Inject

Results and discussion

To enable determination of the amino acid profile of beef burger patties and different plant-based burger patties, amino acids were analyzed using reversed-phase LC with FLD and automated precolumn derivatization following acidic hydrolysis of sample proteins. The LC-FLD analysis with precolumn derivatization of amino acids using the injector program has been described in detail in a previous application note.[4]

Figure 1 shows the tenfold analysis of a calibration solution containing 22.5 pmol/µL of amino acids and 50 pmol/µL of internal standards. Twenty amino acids and the two internal standards norvaline and sarcosine were successfully separated within a run time of 17 minutes. Excellent retention time and peak area precision was obtained, showing values below 0.1% RT RSD and below 1.0% area RSD for most compounds (N = 10; see Table 3).

Repeatability, sensitivity, and calibration results obtained during the analysis of amino acid calibration solutions are presented in Table 3. Excellent sensitivity with limit of detection (LOD) values below 0.2 pmol on column was observed for all amino acids except cystine. The higher LOD obtained for cystine can be explained by low fluorescence of the adduct formed with the OPA reagent.[5] Calibration was performed in the range of 0.45 to 90 pmol/µL and showed excellent R^2 values above 0.999 for all compounds.

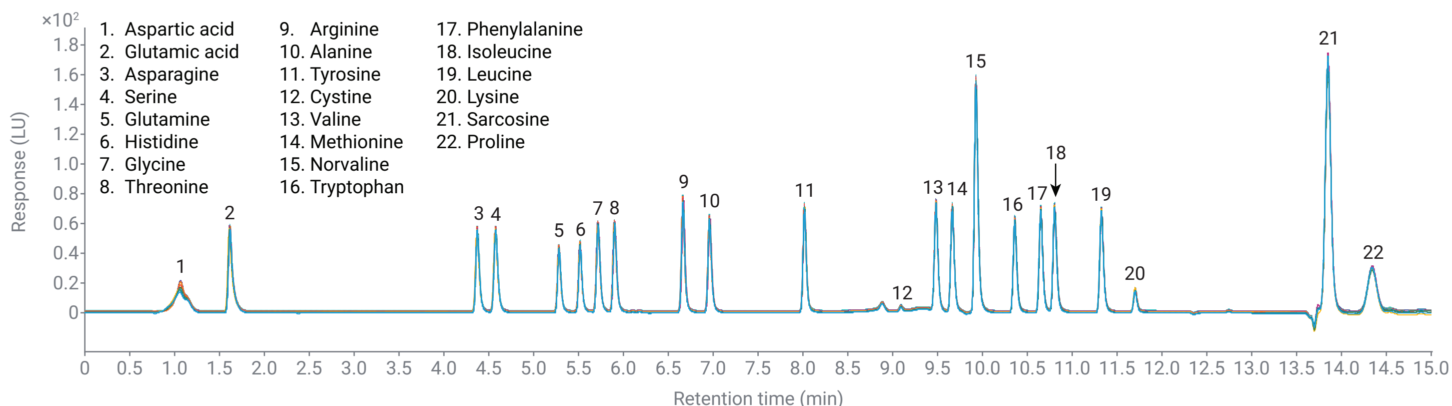

Figure 1. Tenfold analysis of a calibration solution containing 22.5 pmol/µL of amino acids and 50 pmol/µL of internal standards.

Table 3. Repeatability, sensitivity, and calibration results obtained during amino acid analysis. Repeatability calculations are based on 10 consecutive analyses of a calibration solution containing 22.5 pmol/µL of amino acids and 50 pmol/µL of internal standards. Limit of detection (LOD) is calculated for an S/N value of 3.

Peak No.	Compound	Retention Time (min)	RT RSD (%)	Area RSD (%)	LOD (pmol on column)	Calibration Range (pmol/µL)	Calibration Type	R^2
1	Aspartic acid	1.07	0.60	0.58	0.12	0.45 to 90	Linear	0.99999
2	Glutamic acid	1.63	0.20	0.30	0.07	0.45 to 90	Linear	0.99999
3	Asparagine	4.38	0.05	0.26	0.06	0.45 to 90	Linear	1.00000
4	Serine	4.58	0.05	0.24	0.06	0.45 to 90	Linear	0.99999
5	Glutamine	5.29	0.04	0.21	0.08	0.45 to 90	Linear	0.99999
6	Histidine	5.53	0.03	0.60	0.07	0.45 to 90	Quadratic	0.99999
7	Glycine	5.73	0.03	0.32	0.05	0.45 to 90	Linear	0.99999
8	Threonine	5.91	0.03	0.24	0.06	0.45 to 90	Linear	1.00000
9	Arginine	6.67	0.04	0.27	0.05	0.45 to 90	Linear	0.99999
10	Alanine	6.97	0.03	0.23	0.06	0.45 to 90	Linear	0.99998
11	Tyrosine	8.02	0.02	0.26	0.05	0.45 to 90	Linear	0.99999
12	Cystine	9.10	0.01	1.08	1.98	4.5 to 90	Quadratic	0.99989
13	Valine	9.49	0.01	0.24	0.05	0.45 to 90	Linear	0.99999
14	Methionine	9.67	0.01	0.23	0.06	0.45 to 90	Linear	0.99993
15	Norvaline*	9.93	0.01	0.23	NA	NA	NA	NA
16	Tryptophan	10.37	0.01	0.44	0.06	0.45 to 90	Linear	0.99993
17	Phenylalanine	10.66	0.01	0.28	0.06	0.45 to 90	Linear	0.99999
18	Isoleucine	10.81	0.01	0.32	0.05	0.45 to 90	Linear	0.99996
19	Leucine	11.33	0.01	0.40	0.05	0.45 to 90	Linear	0.99996
20	Lysine	11.71	0.01	3.22	0.17	0.45 to 90	Quadratic	0.99925
21	Sarcosine*	13.85	0.01	2.57	NA	NA	NA	NA
22	Proline	14.35	0.01	3.05	0.04	0.45 to 90	Quadratic	0.99997

* Internal standard

To investigate suitability of the method for the analysis of the amino acid profile of a protein, BSA was hydrolyzed, and the recovery rates of the individual amino acids were determined. Figure 2 and Table 4 show the analysis and the determined recovery rates.

As mentioned previously, acidic hydrolysis using 6 M HCl at 110 °C for 24 hours leads to the conversion of asparagine, glutamine, and cysteine to aspartic acid, glutamic acid, and cystine, respectively, and tryptophan is decomposed during acidic hydrolysis.[3] Tryptophan could be recovered using an alkaline hydrolysis and the determination of asparagine and glutamine could be accomplished using enzymatic hydrolysis.[3]

Recovery rates determined for the individual amino acids range between 85% and 115% for all compounds except cystine (see Table 4). These results demonstrate the suitability of the method for the analysis of the amino acid profile of a protein.

Figure 3 and Table 5 show the results of the amino acid profile analysis of a beef burger patty and three different plant-based burger patties that were obtained from a local supermarket. Differences between the amino acid profiles of the individual samples can be clearly observed. Cystine could be detected in all samples analyzed but was not quantified, as peak areas were below the calibration range.

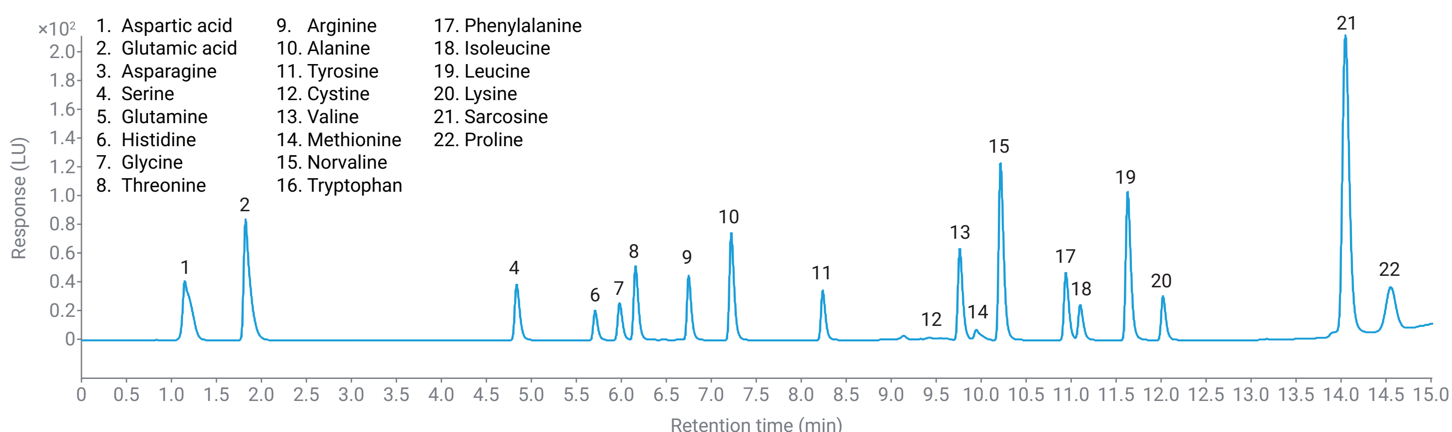

Figure 2. Analysis of the amino acid profile of a hydrolysate of bovine serum albumin (BSA).

Table 4. Recovery rates of the individual amino acids determined during the analysis of a hydrolysate of bovine serum albumin (BSA) (N = 3).

Peak No.	Compound	Recovery Rate (%)
1	Aspartic acid*	115.3
2	Glutamic acid*	104.7
3	Asparagine	Converted to aspartic acid
4	Serine	85.2
5	Glutamine	Converted to glutamic acid
6	Histidine	90.6
7	Glycine	94.3
8	Threonine	86.8
9	Arginine	92.6
10	Alanine	90.6

Peak No.	Compound	Recovery Rate (%)
11	Tyrosine	86.6
12	Cystine	45.5
13	Valine	88.2
14	Methionine	109.5
16	Tryptophan	Not recovered
17	Phenylalanine	91.2
18	Isoleucine	91.0
19	Leucine	89.7
20	Lysine	95.2
22	Proline	95.0

* Aspartic acid and glutamic acid originate from the sum of aspartic and glutamic acid contained in the sample and the conversion of asparagine and glutamine to their respective acids during acidic hydrolysis.

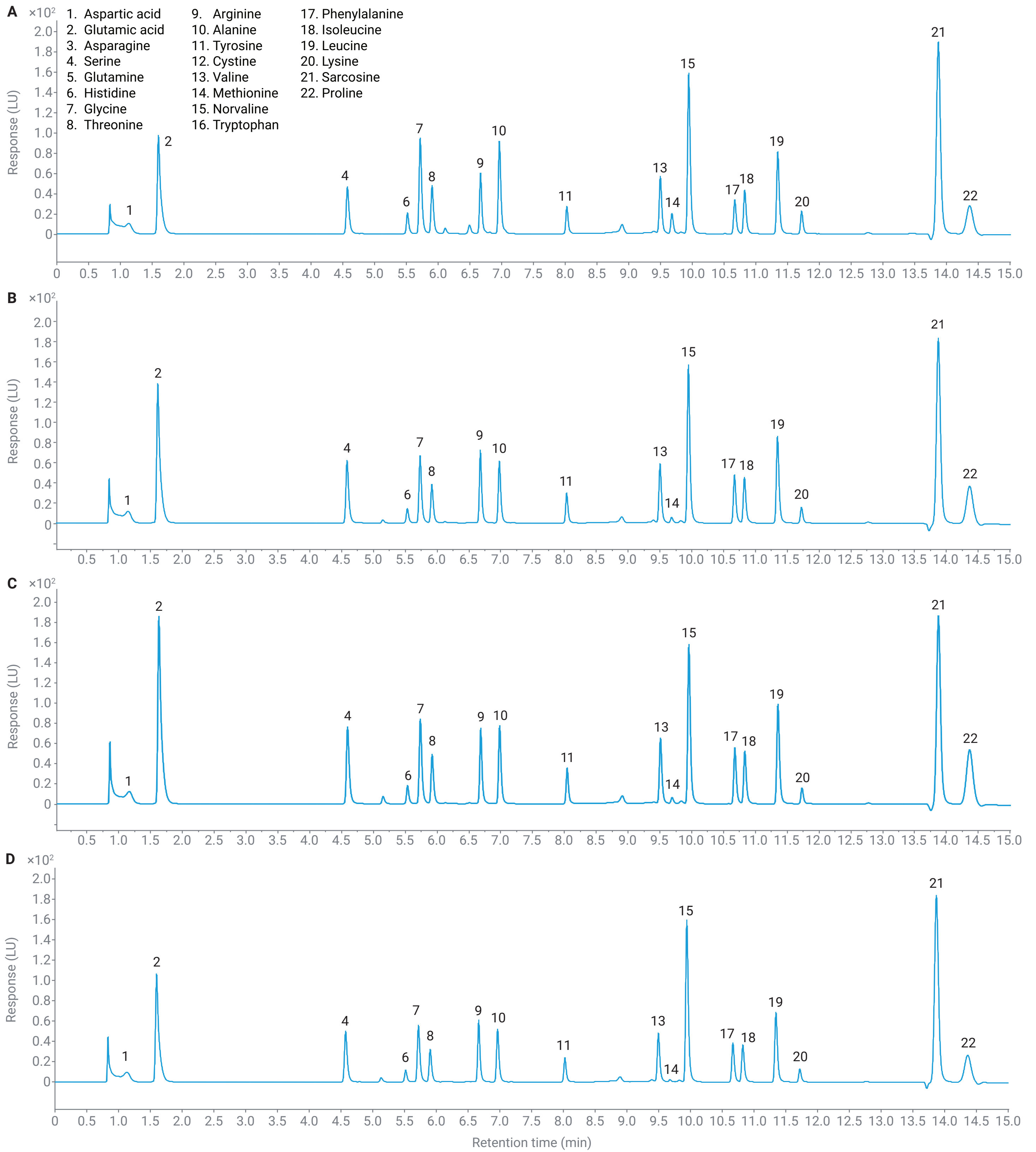

Figure 3. Analysis of the amino acid profile of hydrolysates of beef burger patties (A) and different plant-based burger patties (B–D).

Table 5. Determined amino acid profile of beef burger patties and different plant-based burger patties.

Peak No.	Compound	Percentage of Amino Acids (%)			
		Beef Burger	Plant-Based Burger B	Plant-Based Burger C	Plant-Based Burger D
1	Aspartic acid*	8.8	10.0	9.1	10.3
2	Glutamic acid*	12.2	17.3	19.4	16.5
3	Asparagine	Converted to aspartic acid			
4	Serine	5.5	7.3	7.4	7.3
5	Glutamine	Converted to glutamic acid			
6	Histidine	3.0	2.1	2.2	2.1
7	Glycine	10.7	7.4	7.7	7.7
8	Threonine	5.3	4.2	4.4	4.3
9	Arginine	5.2	6.1	5.1	6.3
10	Alanine	9.7	6.2	6.5	6.6
11	Tyrosine	2.5	2.7	2.7	2.8
12	Cystine	Not quantified			
13	Valine	5.1	5.2	4.7	5.3
14	Methionine	1.8	0.5	0.4	0.3
16	Tryptophan	Not detected			
17	Phenylalanine	3.3	4.5	4.3	4.5
18	Isoleucine	4.2	4.3	4.1	4.4
19	Leucine	8.1	8.4	7.9	8.4
20	Lysine	8.8	6.3	4.9	6.2
22	Proline	5.9	7.6	9.1	7.0

* Aspartic acid and glutamic acid originate from the sum of aspartic and glutamic acid contained in the sample and the conversion of asparagine and glutamine to their respective acids during acidic hydrolysis.

According to literature, alanine, glycine, and methionine are less abundant in plant-based burgers compared to meat burgers, whereas glutamic acid is more abundant in plant-based burgers.[6] These differences in the amino acid profile could also be observed in the current analysis (see Table 5).

Among the plant-based burger patties analyzed, burgers B and D were based on pea protein, whereas burger C was based on soy and wheat protein. For burger C, a slightly higher percentage of glutamic acid and a slightly lower percentage of lysine was determined compared to burgers B and D (see Table 5). This result is consistent with a higher amount of glutamic acid and a lower amount of lysine found in soy protein compared to pea protein.[7,8]

Conclusion

The analysis of amino acids was performed using reversed-phase LC with fluorescence detection following automated precolumn derivatization using the injector program of the Agilent 1260 Infinity II Multisampler. This enabled successful determination of the amino acid profiles of beef burger patties and different plant-based burger patties. Excellent precision and sensitivity were obtained using the Agilent 1260 Infinity II LC. Automation of derivatization also removed the need for manual liquid handling steps, reducing sources of error and saving time and cost generated by manual work.

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Application Note

Food Testing & Agriculture

Quantitation of Amino Acids in Soy Flour, Dried Cow's Milk Powder, and Corn Silage by Triple Quadrupole LC/MS/MS

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Abstract

This application note describes a method for the detection, identification and quantitation of underivatized amino acids in ruminant feed ingredients and dried cow milk powder using an Agilent 1290 Infinity II LC coupled to an Agilent 6470 triple quadrupole LC/MS. The sample preparation procedure involves three specific protein/peptide hydrolysis methods. Released amino acids are separated using hydrophilic interaction chromatography (HILIC). The method was applied to soy flour, dried cow's milk powder, and corn silage, achieving great sensitivity, linearity, and accuracy.

Introduction

Amino acids are the organic structural units which form proteins and are often called the building blocks of life. The general structure of amino acids contains a basic amino group ($-NH_2$) and carboxyl group (–COOH). It is challenging to accurately separate, identify, and quantify amino acids in food and feed products due to interference from endogenous components in the sample. An important sample preparation step for analysis of bound amino acids is protein/peptide hydrolysis, which commonly involves hydrolytic digestion in 6 N hydrochloric acid at approximately 110 °C for 24 hours. Methionine and cystine are present in low concentrations and undergo oxidation to various oxidized derivatives during acid hydrolysis. Controlled oxidation of methionine to methionine sulfone and cystine to cysteic acid are required using performic acid prior to acid hydrolysis. Tryptophan is also present in low concentrations but is extensively degraded during acid hydrolysis, so it is normal to use alkaline hydrolysis.[1,2]

Released amino acids are traditionally separated using ion-exchange or reversed-phase chromatography with ion-pairing reagents, then analyzed using diode array detection or fluorescence detection after derivatization. LC/MS/MS has become increasingly popular for amino acid detection due to greater sensitivity, high selectivity, quantitative accuracy and high throughput. As a consequence of the selectivity and specificity of the mass spectrometer, the need for derivatization during sample preparation and need for ion-pairing reagents in LC detection are eliminated, which also increases reproducibility and robustness in the analysis.

In this study, a fast and sensitive UHPLC-MS/MS method was evaluated to provide identification and accurate quantification of amino acids in complex food and feed matrices. The postextraction matrix-matched standard was included to evaluate any effect of the matrix on recoveries and accuracy of detection. Method criteria for data acceptance were established.

Experimental

Equipment

The LC/MS analysis was performed using an Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II multisampler (G7167B), an Agilent 1290 Infinity II high-speed pump (G7120A), and an Agilent 1290 Infinity II multicolumn thermostat (G7116B) coupled to a 6470 triple quadrupole LC/MS (G6470A). The system was controlled by Agilent MassHunter Acquisition software version 10.1. Data processing was performed with Agilent MassHunter quantitative analysis software version 10.1 and Agilent MassHunter qualitative analysis software version 10.0.

Samples and standards

The sample matrices in this study included soy flour (NIST Standard Reference Material 3234), dried cow's

Chromatographic conditions

Parameter	Setting
Analytical Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.7 µm, 2.1 × 150 mm, PEEK-lined, (p/n 673775-924)
Column Oven	25 ±2 °C
Injection Volume	1 µL
Run Time	14 min
Autosampler	15 ±2 °C
Mobile Phase A	10 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	10 mM ammonium formate + 0.1% formic acid in 90% acetonitrile
Seal Wash	90/10 Water/IPA
Needle Wash	50/50 MeOH/H_2O
Gradient	Time (min) / Flow (mL/min) / %A / %B: 0 / 0.4 / 0 / 100 5.0 / 0.4 / 20 / 80 6.0 / 0.4 / 30 / 70 7.0 / 0.4 / 50 / 50 9.0 / 0.4 / 80 / 20 10.0 / 0.4 / 80 / 20 10.5 / 0.4 / 0 / 100 14.0 / 0.4 / 0 / 100

MS parameters

Parameter	Setting
MS Acquisition	dMRM
Ion Source Type	Agilent Jet Stream Electrospray ionization (AJS ESI ±)
Drying Gas Temperature	330 °C
Drying Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Heater	390 °C
Sheath Gas Flow	12 L/min
Capillary	2,000 V (ESI ±)
Nozzle Voltage	0 V (ESI ±)
Precursor Ion and Production Ion Resolution	Unit
Compound-Specific Conditions	See Table 1

milk powder, and dried, ground corn silage. An amino acid standard mix (part number 5061-3330) including alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine was obtained from Agilent Technologies Inc. (Santa Clara, CA, USA); methionine sulfone, cysteic acid and tryptophan were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA). The samples and standards were stored at 5 °C.

Method

Description

The following steps are a detailed description of sample preparation. Table 1 gives the analyte-specific LC/MS conditions.

1. Weigh 0.3 ±0.015 g sample into a 40 mL glass tube with PTFE cap. Add the spiking solution (an amino acid composite standard solution) for spiking samples.

For the detection of cystine and methionine, follow steps 2 through 5:

2. Keep the samples and performic acid on ice for ~30 minutes. Add 5 mL of performic acid into sample. Swirl gently.
3. Store samples for at least 16 hours in an ice bath in refrigerator.
4. Under a fume hood, with samples on ice, add ~0.8 to 1 g of sodium metabisulfite to sample and swirl gently for at least 15 minutes.
5. Add 5 mL of 12 N HCl. Cap and vortex.

For the detection of tryptophan, follow step 6:

6. Add 15 mL 4 N NaOH to sample. Cap and vortex.

Table 1. Analyte-specific LC/MS conditions: precursor to product ion transitions, fragmentor, collision energies (CE), and retention times (RT).

Compound Name	Precursor Ion	Product Ion	RT (min)	Delta RT (min)	Fragmentor	Collision Energy
Alanine	90.1	44.1	6.0	1.2	40	9
Alanine	90.1	45.1	6.0	1.2	40	40
Arginine	175.1	116.1	7.7	1.5	105	2
Arginine	175.1	70.1	7.7	1.5	105	8
Arginine	175.1	60.1	7.7	1.5	105	4
Aspartic Acid	134.1	88.0	7.2	1.2	75	0
Aspartic Acid	134.1	74.0	7.2	1.2	75	4
Aspartic Acid	134.1	70.0	7.2	1.2	75	6
Cystine	241.0	152.0	7.7	1.5	105	0
Cystine	241.0	120.0	7.7	1.5	105	0
Cystine	241.0	74.1	7.7	1.5	105	25
Glutamic Acid	148.1	84.0	6.7	1.2	85	6
Glutamic Acid	148.1	56.1	6.7	1.2	85	22
Glutamic Acid	148.1	41.0	6.7	1.2	85	18
Glycine	76.0	48.0	6.4	1.2	40	0
Glycine	76.0	30.0	6.4	1.2	40	12
Histidine	156.1	110.1	7.6	1.2	95	4
Histidine	156.1	95.1	7.6	1.2	95	6
Isoleucine	132.1	86.1	4.6	1.2	85	0
Isoleucine	132.1	44.1	4.6	1.2	85	16
Isoleucine	132.1	41.0	4.6	1.2	85	18
Isoleucine	132.1	30.0	4.6	1.2	85	6
Leucine	132.1	86.1	4.4	1.2	85	0
Leucine	132.1	44.1	4.4	1.2	85	14
Leucine	132.1	41.0	4.4	1.2	85	25
Leucine	132.1	30.0	4.4	1.2	85	4
Lysine	147.1	130.1	7.9	1.5	85	0
Lysine	147.1	84.1	7.9	1.5	85	6
Methionine	150.1	104.1	4.7	1.2	75	0
Methionine	150.1	61.0	4.7	1.2	75	14
Methionine	150.1	56.1	4.7	1.2	75	6
Methionine	150.1	28.0	4.7	1.2	75	26
Phenylalanine	166.1	120.1	4.1	1.2	85	4
Phenylalanine	166.1	103.1	4.1	1.2	85	22
Phenylalanine	166.1	91.1	4.1	1.2	85	32
Phenylalanine	166.1	77.0	4.1	1.2	85	36
Proline	116.1	70.1	5.3	1.2	85	6
Proline	116.1	43.1	5.3	1.2	85	25
Serine	106.1	88.1	6.5	1.2	65	8
Serine	106.1	42.1	6.5	1.2	65	24
Threonine	120.0	74.1	6.0	1.2	75	0
Threonine	120.0	56.1	6.0	1.2	75	6

For the detection of the remaining amino acids, follow step 7:

7. Add 5 mL of 6 N HCl. Cap and vortex.

Perform the following steps for all above samples:

8. Place samples in a 110 °C heat block for at least 24 hours.
9. Allow samples to cool to room temperature.
10. Quantitatively transfer the samples to a 50 mL centrifuge tube with Milli-Q water. Bring to the maximum volume mark. Mix well.
11. Filter ~1 mL of the sample through a 0.2 µm nylon syringe filter into a 1.8 mL microcentrifuge tube.
12. Dilute further as needed with 0.1 N HCl.
13. Prepare the postspiked sample (post spike an amino acid composite standard solution to the diluted sample extract before injection to evaluate the matrix effect) along with the diluted sample.
14. The samples are now ready for LC/MS/MS injection using both positive/negative ESI modes.

Tryptophan	205.1	187.9	4.2	1.2	50	16
Tryptophan	205.1	146.0	4.2	1.2	50	23
Tryptophan	205.1	117.9	4.2	1.2	50	10
Tyrosine	182.1	136.1	5.0	1.2	95	0
Tyrosine	182.1	119.1	5.0	1.2	95	10
Tyrosine	182.1	91.1	5.0	1.2	95	22
Tyrosine	182.1	77.0	5.0	1.2	95	34
Valine	118.1	72.1	5.2	1.2	75	0
Valine	118.1	55.1	5.2	1.2	75	14
Cysteic Acid*	168.0	150.9	6.6	1.2	90	12
Cysteic Acid*	168.0	80.9	6.6	1.2	90	20
Methionine* Sulfone	180.0	79.1	5.4	1.2	70	30
Methionine* Sulfone	180.0	64.0	5.4	1.2	70	45

* In ESI negative mode

Evaluation procedure

The method performance was evaluated by analyzing a NIST soy flour (standard reference material 3234) sample. The reference mass fraction values for amino acids are listed in Table 2. The reagent blanks (0.1% HCl solution) were spiked with amino acids at 100 to 200 µg and went through the hydrolysis/dilution to confirm the method extraction efficiency without matrix. The quantitation was performed using an external calibration curve with 1/x weight and single point postmatrix spike correction.

Evaluation criteria

Specificity:

- The relative error (RE %) of retention time of each analyte peak to the average of standard peaks is less than 5%.
- The ion ratio is within the tolerance of 30%.

Linearity and range:

- Calibration curve has R^2 >0.99.
- Calculated working standard values should be within ±30% of the theoretical value.
- The calibration standards should bracket the analyte concentration level.

Accuracy:

- The test result for each amino acid in soy flour is within ±30% deviation of the reference mass fraction value (see Table 2), which is considered the true value.
- The recovery for prespike of amino acid contents in the reagent blank is within 80 to 120%.
- The postspike matrix recovery is within 50 to 150% for result correction (due to the variations in sample preparation, analyte detection, and instrument performance, the criteria should be set by each individual lab).

Table 2. Mass fraction values for amino acids in NIST soy flour standard reference material 3234.

Compound Name	Mass Fraction g/100 g
Alanine	2.28 ±0.16
Arginine	3.72 ±0.31
Aspartic Acid	6.0 ±1.2
Cystine	0.74 ±0.15
Glutamic Acid	10.2 ±1.4
Glycine	2.22 ±0.15
Histidine	1.22 ±0.089
Isoleucine	2.31 ±0.23
Leucine	4.03 ±0.42
Lysine	3.20 ±0.25
Methionine	0.69 ±0.13
Phenylalanine	2.54 ±0.13
Proline	2.71 ±0.23
Serine	2.69 ±0.32
Threonine	2.02 ±0.11
Tryptophan	0.66 ±0.14
Tyrosine	1.76 ±0.43
Valine	2.45 ±0.41

Results and discussion

Column selection

In this study, an Agilent InfinityLab Poroshell HILIC-Z column was used for underivatized amino acids separation.[3] Excellent chromatographic performance in terms of resolution, peak shape and sensitivity were achieved using HILIC column with low pH mobile phase additives. See Figure 1 for the elution profile of amino acids. Baseline separation of leucine and isoleucine isomers was also achieved.

Specificity

A dynamic multiple reaction monitoring (dMRM) acquisition method was used for amino acid identification and quantitation. Monitoring MS/MS transitions with evaluation of the ratio for their relative product ion intensities and RT of analyte peaks enables the target analyte to be distinguished from potential interferences in quantitative analysis. Figure 1 shows an example of an extracted ion chromatogram of a 100 ng/mL working standard in 0.1% HCl. Figure 2 shows that no amino acids are present in the reagent blank at a level greater than 30% of the lowest calibration standard.

Range and linearity

The method was evaluated over the concentration range of 1 to 2,500 ng/mL. To evaluate the linearity of the method, nine working standard (WS) solutions of amino acids were made at 1, 5, 10, 20, 50, 100, 500, 1,000, and 2,500 ng/mL. The calibration curve residuals were ≤30% for WS1 to WS9. Figure 3 demonstrates the statistical data of the calibration curve residuals. The linearity was determined by using a linear calibration with a 1/x weighting factor. The coefficients of determination (R^2) value were >0.99. Table 3 lists the data of the linear concentration range and coefficients of determination.

Approaches for accurate quantitation

Interfering substances in the matrix can be observed and may affect the electrospray ionization process, causing suppression or enhancement of the analyte signal. While good sample preparation and cleanup can mitigate many of these interferences, some may still remain. Currently, there are no guidelines for dealing with matrix effects due to variations in method and instrument performance. However, matrix effects need to be compensated. A postspike matrix-matched standard can address the matrix effect and any other matrix interactions for quantitation purposes when an internal standard is not available or not easy to obtain.[4] Postspike recovery was determined by fortifying samples after extraction with the analyte composite standard solution. The results were corrected using postspike recovery if it was within 50 to 150%.

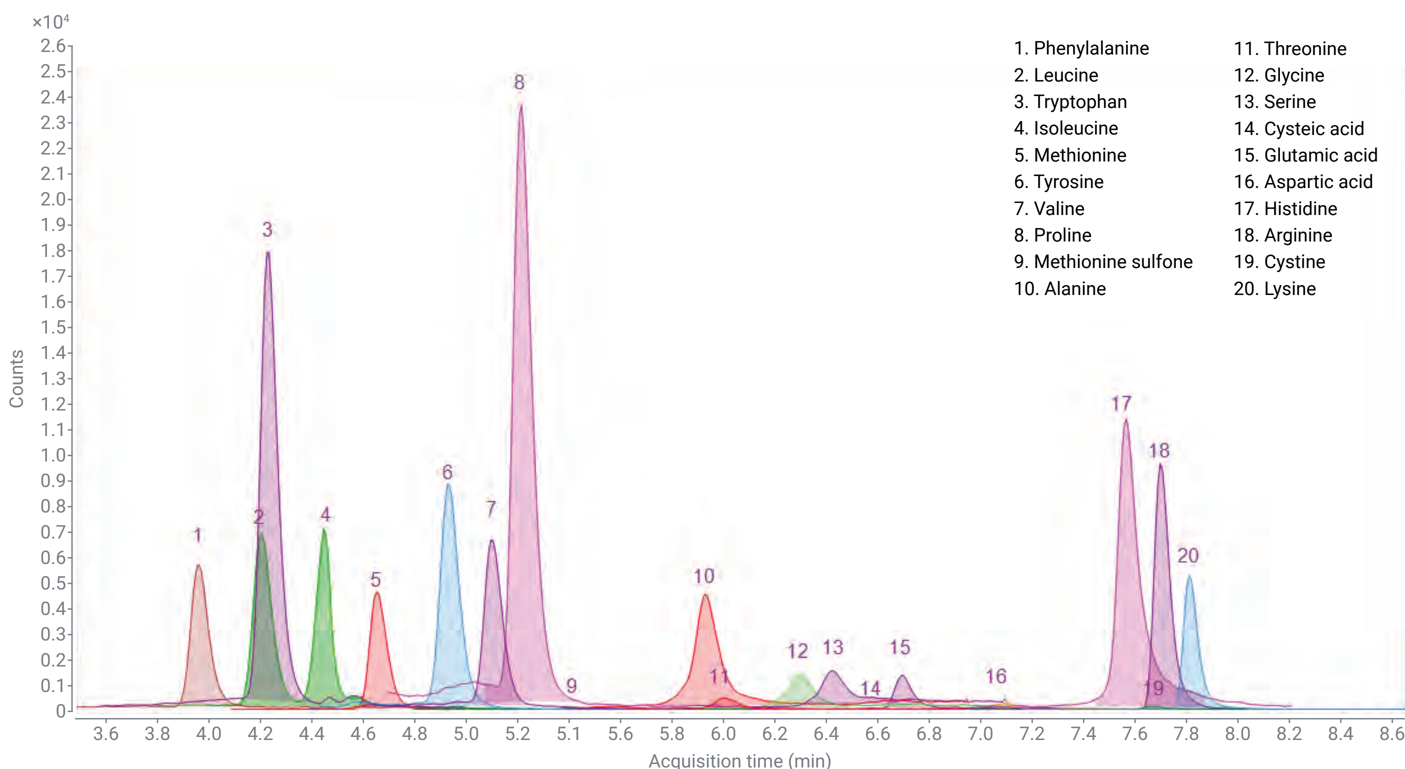

Figure 1. HILIC elution profile of amino acids in a 100 ng/mL working standard mix in 0.1 N HCl, 1 µL injection volume.

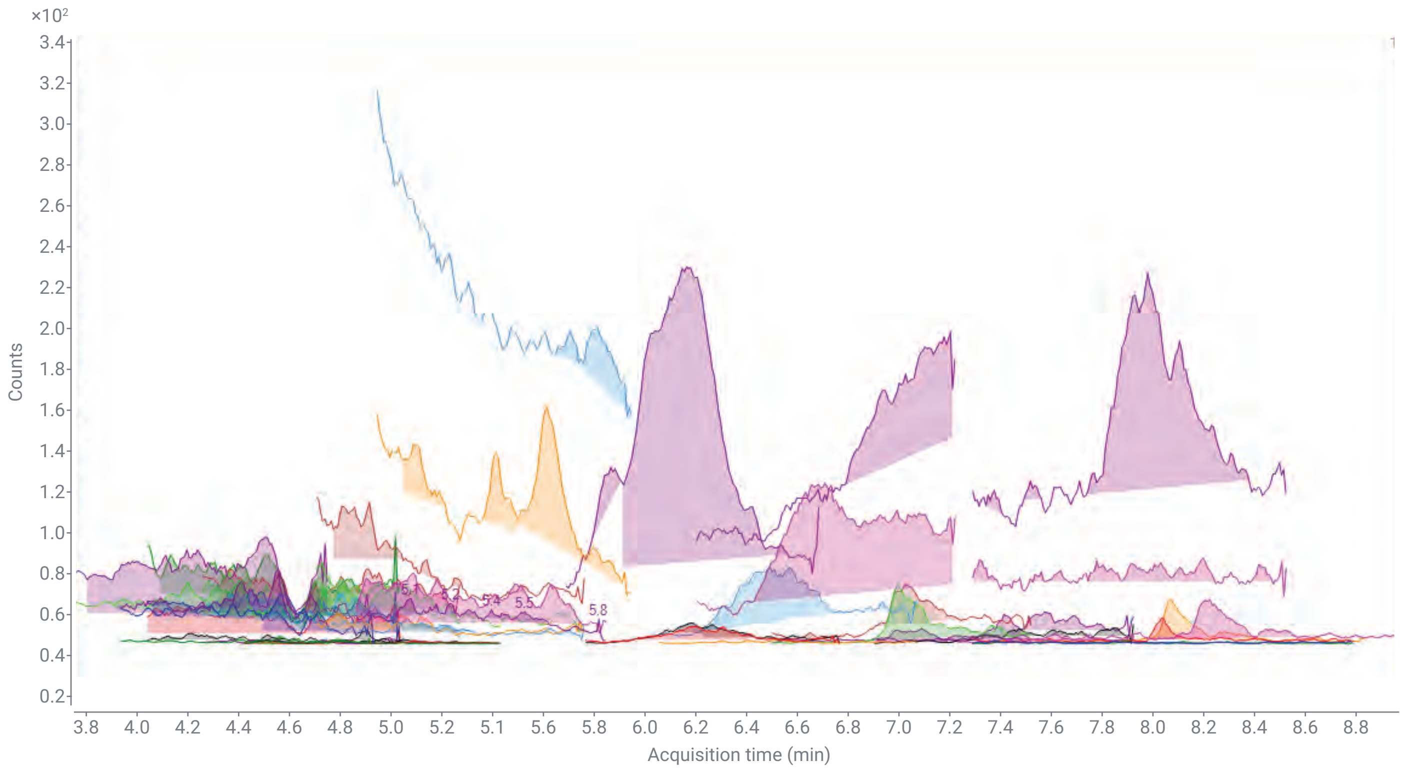

Figure 2. Extracted ion chromatogram of a solvent blank, 0.1% HCl.

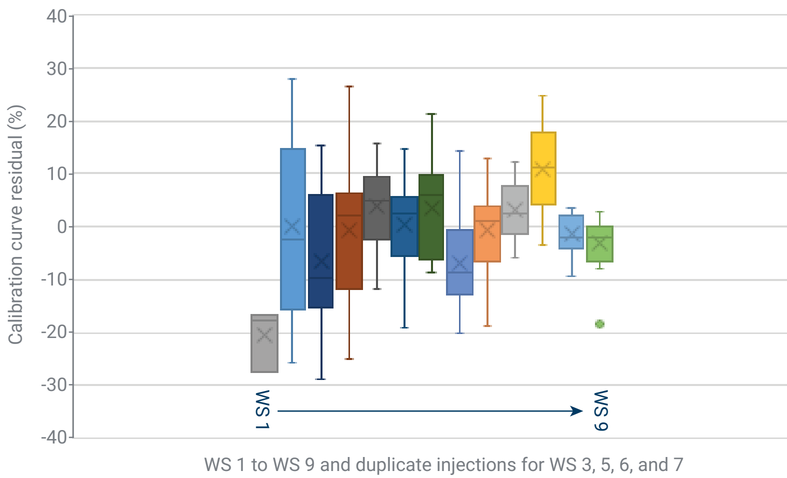

Figure 3. Calibration curve residual for working standards.

Table 3. The linear concentration range and coefficients of determination R^2.

Compound Name	Range (ng/mL)	R^2
Alanine	10 to 1,000	0.9980
Arginine	5 to 1,000	0.9973
Aspartic Acid	20 to 2,500	0.9972
Cysteic Acid	20 to 2,500	0.9988
Cystine	1 to 2,500	0.9917
Glutamic Acid	5 to 2,500	0.9977
Glycine	20 to 1,000	0.9947
Histidine	10 to 2,500	0.9989
Isoleucine	5 to 1,000	0.9949
Leucine	5 to 1,000	0.9906
Lysine	5 to 1,000	0.9988
Methionine sulfone	5 to 1,000	0.9964
Methionine	1 to 1,000	0.9913
Phenylalanine	1 to 1,000	0.9916
Proline	5 to 1,000	0.9992
Serine	10 to 1,000	0.9912
Threonine	10 to 1,000	0.9969
Tryptophan	5 to 1,000	0.9978
Tyrosine	5 to 1,000	0.9939
Valine	5 to 1,000	0.9980

Sample tests

Prespike recoveries were obtained for all amino acids (within 90 to 115% except 81.5% for methionine) in reagent blank samples, indicating that significant losses in analyte amounts are not observed during hydrolysis and extraction. Table 4 shows the recovery for each amino acid.

The evaluated method was applied to a variety of general agricultural products, including soy flour, dried cow's milk powder, and corn silage. The high sensitivity of LC/MS/MS allows a large dilution after sample extraction. The postspike recoveries for all amino acids fell into the accepted range (93% of postspike recoveries were within 70 to 130%) and the results were corrected. The corrected results using matrix-matched standards for all amino acids were in great agreement with the NIST mass fraction values (deviation between −2.4 to 18.3%) with the exception of cystine and methionine, which were at the low-end. Sample results and postspike recoveries for each analyte in each matrix are shown in Table 5.

Table 4. Spike recovery for reagent blank.

Compound Name	Reagent Blank Prespike Recovery (%)
Alanine	90.9
Arginine	104
Aspartic Acid	103
Cystine*	111
Glutamic Acid	104
Glycine	91.6
Histidine	107
Isoleucine	92.0
Leucine	106
Lysine	92.9

Compound Name	Reagent Blank Prespike Recovery (%)
Methionine*	81.5
Phenylalanine	108
Proline	102
Serine	100
Threonine	90.6
Tryptophan	110
Tyrosine	95.0
Valine	95.1

* Cystine and methionine were spiked into the reagent blank, converted to, and calculated from cysteic acid and methionine sulfone.

Table 5. Sample results for soy flour, dried cow's milk powder, and corn silage.

	Soy Flour				Dried Cow's Milk Powder		Corn Silage	
Compound Name	Mass fraction from NIST (g/100 g)	Results on DM* (g/100 g)	Postspike Recovery (%)	Deviation from NIST Value (%)	Results on DM (g/100 g)	Postspike Recovery (%)	Results on DM (g/100 g)	Postspike Recovery (%)
Alanine	2.28	2.12	115	−7.0	0.77	102	0.51	120
Arginine	3.72	3.54	103	−4.7	0.83	97.2	0.10	107
Aspartic Acid	6.00	5.33	127	−11.2	1.60	116	0.39	105
Glutamic Acid	10.20	9.95	122	−2.4	5.04	111	0.73	132
Glycine	2.22	2.10	131	−5.5	0.42	111	0.27	104
Histidine	1.22	1.45	110	18.3	0.92	101	0.12	111
Isoleucine	2.31	2.17	113	−6.3	1.12	106	0.14	148
Leucine	4.03	4.45	116	10.4	2.97	88.7	0.78	91.1
Lysine	3.20	3.56	113	11.2	2.58	102	0.16	108
Phenylalanine	2.54	2.79	115	9.8	1.27	98.2	0.25	107
Proline	2.71	2.62	109	−3.4	2.47	95.4	0.39	112
Serine	2.69	2.53	142	−6.0	1.28	106	0.23	110
Threonine	2.02	1.83	124	−9.6	1.04	101	0.23	98.9
Tryptophan**	0.66	0.64	110	−2.9	0.28	112	0.025	116
Tyrosine	1.76	1.51	120	−14.1	0.97	106	0.10	112
Valine	2.45	2.16	115	−11.8	1.51	96.8	0.31	105
Cysteic Acid	–	1.58	88.6	–	0.28	116	0.090	104
Cystine***	0.74	1.12	–	51.4	0.20	–	0.064	–
Methionine Sulfone	–	1.16	82.1	–	0.65	109	0.12	89.6
Methionine***	0.69	0.95	–	38.0	0.53	–	0.10	–

* DM: Dry matter: 93.24% for soy flour; 95.85% for dried cow's milk powder; 91.45% for corn silage.

** Tryptophan was analyzed after a sample was hydrolyzed using 4 N NaOH.

*** Cystine and methionine were analyzed after a sample was oxidized using performic acid and then hydrolyzed using 6 N HCl. Cystine and methionine results were calculated from cysteic acid (note: cystine in the sample was converted to two cysteic acids) and methionine sulfone, respectively.

Conclusion

A rapid, sensitive, and accurate UHPLC-MS/MS method for the identification and quantitation of underivatized amino acids in complex agricultural products was presented. The method used an Agilent 1290 Infinity II LC stack coupled to an Agilent 6470A triple quadrupole LC/MS with Agilent MassHunter workstation software. The need for a derivatization step in sample preparation and the use of ion-pairing reagents in LC detection are eliminated. The evaluation demonstrated that the method can achieve excellent specificity, linearity, and accuracy.

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Application Note

Food Testing & Agriculture

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 750 µL of dry

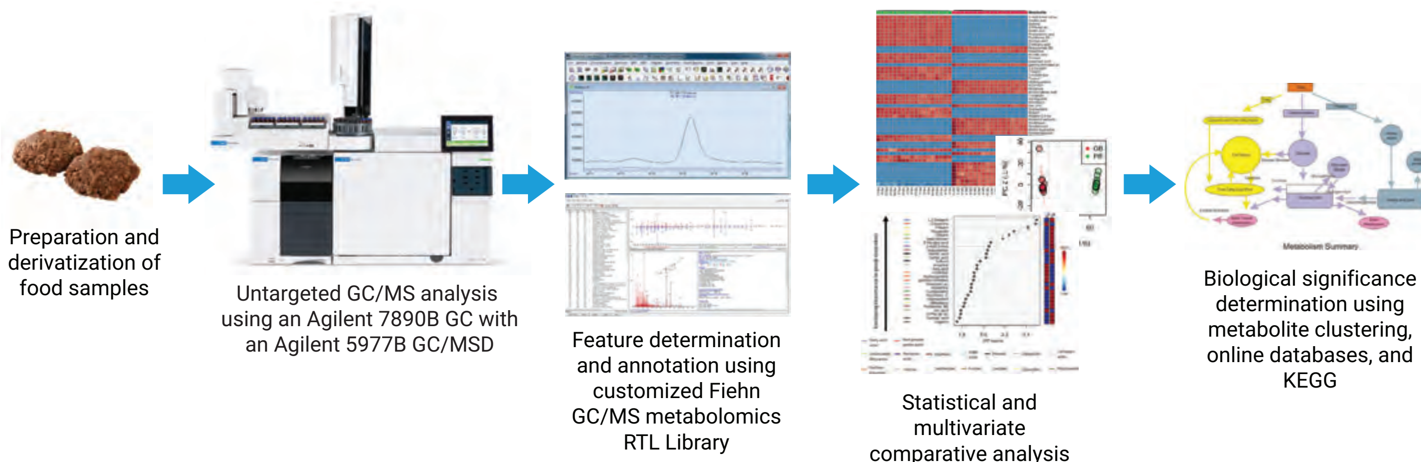

Figure 1. Overview of GC/MS-based metabolomics workflow used to compare the chemical profiles of plant-based and traditional meats.

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 GC retention time (RT), and EI mass spectral fragmentation pattern

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

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 the PB were amino acids, nonprotein amino acids, saccharides, saturated fatty acids, dicarboxylic acids, phenols, dipeptides, sugar alcohols, vitamins, glycerides,

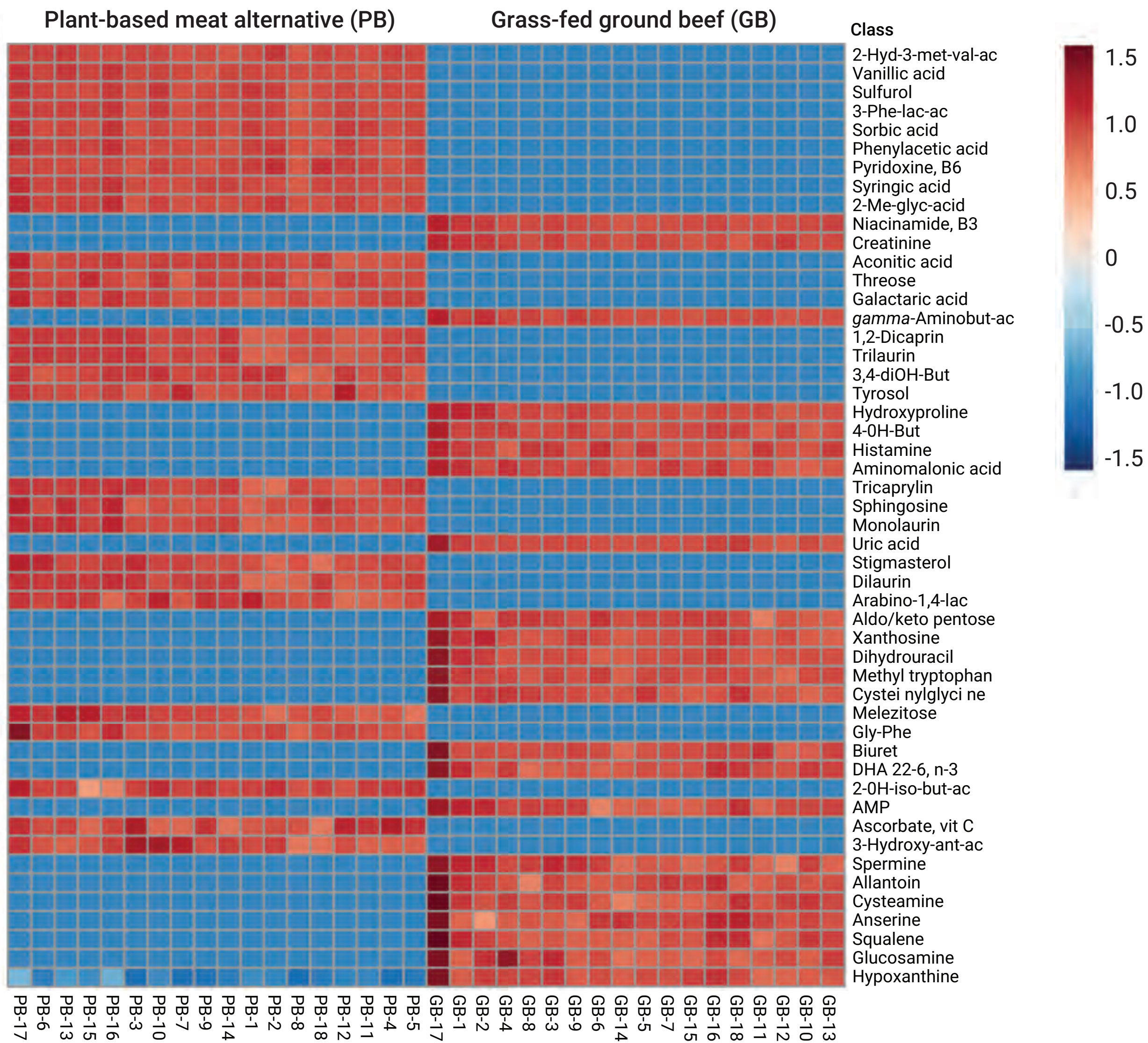

Figure 2. MetaboAnalyst-generated heat map of the top 50 compounds ranked by p values (lowest to highest) that were significantly different ($p < 0.05$) between the GB and the PB. Red (intensity ranges from 0 to 1.5) indicates the higher abundance (upregulation) of a compound, while blue (intensity ranges 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]

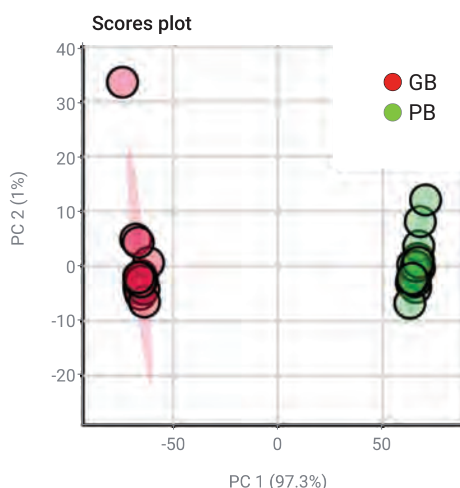

Figure 3. MetaboAnalyst-generated score plot created using unsupervised PCA. Figure courtesy of Van Vliet, S. *et al.*[3]

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]

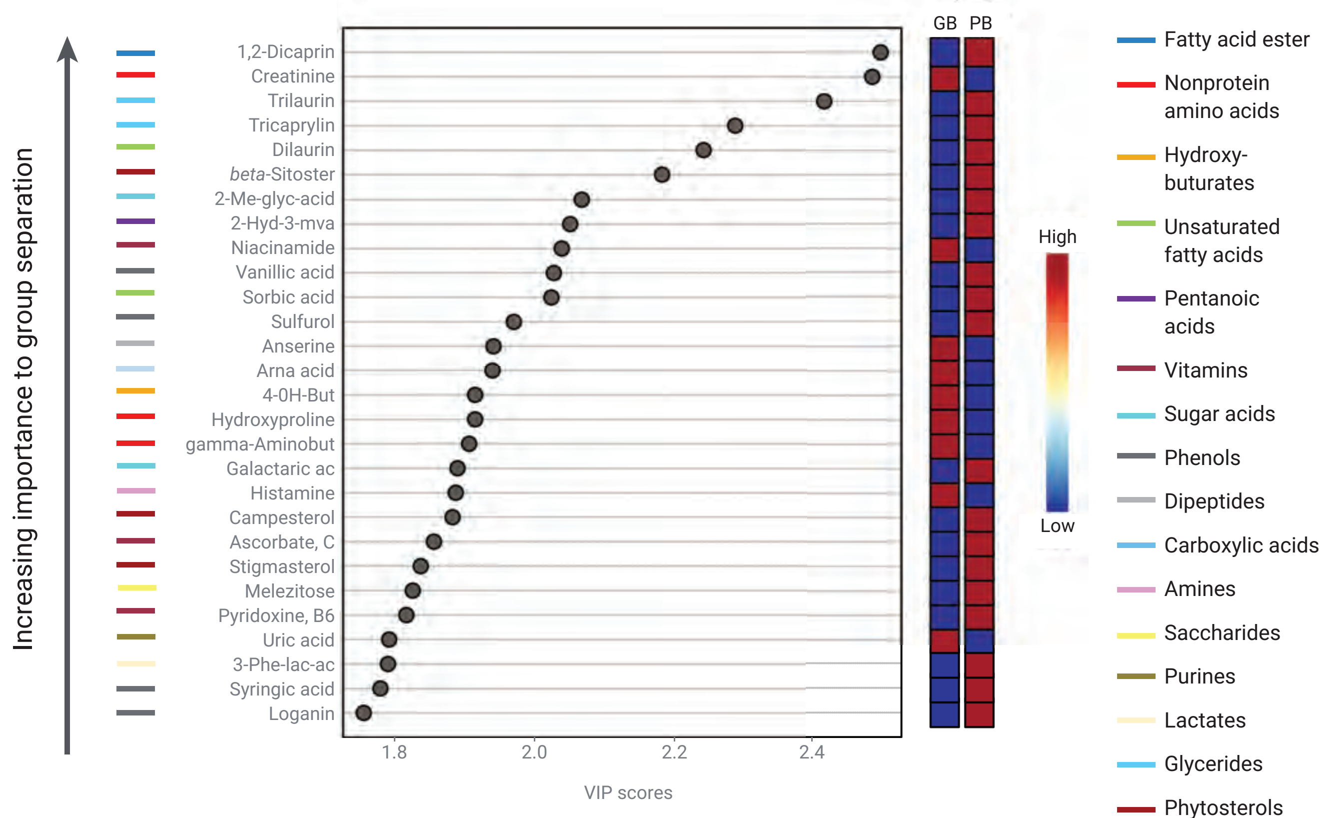

Figure 4. VIP plot generated from the PLS-DA models shows compounds ranked according to their prognostic importance (VIP scores) in separating the chemical profiles of GB and PB. The boxes on the right of the plot show the relative concentrations (blue: low to red: high) of each compound in the GB and 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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Application Brief

Foods

Agilent

Trusted Answers

Characterizing the Elemental Content of Alternative Proteins Using ICP-MS

IntelliQuant screening in helium mode complements quantitative analysis using Agilent 7850 ICP-MS

Authors

Peter Riles and Ed McCurdy

Agilent Technologies, Inc.

Figure 1. Insects such as crickets could be a sustainable alternative source of protein.

Alternative sources of protein

Demand for alternative (non-animal) protein sources is projected to increase rapidly due to increasing population, pressure on land use, public concern about health, and the environmental and animal welfare aspects of intensive livestock farming (*1*). Cultured meat is one possible protein source being investigated (*2*), while foods based on plant, fungi, algae, and insect protein (Figure 1) are also being developed or are already available.

The sample preparation methods, analytical techniques, and quality control (QC) protocols defined in existing food quality and safety regulations, such as FDA EAM 4.7, can be applied to novel foodstuffs (*2*). But new food types and manufacturing processes can lead to the presence of unexpected contaminants that may not be covered in existing regulations. ICP-MS is a valuable tool for food producers, regulators, and consumers, as it can provide full elemental characterization, as well as accurate quantitative determination of all regulated elements.

Elemental screening using ICP-MS with He mode to control polyatomic ion overlaps

Agilent ICP-MS instruments—whether single or triple quadrupole—include the unique combination of an exceptionally robust plasma (CeO/Ce ratio <1.5%), and the ORS^4 collision/reaction cell for the most effective interference control in helium (He) mode. The robust plasma provides unmatched matrix tolerance, ensuring long-term stability and minimal routine maintenance, while also increasing ionization, reducing the formation of many spectral overlaps, and minimizing matrix suppression.

The Agilent ORS^4 operates in He mode with kinetic energy discrimination (KED) to provide a simple, universal method to filter out matrix-derived polyatomic ions. He KED on the ORS^4 ensures consistent, accurate results in varied sample types, extending the number of trace analytes that can be measured reliably in unknown samples. He KED also gives access to many secondary or qualifier isotopes for data confirmation. In EAM 4.7, He KED is the only cell mode permitted for single quadrupole ICP-MS. Reaction gases are not allowed, because of the risk of errors due to spectral overlap from cell-formed reaction product ions.

Characterizing alternative proteins

In this work, an Agilent 7850 ICP-MS was used to analyze four commercially available alternative protein products:

- Cricket protein powder
- Reishi mushroom
- Almond flour
- Besan (chickpea) flour

The powdered samples were digested (0.5 to 50 g) in a microwave oven. A mix of HNO_3 and HCl was used to ensure stability of all the elements, including Hg. Potential Cl-based polyatomic interferences were removed in the standard He cell mode. The 12 elements defined in EAM 4.7 were quantified, together with the nutrient/mineral elements, Na, Mg, P, K, Ca, and Fe. The mineral elements are often measured using ICP-OES, but Agilent ICP-MS systems have an exceptionally wide detector dynamic range, so these high concentration elements can be measured in the same run as the trace analytes.

The results shown in Table 1 reveal large differences in the levels of some of the elements. For example, the reishi mushroom powder contained 15.5 mg/kg (ppm) Cr, compared to less than 0.2 ppm in the other samples.

Table 1. Agilent 7850 ICP-MS concentrations for EAM 4.7 specified elements (in bold) and mineral elements in four alternative proteins. Results in original dried sample in µg/kg (ppb) except where indicated.

Element	Cricket Protein	Reishi Mushroom	Almond Meal	Besan Flour
Na (mg/kg)	3440	19.7	5.14	23
Mg (mg/kg)	1160	439	2880	1030
P (mg/kg)	9180	1030	5580	2490
K (mg/kg)	10100	1860	7190	8610
Ca (mg/kg)	1190	709	2370	522
Cr	**173**	**15500**	**58.2**	**61.3**
Mn (mg/kg)	**36.6**	**82.4**	**25.3**	**14.7**
Fe (mg/kg)	53.3	226	40.9	55.5
Ni	**214**	**2590**	**716**	**2210**
Cu (mg/kg)	**29.1**	**4.71**	**10.8**	**8.34**
Zn (mg/kg)	**212**	**7.27**	**29.7**	**33**
As	**36.4**	**86.5**	**23.2**	**7.76**
Se	**387**	**47.2**	**26.3**	**133**
Mo	**730**	**75.7**	**439**	**679**
Cd	**11.8**	**138**	**12.1**	**0.709**
Hg	**2.87**	**52.2**	**1.58**	**1.14**
Tl	**3.19**	**2.18**	**3.3**	**0.867**
Pb	**80.5**	**209**	**12.2**	**14.2**

The 18 quantitative elements represent a tiny fraction of the information available from an Agilent ICP-MS system operating in He KED mode. Built-in preset methods provided with Agilent ICP-MS MassHunter software include a He mode Quick Scan acquisition, which collects data for every mass, with only two seconds of additional acquisition time.

He mode attenuates all common polyatomic ion overlaps, so the spectrum is simple, making it easy to associate each measured peak with an analyte. Secondary (qualifier) isotopes can be used to confirm the identity of unexpected elements, based on the isotope abundance template fit, as shown in Figure 2. Quick Scan spectra are automatically processed by IntelliQuant, giving semiquantitative results for all measurable elements without needing element-specific standards.

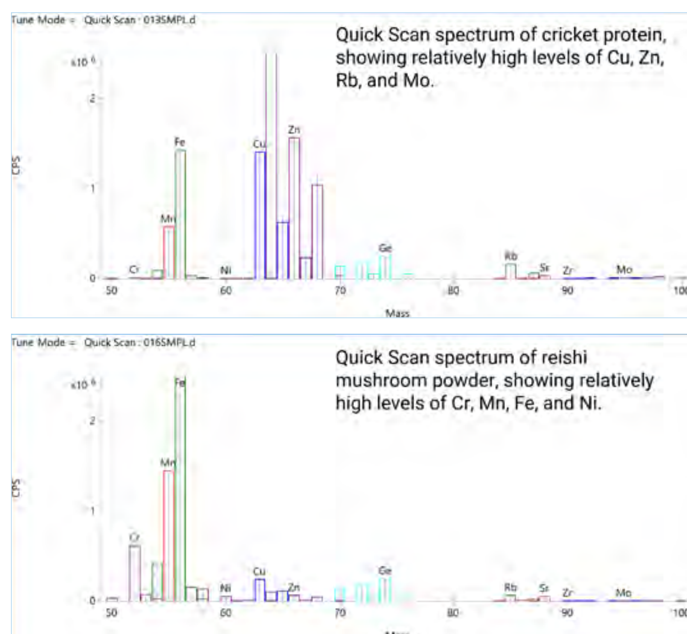

Figure 2. Mass 50 to 100 from the Quick Scan spectra for cricket protein (top) and reishi mushroom (bottom). Same intensity scale used for both. Quick Scan identifies unexpected elements, and the identity is confirmed by the isotope template match. IntelliQuant gives semiquantitative concentrations without requiring element-specific standards.

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Safety

Chapter 4

Application Note

Food and Beverage Testing

Quantitation of Over 1,000 Pesticide Residues in Tomato According to SANTE 11312/2021 Guideline

Using LC/MS/MS and GC/MS/MS detection

Authors

Peter Kornas and Teresa Klink
Agilent Technologies, Inc.

Abstract

A comprehensive multiresidue workflow was developed and validated for the simultaneous quantitation of over 1,000 pesticide residues in tomato to accelerate and simplify routine laboratory food testing. The workflow analyzes a wide range of pesticide residues simultaneously in 20 minutes and uses a single sample preparation method for both LC/MS/MS and GC/MS/MS analyses, leading to increased turnaround time, simplified analysis, and lower laboratory costs.

The workflow includes sample preparation, chromatographic separation, mass spectrometric (MS) detection, data analysis, and data interpretation using Agilent LC/MS/MS and GC/MS/MS systems. For sample preparation, the Agilent QuEChERS extraction kit was used without further cleanup. Compound transitions and associated optimized parameters were developed based on the Agilent pesticide MRM databases for both LC/MS and GC/MS workflows.

Workflow performance was evaluated and verified according to the SANTE 11312/2021 guideline based on instrument limit of detection (LOD), calibration curve linearity, recovery, and precision using matrix-matched calibration standards from 0.5 to 100 µg/L. Over 98% of analytes demonstrated linearity with $R^2 \geq 0.99$. Method precision was assessed using recovery repeatability (RSD_r). At the 10 µg/kg level, RSD_r values of 98% of compounds were within the limit of 20%. The mean recoveries of the six technical replicates were within the limits of 40 to 120% for 98% of target analytes.

Introduction

Pesticides play an important role in the agriculture and food industries to improve crop yield and food production. Residues of pesticides remaining in or on commodities such as fruits, vegetables, or cereals can cause adverse health effects as well as environmental concerns. Regulatory agencies have set maximum residue levels (MRLs) for hundreds of pesticides and their metabolites. Most MRLs are set at low parts per billion (ppb) levels, which poses significant challenges, especially if hundreds of analytes are screened and quantified simultaneously in complex food matrices. In Europe, pesticide testing laboratories adhere to the SANTE 11312/2021 guideline.[1] This guideline ensures a consistent approach for controlling MRLs that are legally permitted in food or animal feed. Due to the vast number of pesticides, the analysis is very elaborate, often requiring multiple analytical approaches and laboratory-intensive workflows, resulting in high operating costs and slow turnaround times.

In this study, an accurate and reliable analysis of over 1,000 pesticide residues in tomato was developed using a single QuEChERS extraction for sample preparation. As shown in the Venn diagram (Figure 1), 764 analytes were analyzed by LC/MS/MS and 341 analytes were analyzed by GC/MS/MS. The GC/MS/MS analysis included 84 analytes that can also be determined using LC/MS/MS; thus, this workflow covers a total of 1,021 unique substances.

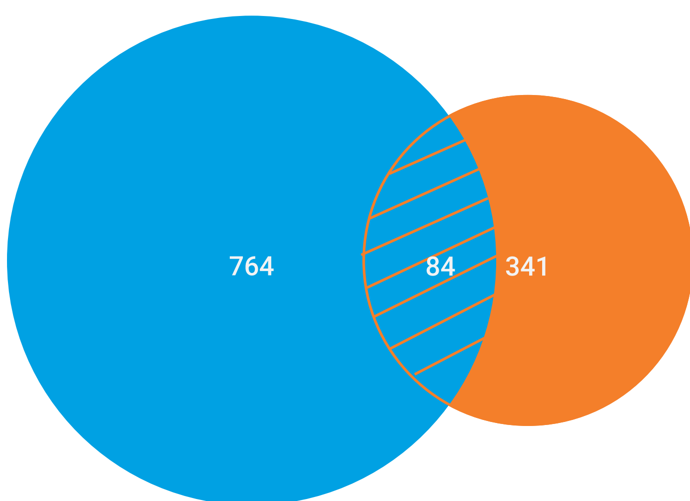

Figure 1. Venn diagram of compounds analyzed using LC/MS/MS (blue) and GC/MS/MS (orange).

This workflow, including sample preparation, chromatographic separation, MS detection, targeted quantitation, and results interpretation, helps streamline routine pesticide analysis and therefore accelerates lab throughput and productivity. Details of sample preparation procedures, instrumentation setup, and data analysis parameters are discussed, enabling the quantification and confirmation of pesticide residues.

Experimental

Chemicals and reagents

Agilent LC/MS-grade acetonitrile (ACN), methanol (MeOH), water, and ammonium formate were used in the study. LC/MS-grade formic acid was purchased from VWR. All other solvents used were HPLC grade and from VWR and Merck.

Standards and solutions

The following ready-to-use and custom premixed pesticide standards were acquired:

- Agilent LC/MS pesticide comprehensive test mix (part number 5190-0551)
- Agilent custom pesticide test mix (part numbers CUS-00000635 to CUS-00000643)
- Agilent custom organic standard (part number CUS-00004663)
- AccuStandard custom pesticide standard (part numbers S-96086-01 to S-96086-10), amchro GmbH, Hattersheim, Germany
- Agilent GC pesticide standard 1 to 10, and 12 (part numbers PSM-100-A to -J, and -L)
- Agilent GC pesticide standard no. 1 and 2 (part numbers PSM-105-A and -B)

Other single standards, either as standard solution or powders, were purchased from AccuStandard (amchro GmbH, Hattersheim, Germany) and LGC (LGC Standards GmbH, Wesel, Germany).

When single standards were purchased as powders, single stock solutions with a concentration of 1,000 mg/L were prepared in acetone and stored at –20 °C.

Intermediate standard mixes were prepared from stock solutions and used for preparation of prespiked quality control (QC) samples, solvent calibration standards, and matrix-matched calibration. Calibration standards were prepared freshly and stored in a refrigerator at 4 °C if not used immediately.

Sample preparation

Pesticide-free and organic-labeled tomatoes were obtained from local grocery stores. The tomatoes were homogenized using a domestic blender and stored in the refrigerator at 4 °C before analysis.

The following products and equipment were used for sample preparation:

- Agilent Bond Elut QuEChERS EN extraction kit (part number 5982-5650CH)
- Vortex mixer (VWR International GmbH, Darmstadt, Germany)
- Centrifuge UNIVERSAL 320 R (Andreas Hettich GmbH, Tuttlingen, Germany)

Samples of 10 ± 0.1 g of homogenized tomato were weighed into a 50 mL tube. Prespiked QC samples were fortified by spiking 200 µL of working standards (500 µg/L) to give a final concentration of 10 µg/kg. After spiking, the samples were capped tightly, vortexed, and equilibrated for 15 to 20 minutes. QuEChERS extraction was then performed and the samples were centrifuged. An aliquot of this extract was directly used for LC/MS/MS analysis. Before GC/MS/MS analysis, an aliquot of the extract was diluted by a factor of 5 with ACN. The preparation procedure is illustrated in Figure 2.

Preparation of matrix-matched calibration standards

Matrix-matched calibration standards (postspiked standards) were used and prepared for the assessment of workflow performance. A matrix blank was prepared using an unfortified, blank sample of tomato. Preparation of matrix-matched calibration levels was performed by mixing intermediate standard solutions with matrix blank extract. These solutions were used for LC/MS/MS analysis directly and diluted by a factor of 5 before GC/MS/MS analysis. The matrix-matched standard at 10 ppb was used to evaluate the matrix effect (ME) by comparing responses with the corresponding solvent standard.[1]

Instrumentation

The LC/MS/MS study was performed using an Agilent 1290 Infinity II LC system coupled to an Agilent 6470B triple quadrupole LC/MS. The modules of the LC/MS system included:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II autosampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6470B triple quadrupole LC/MS (G6470B)
- Agilent pesticide dynamic MRM database (G1733CA)
- Agilent MassHunter software (version 10.1)

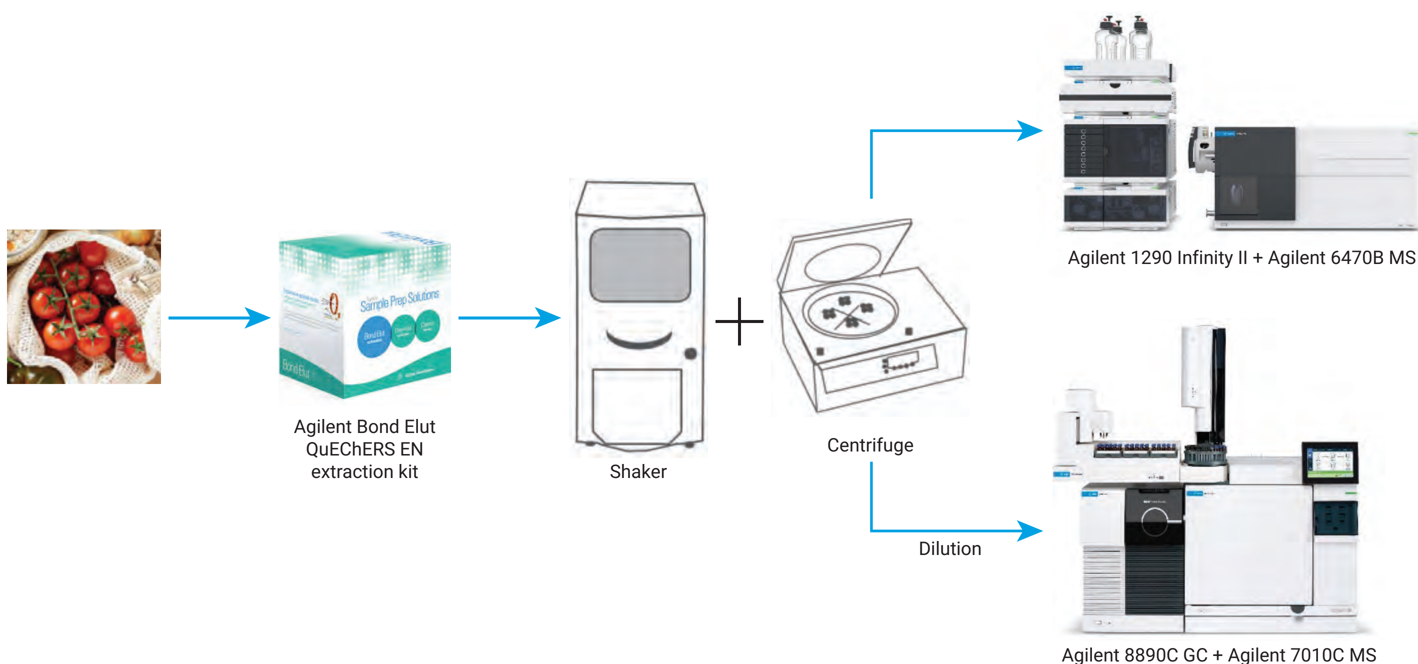

Figure 2. Sample preparation procedure using the Agilent Bond Elut QuEChERS EN extraction kit for sample cleanup before analysis.

The coupled 6470 triple quadrupole LC/MS was equipped with an Agilent Jet Stream (AJS) electrospray ion source and was operated in dynamic MRM (dMRM) mode.

The main LC and MS parameters are listed in Table 1. Please refer to the Agilent application note by Kornas for the detailed LC/TQ configuration.[2]

Table 1. LC and MS conditions.

Parameter	Value
LC	
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902)
Column Temperature	40 °C
Injection Volume	2 µL
Autosampler Temperature	6 °C
Mobile Phase A	5 mM ammonium formate in water with 0.1% formic acid
Mobile Phase B	5 mM ammonium formate in methanol with 0.1% formic acid
Flow Rate	0.4 mL/min
Gradient	Time (min) A(%) B(%) 0 95 5 3 70 30 17 0 100 20 0 100
Postrun Time	3 min
Needle Wash	Multiwash
MSD	
Ionization Mode	Simultaneous positive/negative ESI with Agilent Jet Stream (AJS)
Scan Type	Dynamic MRM (dMRM)
Gas Temperature	200 °C
Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	2,500 V (+)/3,000 V (–)
Nozzle Voltage	0 V
Total MRMs	1,590
Min/Max Dwell Time	0.52 ms/242.30 ms

The GC/MS/MS study was performed using an Agilent 8890 GC and Agilent 7010C triple quadrupole GC/MS system. The modules of the GC/MS system included:

- Agilent 8890 GC (G3540A)
- Agilent 7693A automatic liquid sampler (G4513A and GG4520A)
- Agilent 7010C triple quadrupole GC/MS (G7012C)
- Agilent MassHunter pesticide & environmental pollutant (P&EP) MRM database 4.0 (G9250AA)[4]
- Agilent MassHunter software (MassHunter acquisition version 10.2 and MassHunter Quantitative Analysis version 12.0)

The GC was configured with the Agilent 7693A automatic liquid sampler (ALS) and 150-position tray. The system used a multimode inlet (MMI). Chromatographic separation was performed using the conventional 15 m × 15 m midcolumn backflush configuration described in the P&EP database. Therefore, two Agilent HP-5ms Ultra Inert (UI) GC columns (part number 19091S-431UI) were used, and midcolumn backflush capability was provided by the Agilent Purged Ultimate Union (PUU) installed between the two identical 15 m columns, and the pneumatic switching device (PSD) module on the 8890 GC. The acquisition method was retention time locked to match the retention times in the MassHunter P&EP 4.0.

The main GC and MS parameters are listed in Table 2. Please refer to the Agilent application note by Klink for the detailed GC/TQ configuration.[3] All data were acquired in dynamic MRM (dMRM) mode.

Table 2. GC and MS conditions.

Parameter	Value
GC	
Columns	Agilent HP-5ms, 15 m × 0.25 mm, 0.25 µm film thickness (two) (p/n 19091-431UI)
Carrier	Helium
Column 1 Flow	0.94 mL/min
Column 2 Flow	1.14 mL/min
Injection Volume	1 µL, solvent vent
Inlet Liner	Agilent Ultra Inert dimpled liner (p/n 5190-2297)
MMI Temperature Program	60 °C for 0.06 min, 720 °C/min to 280 °C and hold
Oven Temperature Program	60 °C for 1 min, 40 °C/min to 170 °C, 10 °C/min to 310 °C and hold for 3 minutes
Run Time	20.75 minutes
Transfer Line Temperature	280 °C
Backflush Conditions	1.5 min postrun, 310 °C oven temperature
MSD	
Source	High-efficiency source (HES)
Vacuum Pump	Performance turbo
Quad Temperature (MS1 and MS2)	150 °C
Source Temperature	280 °C
Mode	dMRM
EM Voltage Gain Mode	10
Total MRMs (dMRM Mode)	2,093
Min/Max Dwell Time	1.2 ms/100.2 ms

Results and discussion

Development of multicompound methods

A major part of this study was the development of dMRM transitions for all pesticides from the Agilent databases. For LC/MS/MS, the Agilent pesticide dynamic MRM database was used. MRM transitions as well as fragmentor voltages, collision energies, and ionization polarity were optimized using the Agilent MassHunter Optimizer software by flow injection. Approximately 1,600 MRM transitions from 764 pesticides were stored in the final dMRM method. Typical chromatographic peak widths were between 8 to 12 seconds. The selected cycle time of 490 ms ensured that sufficient data points were collected across the chromatographic peaks for reproducible quantitation and confirmation of results.

For GC/MS/MS, most of the compounds were already listed in the MassHunter P&EP database.[4] Compounds whose MRM transitions were not listed in this database were developed using the MassHunter Optimizer for GC/TQ. Starting with a GC method that provides good chromatographic compound separation, the MassHunter Optimizer first identifies precursor ions and product ions, then optimizes collision energies for each promising precursor-product combination to identify the best MRM parameters. Around 2,100 MRM transitions from 341 pesticides were stored in the final dMRM method. The selected cycle time of 300 ms ensured that sufficient data points were collected across the chromatographic peaks for reproducible quantitation and confirmation of results. The GC acquisition method was retention time locked to match the retention times in the Agilent P&EP database, which was used to seamlessly create the MS method. The use of P&EP increased the ease and speed of setting up a targeted dMRM method. Retention time locking allows a new column or instrument to have retention times that match the MRM database or an existing method exactly, allowing methods to be easily ported from one instrument to another and across instruments globally. This simplifies method maintenance and system setup.

Two or three target specific MRM transitions were selected per pesticide in each method to satisfy the regulatory requirements for identification and confirmation by LC/MS/MS and GC/MS/MS, respectively.[1]

Data were acquired in dynamic MRM (dMRM) mode, which enables the capability for large multi-analyte assays and to accurately quantitate narrow peaks by an automated and most-efficient dwell time distribution. Furthermore, dMRM enables the analyst to add and remove additional analytes with ease.

Matrix effect assessment

Effects caused by the sample matrix are frequent and cause suppression or enhancement of the MS detection system response.[1] ME was assessed by the ratio of target response in matrix-matched standards to that in corresponding solvent standards. Typically, there is no strict requirement on acceptance ME criteria, because ME can be corrected by the matrix-matched calibration curve. However, ME is an important parameter for method sensitivity and reliability assessment, and less than 20% signal suppression or enhancement is usually considered as insignificant ME.[1] In this study, ME was investigated using a 10 µg/L standard in tomato extract (postspiked standard) and the response was compared to the corresponding solvent standard. The 10 µg/L standard was chosen, as this is the lowest MRL for pesticides and their metabolites.

More than 45% of the 1,021 targets in tomato showed significant ME at 10 µg/L.

Based on the results of the ME assessment, matrix-matched calibration standards were used to compensate MEs in this study.

Verification of workflow performance

The workflow performance criteria were verified based on linearity, method sensitivity, recovery, and precision. The batch included solvent blank, matrix-matched calibration standards, matrix blank, and prespiked QCs. Six technical replicates were prepared for the prespiked QCs.

Linearity

Calibration curves were generated for all compounds using matrix-matched standards ranging from 0.5 to 100 µg/L, and eight calibration points. Linear or quadratic regression with 1/x weight and unspecified origin were used for calibration curve generation. The calibration range was determined based on LOQ sensitivity and selectivity requirements. Results in Figure 3 show that more than 98% of the targets met the calibration curve linearity requirement of $R^2 \geq 0.99$.[1] Only some compounds showed a modified calibration range due to either lack of sensitivity at low calibration levels or detector saturation at high concentration levels.

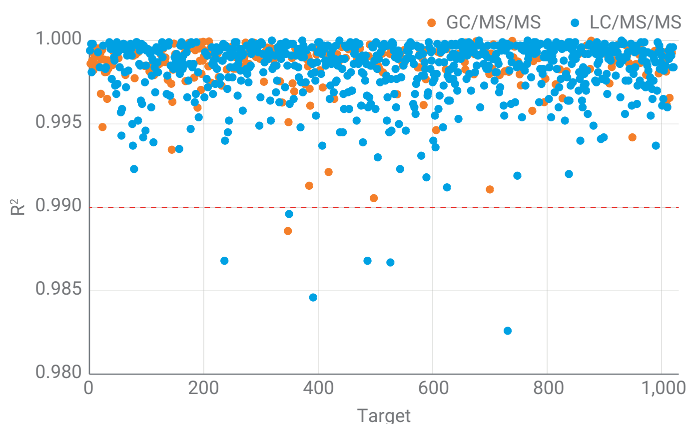

Figure 3. R^2 distribution of linearity curves for 1,021 pesticides, compounds below R^2 = 0.98 are not shown (9 in total).

Instrument limit of detection (LOD)

A sensitive workflow for pesticide residue analysis is beneficial for users to perform routine operations following various regulatory guidelines. Instrument LODs were used to evaluate method sensitivity. Instrument LOD was established based on matrix-matched calibration standards for signal-to-noise ratio (S/N) of 10 and up. The S/N was defined using the peak height and peak-to-peak algorithm embedded in MassHunter Quantitative Analysis software. The noise region was manually chosen and had a minimum length of 0.1 minutes.

More than 97% of target compounds showed an instrument LOD of ≤ 10 µg/L, and, even at a concentration level of 1 µg/L, more than 88% of compounds had an S/N of 10 and up (Figure 4). These results demonstrate the high sensitivity of both systems, the 6470 triple quadrupole LC/MS and the 7010 triple quadrupole GC/MS, against a complex matrix such as a tomato QuEChERS raw extract.

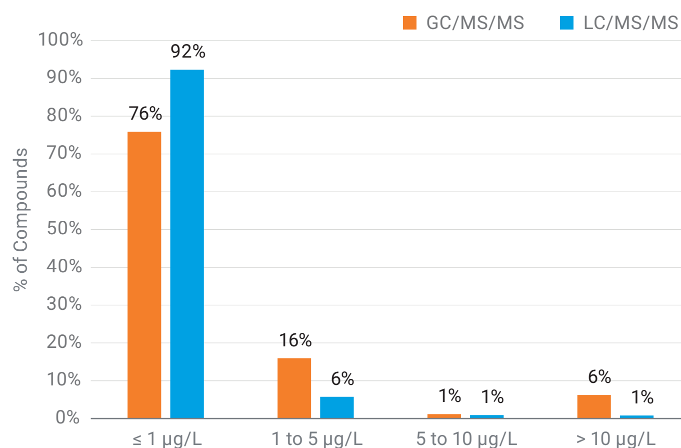

Figure 4. Instrument LOD in tomato QuEChERS raw extract.

Method precision and recovery

Method precision was estimated using recovery repeatability (RSD_r) based on the variation of recovery values from technical replicates of prespiked QC samples that were spiked at 10 µg/kg. The RSD_r was determined by calculating percent relative standard deviation (%RSD) of recovery using these six technical preparations. Typically, the acceptable RSD_r is 20% or less. The RSD_r values of 98% of all targets were within 20%, demonstrating consistent behavior with each technical preparation. These results confirmed the high repeatability of this workflow. Figure 5 shows that the vast majority of compounds had RSD of recovery rates below 20%.

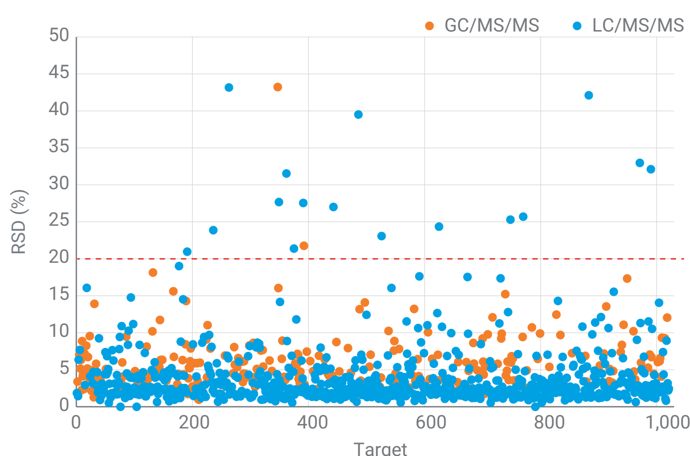

Figure 5. RSD_r of recovery rates at 10 µg/kg in QuEChERS tomato raw extract.

Recovery was used in this experiment to evaluate the capability of a quantitative analytical workflow for over 1,000 pesticides. Recovery was calculated based on analyte response ratios between prespiked QCs and corresponding matrix-matched calibration levels. Mean recovery at 10 µg/kg level was obtained for six technical replicates. According to SANTE 11312/2021, mean recoveries are acceptable within the range of 40 to 120% if they are consistent ($RSD_r \leq 20\%$). Based on these criteria, the mean recovery results for more than 97% of targets in tomato QuEChERS raw extract at 10 µg/kg met the acceptance criteria. The vast majority of compounds (975) were within the recovery range of 70% to 120% and only 26 compounds (3%) were below 70% or above 120%, respectively (Figure 6).

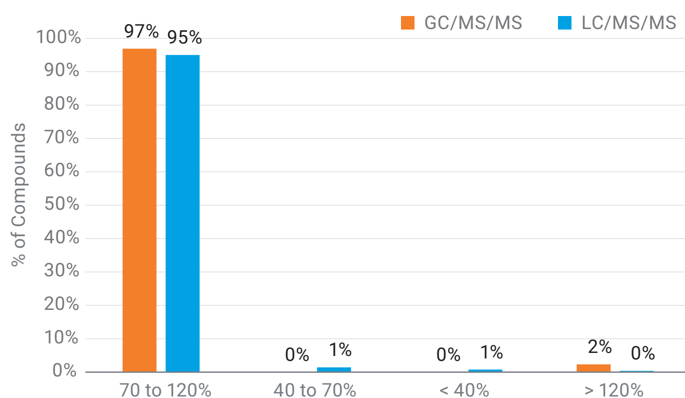

Figure 6. Recovery rates in tomato QuEChERS raw extract ($RSD_r \leq 20\%$).

Combination of methods

The combination of LC/MS/MS and GC/MS/MS allows users to cover the widest range of pesticides and metabolites occurring in food. Due to the molecular structure of this huge class of compounds, it is impossible to analyze various pesticides solely by GC or LC techniques. Exploiting both techniques makes it possible to get a wide coverage of these residues that can potentially endanger human health.

The presented workflow used both techniques and covered in total 764 pesticides analyzed by LC/MS/MS and 341 compounds analyzed by GC/MS/MS. All detailed results can be found in references 2 and 3. Furthermore, the analyses covered pesticide residues (84) that can be analyzed by either technique. This gives a clear benefit when, for example, positive results must be confirmed or higher sensitivity is needed.

In Figure 7, the chromatograms of silafluofen in a spiked matrix sample at 10 µg/kg are shown. The left chromatogram shows that sensitivity using LC/MS/MS was not good enough to get reliable results at MRL of 10 µg/kg. The full Agilent solution allows analysis of this compound using GC/MS/MS, resulting in much better sensitivity (right chromatogram).

The use of the other technique for confirmatory analysis can be demonstrated for bifenthrin. This compound can be reliably quantified using both techniques. The chromatograms in Figure 8 clearly demonstrate that sensitivity is high enough to determine and confirm positive results by either LC or GC technique.

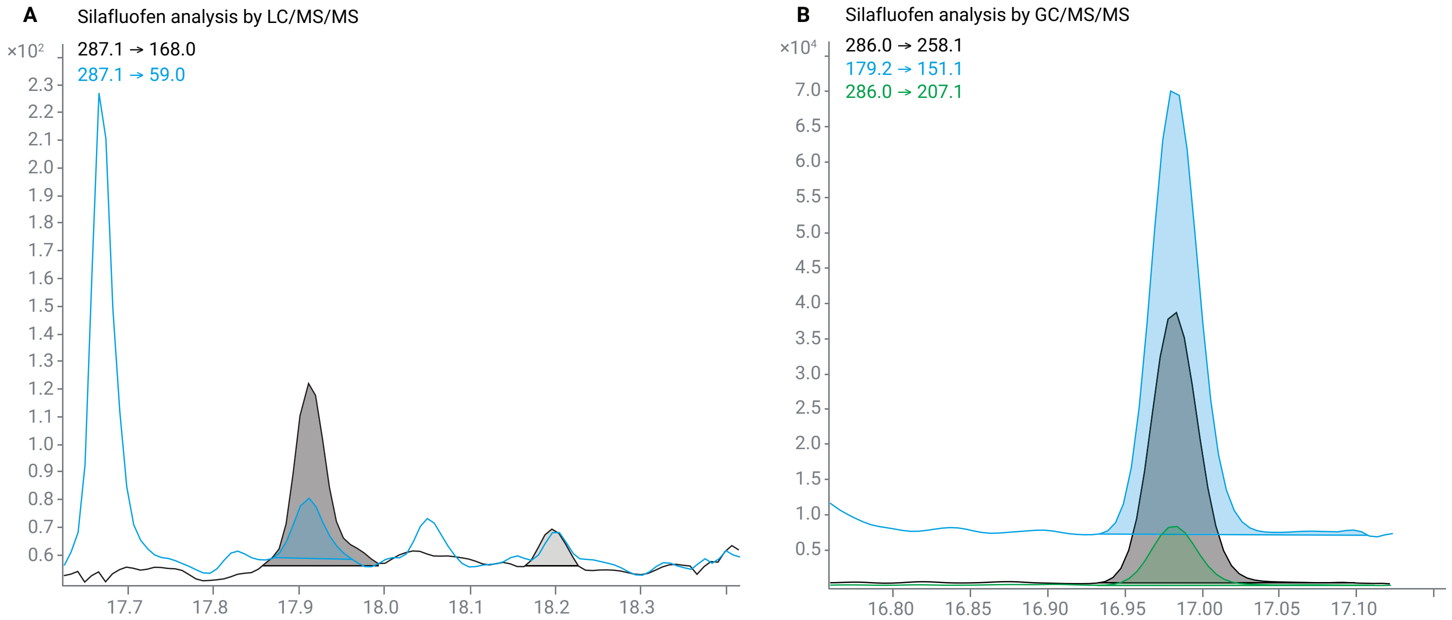

Figure 7. Analysis of silafluofen by LC/MS/MS (A) and GC/MS/MS (B).

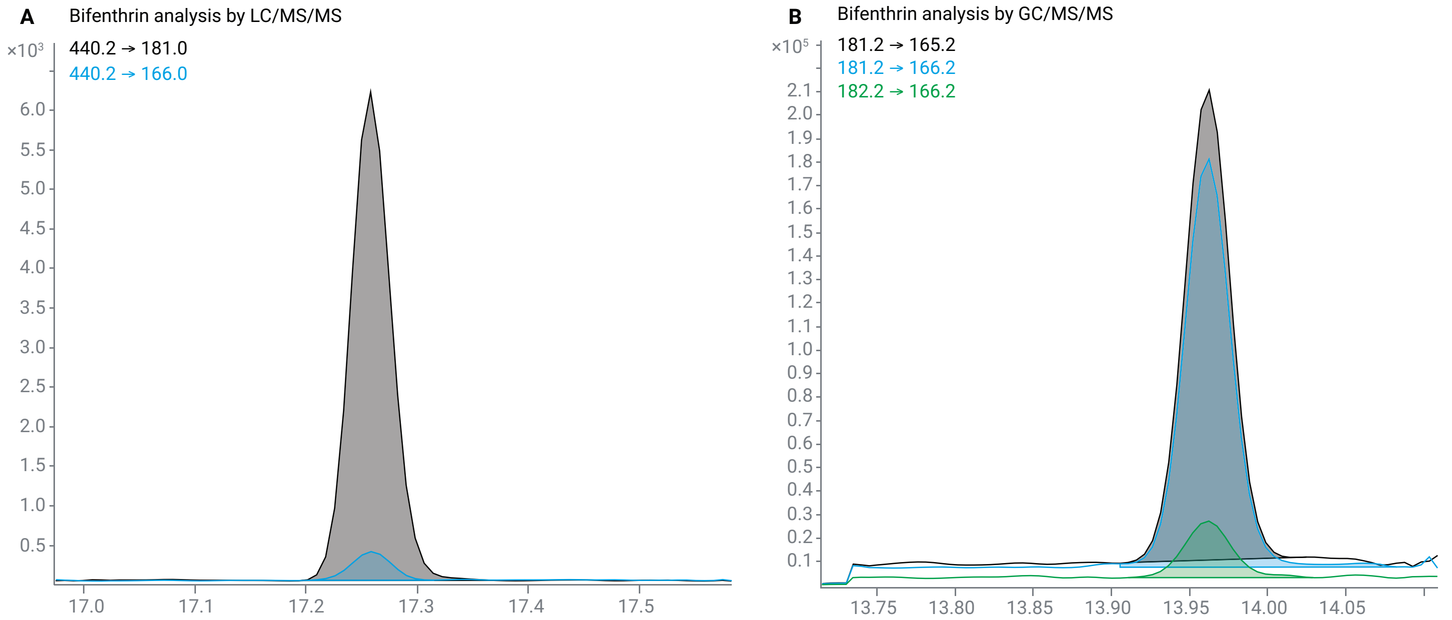

Figure 8. Analysis of bifenthrin by LC/MS/MS (A) and GC/MS/MS (B).

Conclusion

This application note demonstrates the applicability of a sensitive and reproducible workflow for fast and reliable quantitation of more than 1,000 pesticide residues in tomato QuEChERS raw extract conforming to the SANTE 11312/2021 guideline. The simple sample preparation protocol uses the Agilent Bond Elut QuEChERS EN extraction kit for facile extraction without requiring further sample cleanup. A single sample preparation procedure can be used and then split into two aliquots for subsequent analysis by LC/MS/MS and GC/MS/MS.

An Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS was used to quantify 764 pesticides, and an Agilent 8890 GC coupled to an Agilent 7010C triple quadrupole GC/MS was used to quantify 341 pesticide residues with matrix-matched calibration. Both methods had 20-minute run times, and column setups offered good chromatographic separation and even retention time distribution of all targets.

To achieve the most efficient use of instrument cycle time, all data were acquired in dMRM mode. The dMRM methods were created and developed based on the Agilent pesticide MRM databases.

The overall workflow performance was assessed for linearity, instrument LOD, recovery, and precision, demonstrating its suitability for the quantitation of over 1,000 pesticide residues in the same QuEChERS raw extract.

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Application Note

Food & Beverage Testing

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Trusted Answers

Five Keys to Unlock Maximum Performance in the Analysis of Over 200 Pesticides in Challenging Food Matrices by GC/MS/MS

Authors

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Abstract

This application note describes five best practices to enhance analytical performance in the analysis of over 200 pesticides in challenging matrices including spinach, walnut, and cayenne pepper. The novel Agilent Captiva EMR passthrough cleanup procedure following the Agilent QuEChERS extraction enabled a cleaner matrix background. The cleanup and extraction reduced matrix interferences with target analytes and extended the maintenance-free operation time of the instrument. Calibration performance was demonstrated over a wide dynamic range to over four orders of magnitude. It was shown that the Agilent 8890/7000E triple quadrupole GC/MS system achieved excellent linearity over a concentration range of 0.1 to 5,000 ppb. The Agilent 8890/7010C triple quadrupole GC/MS system demonstrated superior sensitivity yielding a higher signal-to-noise ratio at lower concentrations.

Introduction

The global agriculture industry uses over a thousand different pesticides in the production of food. Producers require pesticides to meet the increasing demand for reasonably priced food. This growing demand has increased the use of pesticides and encouraged problematic agricultural practices that have elevated risks in the food supply and the environment. Concerns about trace level chemical pollutants in food are driving the demand for more rapid and reliable methods for the identification and quantitation of chemical residues. The Agilent 8890/7000E and 8890/7010C triple quadrupole GC/MS systems (GC/TQ) are ideally suited to meet this need.

The US Environmental Protection Agency (EPA) sets tolerances as part of the food safety equation.[1] The tolerance corresponds to the maximum residue limit (MRL), which is the maximal level of pesticide residue allowed to remain in or on the treated food commodity. The MRLs may vary over a broad concentration range depending on different pesticides and food commodities. For example, the MRLs established for 68 pesticides regulated in spinach vary from 10 ppb for fludioxonil to 60,000 ppb for boscalid.[2] This range of limits presents a challenge for the analysis, requiring both high sensitivity and the ability to calibrate over a wide dynamic range.

Five key components of successful pesticide analysis discussed in this application note are:

1. Effective sample extraction and matrix cleanup, which allow for minimal matrix background and interferences while maintaining high pesticide recoveries. Also, a robust analytical method that achieves the required method performance while increasing maintenance-free uptime.
2. Evaluation of the matrix in full scan data acquisition mode to ensure the most efficient performance, especially with the high efficiency source (HES).
3. Midcolumn backflushing to extend maintenance-free operation of the system. This technique minimizes column trimming and source cleaning while also allowing reduced analysis time.
4. A leak-free GC/TQ system enables extended GC column life and facilitates maintenance-free consistent and reliable MS performance.
5. Use of the temperature-programmed Agilent multimode inlet (MMI) with a 2 mm dimpled liner (no glass wool) to ensure efficient volatilization of even the most thermally labile compounds.

This application note demonstrates the analysis of over 200 pesticides in three challenging matrices, including a high chlorophyll fresh matrix spinach, a complex dry matrix cayenne pepper, and an oily dry matrix walnut. The achieved wide dynamic ranges with high method sensitivity enabled accurate quantification of pesticides in these matrices, at their MRLs.

Matrix-matched calibrations with R^2 >0.99 over a dynamic range as wide as 0.1 to 5,000 ppb were achieved with the 7000E GC/TQ and 0.1 to 1,000 ppb with the 7010C GC/TQ. The 7010C GC/TQ equipped with the HES enabled superior sensitivity yielding high signal-to-noise ratio even at low concentrations and allowed for accurate quantification at concentrations below 0.1 ppb. However, this was not required in this work as the MRLs for pesticides regulated in the commodities of interest did not require sub-0.1 ppb quantification.

Experimental

GC/TQ analysis

The 8890/7000E and 8890/7010C GC/TQ systems (Figure 1A) were used and configured to achieve the best performance over a wide calibration range. This calibration range encompassed the varying MRLs for pesticides regulated in the analyzed commodities. The GC was configured with the Agilent 7693A automatic liquid sampler (ALS) and 150-position tray. The system used a multimode inlet (MMI) operated in temperature-programmed splitless injection mode. Midcolumn backflush capability was provided by the Agilent Purged Ultimate Union (PUU) installed between two identical 15 m columns, and the 8890 pneumatic switching device (PSD) module (Figure 1B). The instrument operating parameters are listed in Table 1.

Data were acquired in dynamic MRM (dMRM) mode, which enables the capability for large multi-analyte assays and to accurately quantitate narrow peaks by an automated and most-efficient dwell time distribution. The dMRM capability enabled a successful analysis for a large panel of 203 pesticide with 614 total MRM transitions with up to 52 concurrent MRMs (Figure 2). Furthermore, dMRM enables the analyst to add and remove additional analytes with ease. The acquisition method was retention time-locked to match the retention times in the Agilent MassHunter Pesticide & Environmental Pollutant MRM Database (P&EP 4), which was used to seamlessly create the MS method. The use of P&EP 4 increased the ease and speed of setting up a targeted dMRM method. The acquisition method was retention time locked to the P&EP library.

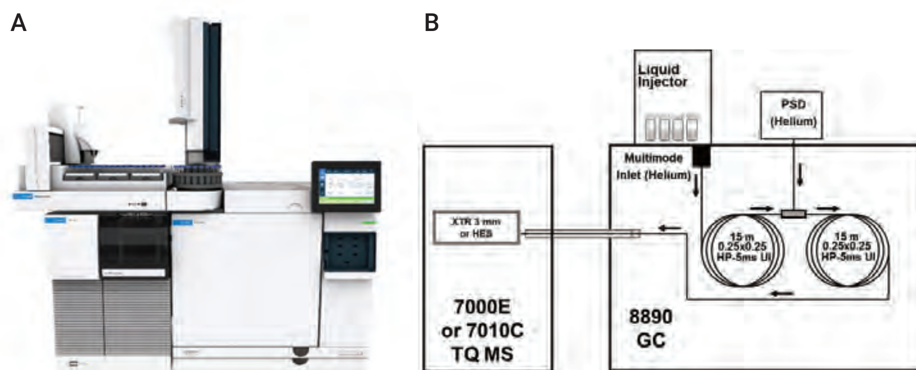


Figure 1. The Agilent 8890/7000E and 8890/7010C GC/TQ system (A) and system configuration (B).

Table 1. Agilent 8890/7000E and 8890/7010C gas chromatograph and mass spectrometer conditions for pesticide analysis.

GC	
Agilent 8890 with fast oven, auto injector, and tray	
Inlet	Multimode inlet (MMI)
Mode	Splitless
Purge Flow to Split Vent	60 mL/min at 0.75 min
Septum Purge Flow	3 mL/min
Septum Purge Flow Mode	Switched
Injection Volume	1.0 µL
Injection Type	Standard
L1 Airgap	0.2 µL
Gas Saver	On at 30 mL/min after 3 min
Inlet Temperature	60 °C for 0.1 min, then to 280 °C at 600 °C/min
Post Run Inlet Temperature	310 °C
Post Run Total Flow	25 mL/min
Carrier Gas	Helium
Inlet Liner	Agilent Ultra Inert 2 mm dimpled liner (p/n 5190-2297)
Oven	
Initial Oven Temperature	60 °C
Initial Oven Hold	1 min
Ramp Rate 1	40 °C/min
Final Temp 1	170 °C
Final Hold 1	0 min
Ramp Rate 2	10 °C /min
Final Temp 2	310 °C
Final Hold 2	2.25 min
Total Run Time	20 min
Post Run Time	1.5 min
Equilibration Time	0.25 min

Column 1	
Type	Agilent HP-5ms UI (p/n 19091S-431UI-KEY)
Length	15 m
Diameter	0.25 mm
Film Thickness	0.25 µm
Control Mode	Constant flow
Flow	1.016 mL/min
Inlet Connection	Multimode inlet (MMI)
Outlet Connection	PSD (PUU)
PSD Purge Flow	5 mL/min
Post Run Flow (Backflushing)	–7.873
Column 2	
Type	Agilent HP-5ms UI (p/n 19091S-431UI-KEY)
Length	15 m
Diameter	0.25 mm
Film Thickness	0.25 µm
Control Mode	Constant flow
Flow	1.216 mL/min
Inlet Connection	PSD (PUU)
Outlet Connection	MSD
Post Run Flow (Backflushing)	8.202

MSD	
Model	Agilent 7000E or 7010C
Source	Inert Extractor Source with a 3 mm lens or HES
Vacuum Pump	Performance turbo
Tune File	Atunes.eiex.jtune.xml or Atunes.eihs.jtune.xml
Solvent Delay	3 min
Quad Temperature (MS1 and MS2)	150 °C
Source Temperature	280 °C
Mode	dMRM or Scan
He Quench Gas	2.25 mL/min
N_2 Collision Gas	1.5 mL/min
MRM Statistics	
Total MRMs (dMRM Mode)	614
Minimum Dwell Time	6.85 ms
Minimum Cycle Time	69.8 ms
Maximum Concurrent MRMs	52
EM Voltage Gain Mode	10
Scan Parameters	
Scan Type	MS1 Scan
Scan Range	45 to 450 *m/z*
Scan Time (ms)	220
Step Size	0.1 amu
Threshold	0
EM Voltage Gain Mode	1

Full scan data acquisition mode was used for the preliminary screening of the matrix extract. This screening was used to evaluate the in-source loading and for monitoring the efficiency of the sample cleanup.

Agilent MassHunter Workstation revisions 10.1 and 10.2 including MassHunter Acquisition software for GC/MS systems 10.2, MassHunter Quantitative 10.1, and MassHunter Qualitative 10 packages were used in this work.

Calibration performance was evaluated using a series of matrix-matched calibration standards ranging from 0.1 to 5,000 ppb, including 0.1, 0.5, 1, 5, 10, 50, 100, 250, 500, 1,000, and 5,000 ppb. The standard α-BHC-d_6 at a final concentration of 20 ppb in vial was used as the internal standard for quantitation of the target pesticides. A linear or quadratic regression fit with a weighting factor of 1/x was applied to all calibration curves.

Sample preparation

A sample preparation workflow chart is shown in Figure 3. The sample preparation included two major steps: sample extraction by traditional QuEChERS extraction, followed with Captiva EMR pass-through clean up. Different Captiva EMR products were used for different matrices based on different matrix challenges. A Captiva EMR–HCF cartridge was used for high-chlorophyll fresh matrix spinach. Captiva EMR–LPD was used for the low pigmented but oily dry matrix walnut. Captiva EMR–GPD was used for a very challenging dry matrix cayenne pepper. The new sample preparation workflow demonstrates a simplified procedure with improvement on both sample matrix removal and targets quantitation data quality.

As shown in Figure 3, samples were first extracted by the traditional QuEChERS EN extraction kit (part number 5892-5650). For fresh spinach, 10 g of homogenized spinach sample was used for extraction. For walnut, 5 g of walnut powder was used, followed with the addition of 10 mL of water and 10 minutes of vortexing. For cayenne pepper, 2 g of cayenne pepper powder was used, followed with the addition of 10 mL water and 10 minutes vortexing. The 10 mL of ACN with 1% acetic acid was then added for extraction, followed with QuEChERS EN extraction. After extraction, 3 mL of crude extract or with 10% of water mixture was transferred to Captiva EMR cartridges for pass-through cleanup. The following cartridges were used: Captiva Enhanced Matrix Removal High Chlorophyll Fresh, with NH_2, (Captiva EMR–HCF1, part number 5610-2088) for spinach, the Captiva Enhanced Matrix Removal Low Pigment Dry (Captiva EMR–LPD, part number 5610-2092) for walnut, and the Captiva Enhanced Matrix Removal General Pigmented Dry (Captiva EMR–GPD, part number 5610-2091) for cayenne pepper. The sample eluent was collected and further dried by anhydrous $MgSO_4$, (part number 5982-0102) and samples were then ready for GC/TQ analysis. The positive pressure manifold 48 processor (PPM-48, part number 5191-4101) was used for Captiva EMR pass-through clean up processing.

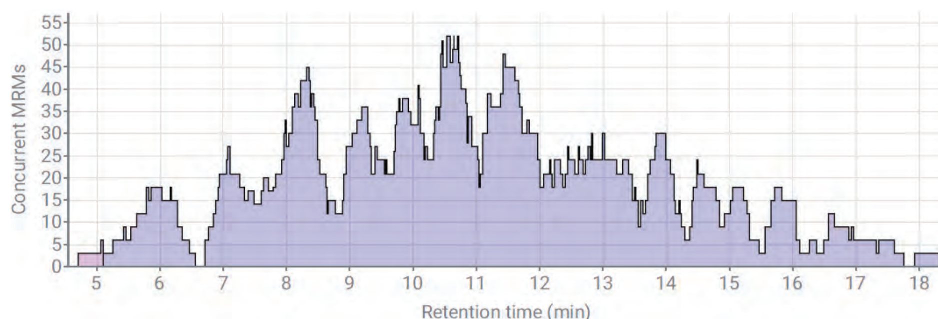


Figure 2. The distribution of 614 MRM transitions with up to 52 concurrent MRMs monitored during the analysis enabling most efficient dwell time distribution.

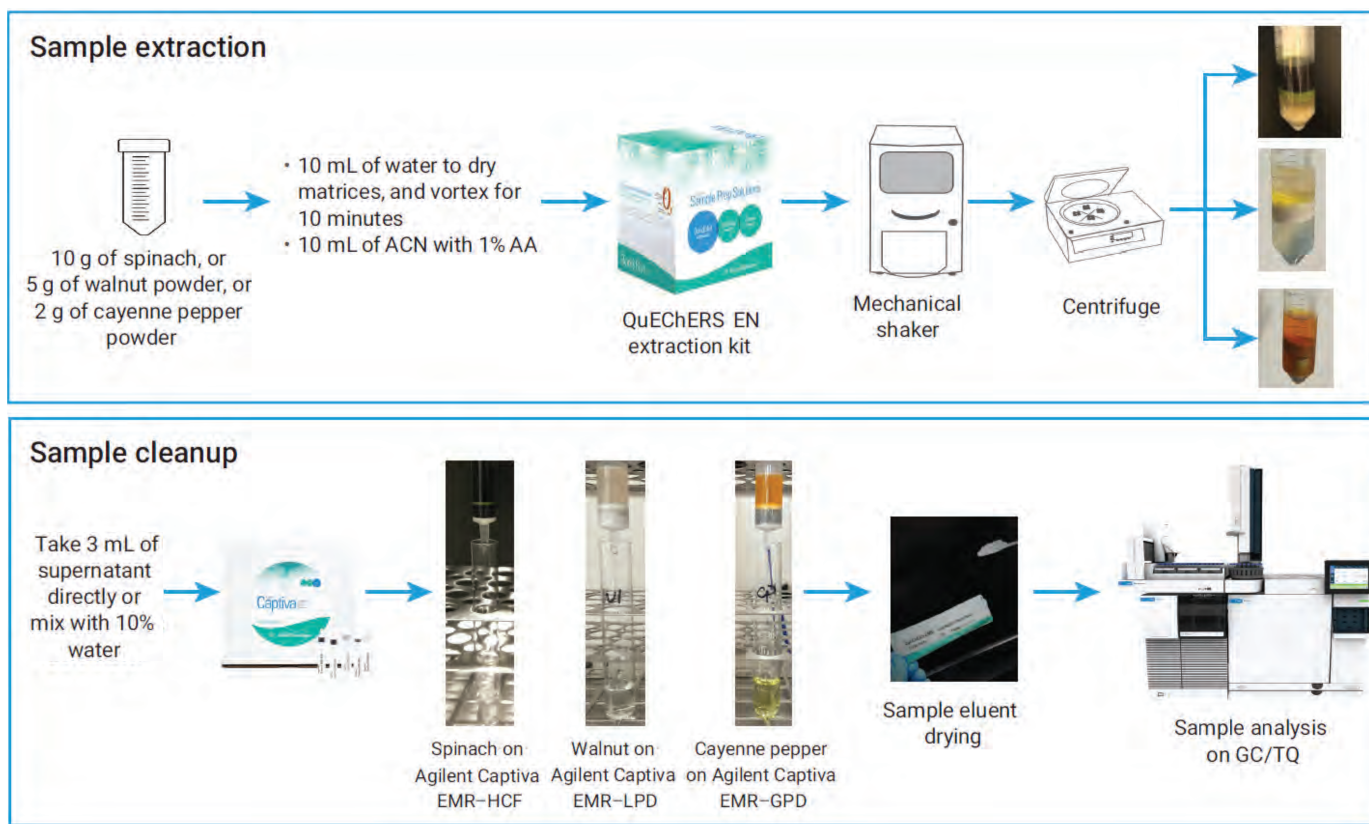


Figure 3. Sample preparation flowchart including traditional QuEChERS extraction, followed with Captiva EMR pass-through cleanup.

Results and discussion

Robust pesticide analysis that supports a high-throughput workflow must provide an extended maintenance-free operation with minimal downtime. The workflow must also meet the required sensitivity that can be at sub-ppb level. It must also enable calibration performance over a wide dynamic range that would encompass the MRLs for the compounds monitored in the commodity, which often vary over a wide dynamic range. The five key strategies outlined in this application note allowed achieving limits of quantification (LOQs) of up to 0.1 ppb while maintaining the calibration performance over a range up to 5,000 ppb for the 7000E and 1,000 ppb for the 7010C. In addition, the strategies would enable minimal instrument downtime limited to liner and septum replacement every ~100 injections.

The work presented in this application note and the system robustness study with 700 consecutive injections described elsewhere[3] resulted in over 1,000 injections of complex matrix extracts including spinach, walnut, and cayenne pepper. During this time, there was no need to perform TQ MS tuning, source cleaning, or GC column trimming.

Sample preparation

Efficient sample extraction and matrix cleanup are the keys to successful pesticide analysis. Analysis of crude QuEChERS extracts, especially of complex pigmented and oily matrices, can significantly increase the need for liner replacement, inlet cleaning, GC column trimming, and MS source cleaning. Such maintenance procedures decrease throughput of the analysis.

Performing an efficient matrix cleanup following QuEChERS extraction reduces in-source matrix loading and interferences with targets, while improving signal-to-noise ratio, accuracy, and reproducibility for target pesticides. Captiva EMR passthrough clean up following the traditional QuEChERS extraction was used in this work. The new sample cleanup protocol is a simplified procedure that demonstrates an improvement on both sample matrix removal and targets overall recovery and reproducibility. As shown in Figure 4, the abundance of TIC signal in full scan data acquisition mode was noticeably reduced for spinach, walnut, and cayenne pepper extracts after clean up when comparing the crude extracts before cleanup.

Matrix screening in full scan data acquisition mode

Performing sample screening in full scan data acquisition mode facilitates the evaluation of in-source matrix loading. Every MS source has a limitation on the amount of material present in the source, at any point of time, to maintain the optimal performance. Quantitation accuracy of the analysis can be significantly compromised if the source is overloaded with matrix. Therefore, it is essential to analyze matrix in full scan mode to evaluate TIC and maintain the optimal GC/TQ performance. The abundance of TIC in full scan mode is recommended not to exceed 7×10^7 counts when analyzing with an EM gain set to 1. Out of the three analyzed matrices, cayenne pepper featured the highest matrix background, although noticeably reduced after the clean up procedure. This evaluation revealed that pesticides that elute between 11 and 12.5 minutes were expected to have sacrificed performance in the cayenne pepper matrix when evaluating sensitivity and the dynamic range. For example, Endosulfan I eluted at 11.273 minutes, and could be quantitated only starting at 5 ppb in the cayenne pepper matrix with both 7000E and 7010C, while spinach and walnut matrices had significantly lower matrix levels coeluting with Endosulfan I, with 0.1 ppb LOQ observed. Best practices on using the Agilent GC/TQ system in full scan data acquisition mode can be found in the application note 5994-3859EN.[4]

Some of the practices that can be employed to lower the matrix background include adequate sample cleanup, sample dilution, and smaller injection volume. The latter two approaches often result in better LOQs, especially with the HES-equipped 7010C GC/TQ system.

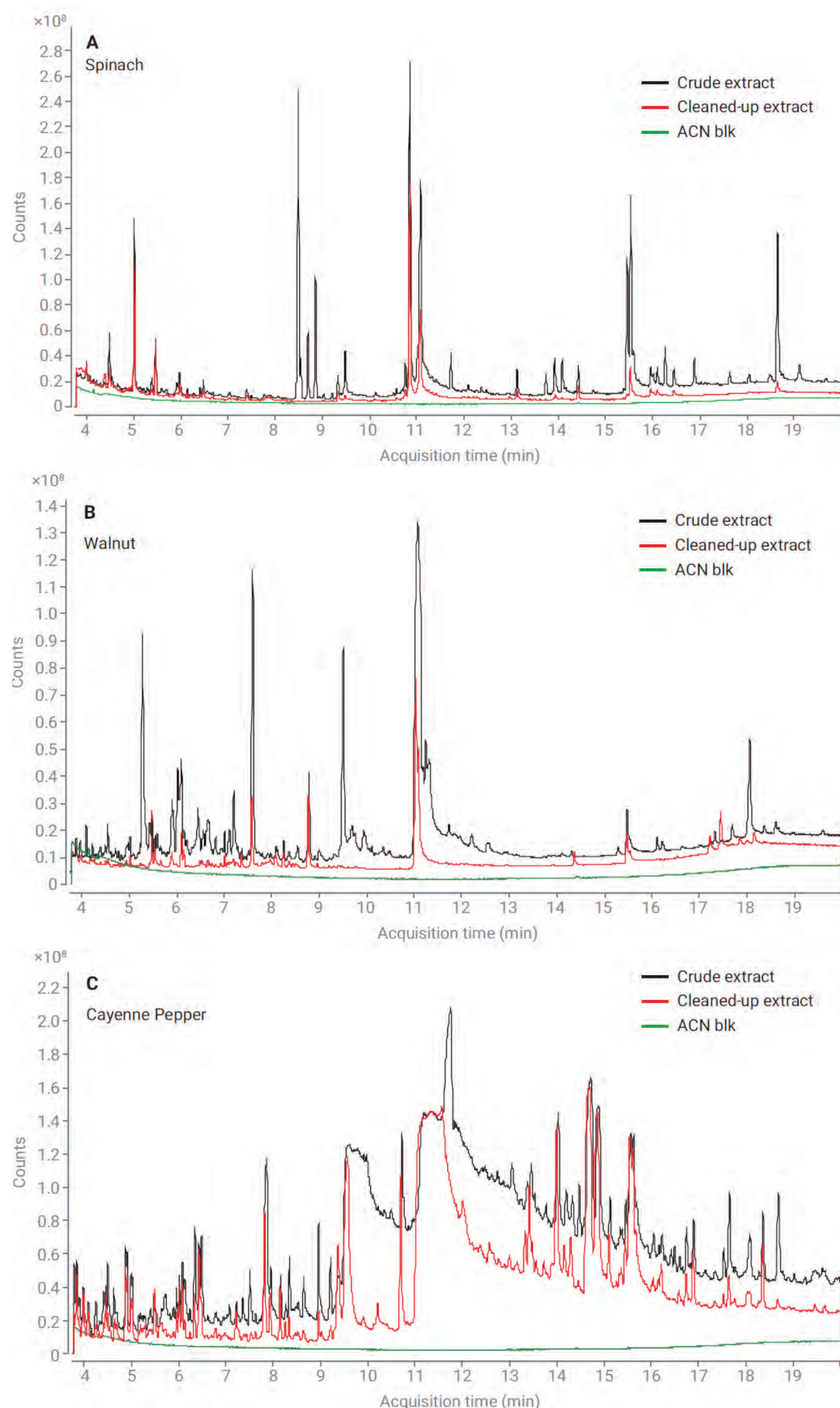


Figure 4. Scan TIC of the spinach (A), walnut (B), and cayenne pepper (C) extracts. The red trace corresponds to matrix sample with Captiva EMR cleanup, and the black trace corresponds to matrix sample without clean up. The green trace corresponds to the acetonitrile solvent blank.

Midcolumn backflushing

The use of the midcolumn backflushing configuration allows the analyst to limit the analysis time to the retention time of the last-eluting compound of interest. Challenging matrices, especially the oily ones, such as walnut, are rich in high-boiling components, with long retention times. These retention times often exceed that for the target pesticides. A common way to avoid ghost-peaks in the subsequent runs was to use an extended column bake-out after the last target analyte eluted from the column. However, this approach has several disadvantages including the deposition of high-boilers and GC column stationary phase into the EI source, contamination of the head of the GC column, a decrease of the column lifetime, and a longer cycle time due to the extended bake-out.

Midcolumn backflush allows the elution of the high boiling matrix components from the column without the sacrifices encountered with the bake-out approach. Midcolumn backflushing is a technique in which the carrier gas flow is reversed after the last analyte has exited the column. After the MS data are collected, the oven is held at the final temperature in post run mode, and the carrier gas flow through the first column is reversed. This reversed flow carries any high boilers that were in the column at the end of data collection. The high boilers are carried out of the head of the column and into the split vent trap (Figure 5A). The ability to reverse the flow is provided by the Agilent Purged Ultimate Union (PUU). The PUU is a tee that is inserted, in this case, between two identical 15 m columns.

During the analysis, a small makeup flow of carrier gas from the 8890 pneumatic switching device (PSD) module is used to sweep the connection. During backflushing, the makeup flow from the PSD is raised to a much higher value, sweeping high boilers backward out of the first column while simultaneously providing forward flow in the second column. For the configuration in this application, the backflushing time was 1.5 minutes. More details about using PSD for backflushing in the 8890 GC system can be found in the application note 5994-0550EN.[5]

The chromatograms shown in Figure 5B illustrate the effectiveness of the backflush technique in reducing cycle time sample carryover. The cycle time was reduced by 50% and the columns did not have to be exposed to the higher bake-out temperatures for an extended time. Using backflush, excess column bleed and heavy residues are not introduced into the MSD, thereby reducing ion source contamination.

In addition, the midcolumn backflushing configuration provides a significant time saving benefit when coupled with the MMI inlet. Maintenance procedures, such as septum and liner change, and column trimming can be performed without the need to cool down MS transfer line and source. When the septum is removed, the PSD provides the carrier gas flowing backward through column 1. The PSD also prevents air from entering the GC columns and the MS. MMI fast cooling capability enables more time savings. As a result, liner and septum replacement, which are the most common maintenance procedures, can be performed in a few minutes.

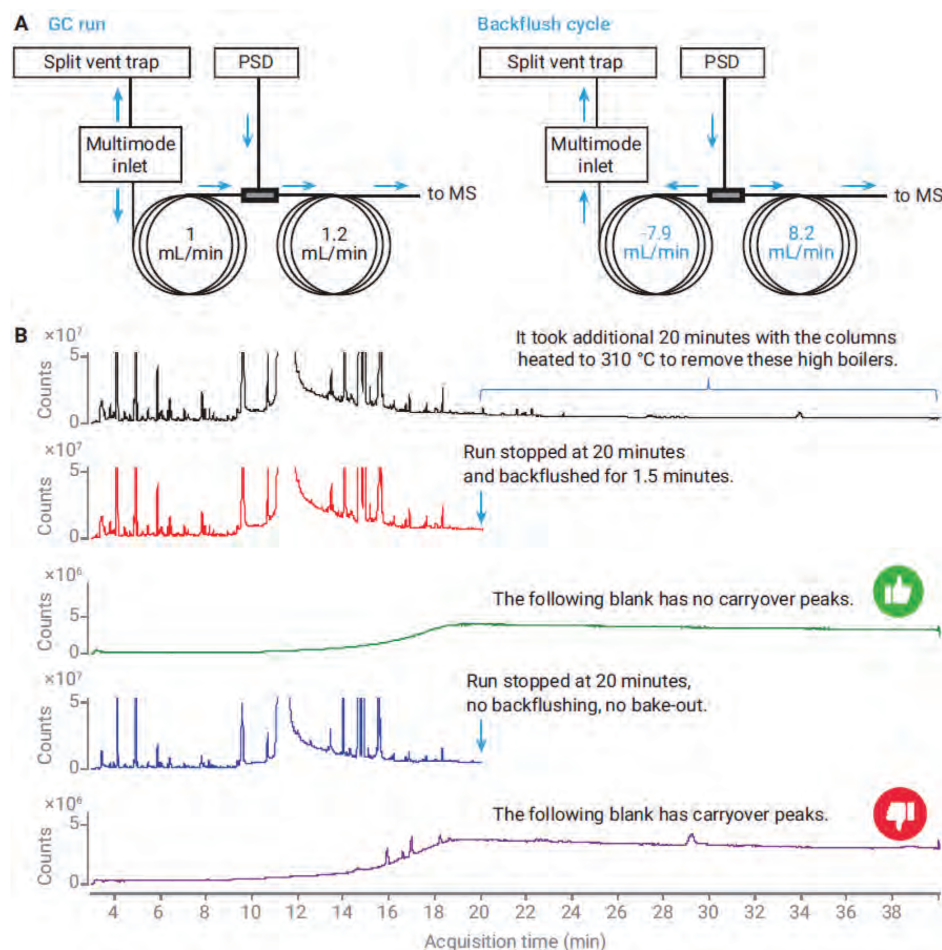


Figure 5. Midcolumn backflush configuration and gas flow during the GC run and the backflush cycle (A); TIC Scan chromatograms of a cayenne pepper extract followed by the analysis of an instrument blank with column bake-out, with backflush and without backflush or bake-out (B).

Leak-free GC/TQ system

Maintaining the GC/MS system leak-free is essential for the long-term performance of the instrument. Undesired leaks reduce the GC column lifetime and lead to oxidation of the EI source degrading its performance. The tools that enable tight connection make installation easy and reproducible and include the self-tightening collared column nuts for GC (Figures 6A and 6B part numbers G3440-81011 and G3440-81013) and CFT gold-plated flexible metal ferrules (Figure 6C, part number G2855-28501).

Figure 6. Self-tightening collared column nuts for the inlet (A) and MS transfer line connection (B) and gold-plated flexible metal ferrules (C).

The self-tightening collared column nuts have an innovative spring-driven piston. The piston continuously presses against the short graphite/polyimide ferrule, maintaining a leak-free seal even after hundreds of temperature cycles of the oven. The addition of the collar makes column installation into the GC inlet and MS transfer line easy and reduces the possibility of variation. The locking collar allows locking the column in place, for accurate and repeatable installation results, time after time. The simplicity of the column installation process with the self-tightening collared column nuts is demonstrated in these videos.[6,7] When MS source maintenance is not required, the collared nut in combination with the column installation tool (part number G1099-20030) allows installation of the column into the MS without opening the side door.

Gold-plated flexible metal ferrules are inert and provide exceptionally reliable sealing. They prevent formation of microleaks at the CFT (PUU) connection and allow for maintaining high sensitivity of the GC/TQ.

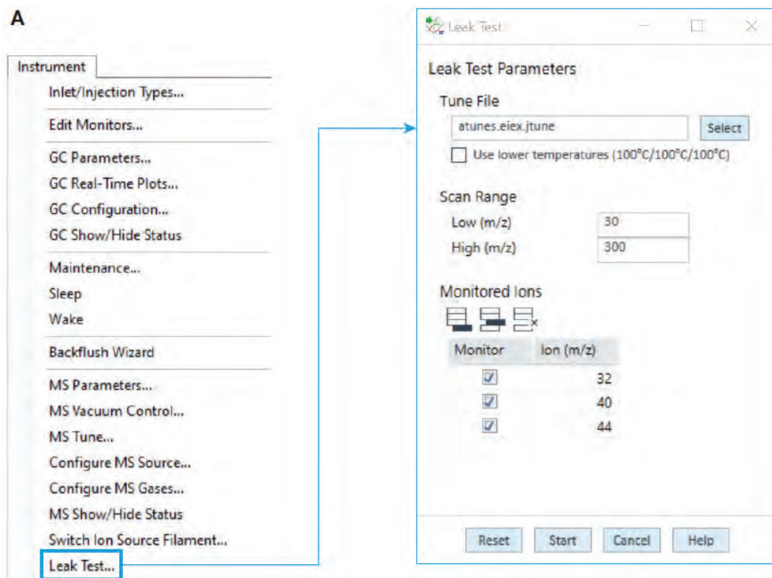
To confirm the leak-free status of the system, the air/water check, or autotune report, are often evaluated to determine how much of a leak is detected by the MS. However, this approach does not help to identify the source of the leak. Additionally, it may miss microleaks like those that may be present at user connections.

The novel leak test functionality is available with the 7000E and 7010C GC/TQ with MassHunter Data Acquisition 10.2 and above. The leak test can identify the source, and monitor the magnitude, of the leak. The tool monitors up to 10 user-specified ions (Figure 7A), including ions from a leak testing gas such as air duster (*m/z* 69 and 83, Figure 7B). The tool plots the corresponding chromatograms including EICs and TIC (Figure 7C).

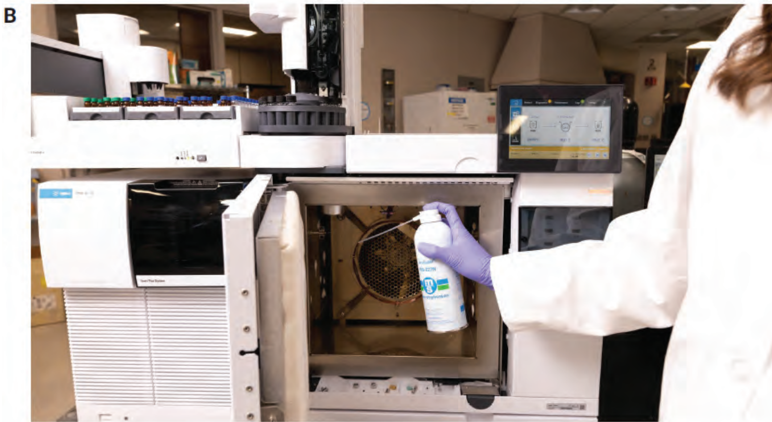
Optimized injection with the temperature-programmable multimode inlet (MMI)

Efficiently volatilizing the sample in the GC inlet is an essential component of a successful GC/MS analysis. Some pesticides, such as captafol, captan, dicofol, folpet, and deltamethrin, are known to be thermally labile. They are anticipated to suffer thermal degradation during injection. Starting the injection at lower temperature of 60 °C and ramping up to 280 °C allows for volatilizing all the target analytes while maintaining their chemical integrity upon introduction to the GC column. Moreover, the ability to program the inlet temperature allows heating up the inlet further to 310 °C during the post run while backflushing. This heating enables the system to bake-out any matrix residue that may remain in the inlet.

A



B



C

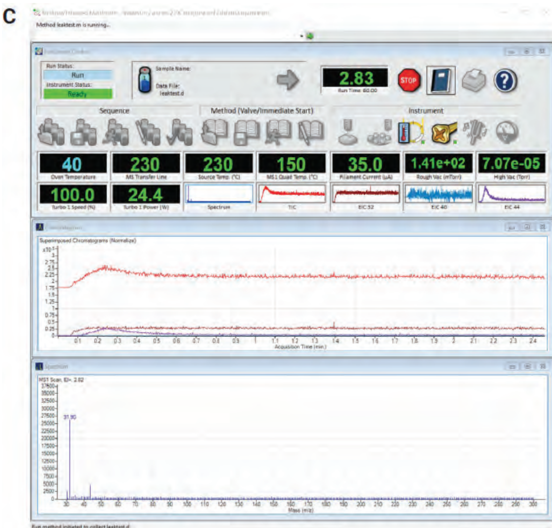


Figure 7. The novel leak testing tool that enables monitoring of the user-specified ions to identify the source and the amount of leak.

The combination of temperature-programmable injection with an Ultra Inert 2 mm dimpled liner resulted in high sensitivity even for challenging pesticides like deltamethrin in a complex walnut matrix. Figure 8A demonstrates the response of deltamethrin, a pesticide with an established MRL in walnut, at 0.5 ppb with the 7000E and the 7010C GC/TQ. The 7010C GC/TQ is equipped with the HES that yields a higher sensitivity resulting in higher signal-to-noise ratio (S/N).

Pentachloronitrobenzene is a pesticide that is commonly analyzed by GC/MS in various food commodities as it has established MRLs in many vegetables and fruits (Crop Group 8 Fruiting Vegetables Group), peanuts, and soybean seeds that vary from 20 ppb to 1 ppm.[8] Pentachloronitrobenzene presents a challenge for LC/MS analysis, so GC/MS analysis is the technique of choice. Figure 8B demonstrates the chromatograms for a selective MRM transition for pentachloronitrobenzene in a walnut extract with the 7000E and the 7010C.

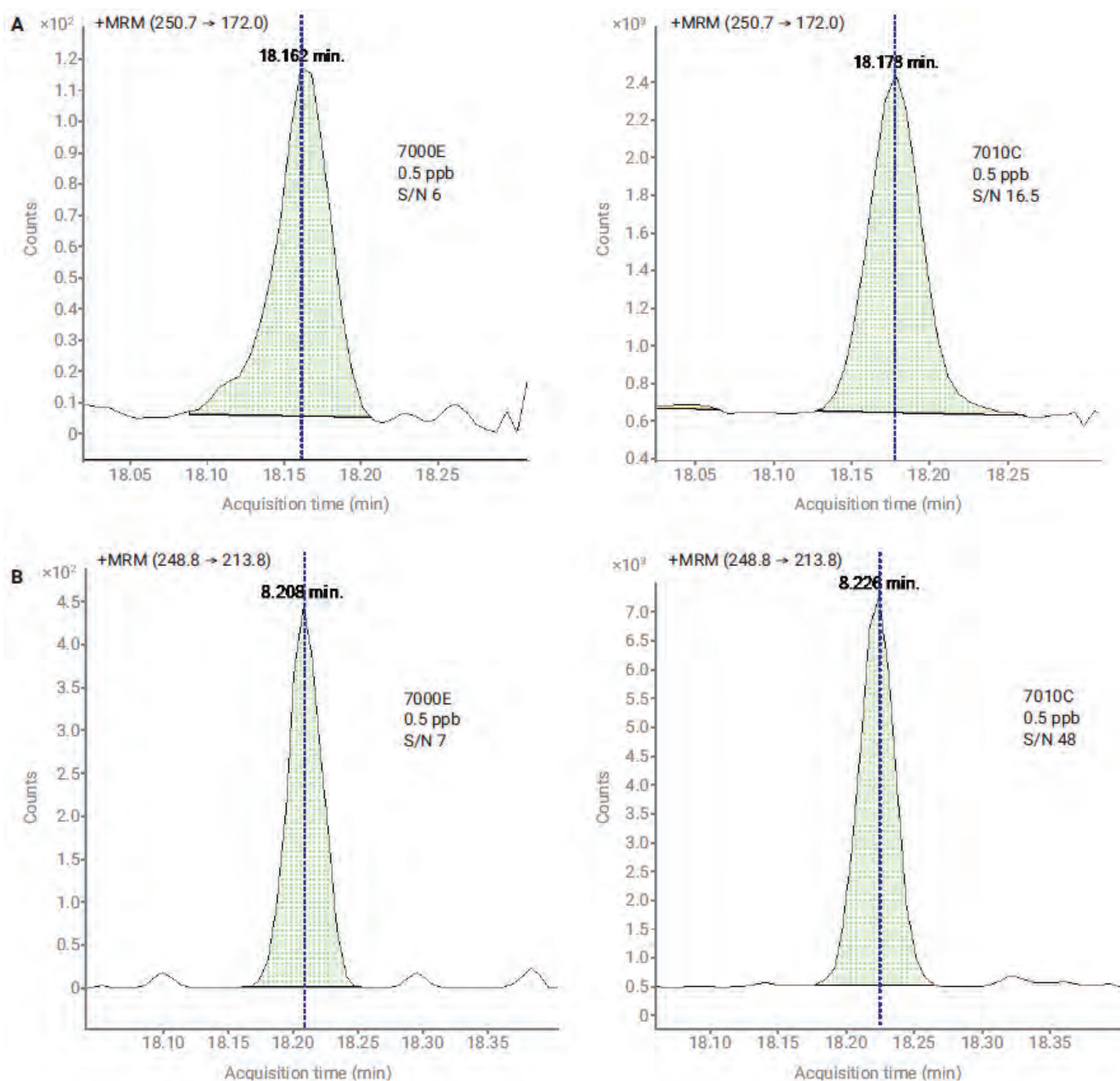


Figure 8. MRM chromatograms for deltamethrin (A) and pentachloronitrobenzene (B) at 0.5 ppb in walnut extract analyzed with the 7000E and the 7010C GC/TQ.

Calibration performance over a wide dynamic range with the 7000E and 7010C GC/TQ

The biggest challenge with the multiresidue analysis of food commodities is that the MRLs established for the pesticides vary over a wide range that may require undesirable sample reinjection. Achieving a broad dynamic calibration range can greatly reduce the need for diluting the sample and repeating the analysis.

Bifenthrin has established MRLs in spinach, walnut, and cayenne pepper that are 200, 50, and 500 ppb, respectively. Figure 9 demonstrates the linear calibration curves acquired with the 7000E over the calibration ranges of 0.1 to 1,000 ppb (R^2 = 0.996) in spinach, 0.1 to 5,000 ppb (R^2 = 0.991) in walnut, and 0.1 to 5,000 ppb (R^2 = 0.995) in cayenne pepper, encompassing the established MRL values.

MRLs for pesticide vary significantly not only across various commodities, but also for various pesticides regulated in one commodity. For example, pyriproxyfen and fludioxonil are monitored in spinach with the MRLs of 3,000 and 10 ppb, respectively. Figure 10A demonstrates that the 7000E GC/TQ maintained linear calibration performance for both pyriproxyfen and fludioxonil in spinach extract from 0.1 to 5,000 ppb, while demonstrating excellent accuracy even at low concentrations (see the zoomed in calibration for fludioxonil).

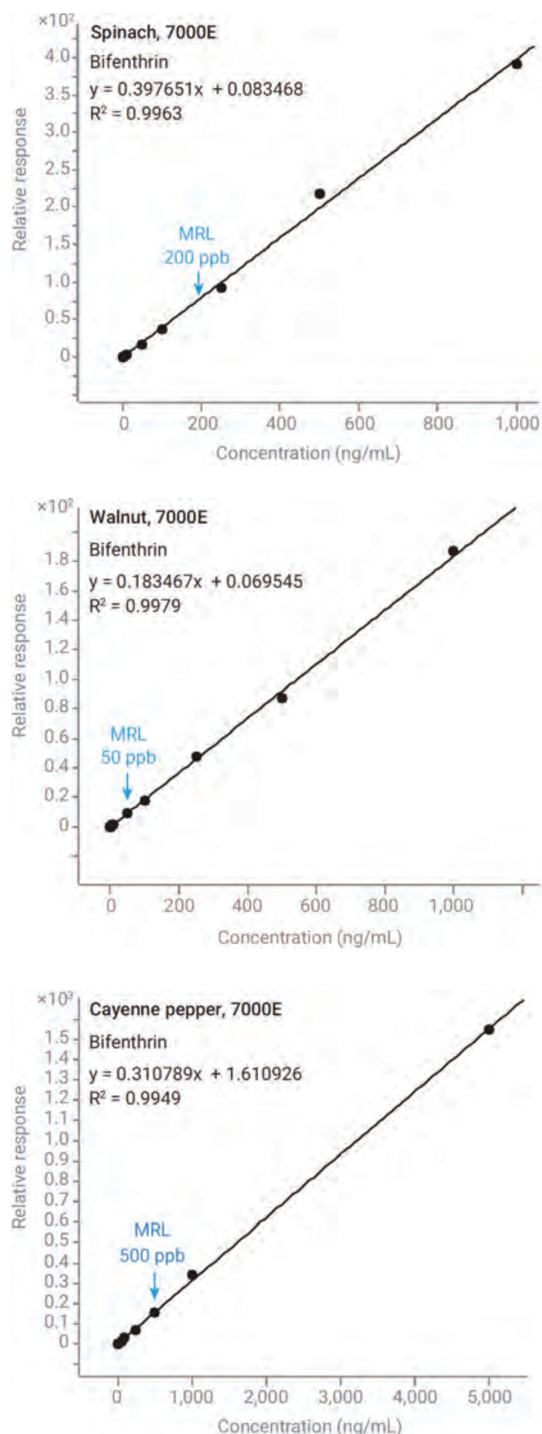


Figure 9. Matrix-matched calibration curves for bifenthrin in spinach, walnut, and cayenne pepper extracts with the 7000E GC/TQ.

As shown in Figure 10B, the 7010C GC/TQ also allowed for achieving a linear calibration curve over a broad range for both pesticides (0.1 to 1,000 ppb). However, the dynamic range of the 7010C would require an extra injection of a diluted sample to accommodate accurate quantitation of pyriproxyfen at its MRL of 3,000 ppb. While the upper limit of the calibration range achieved with the 7010C for pyriproxyfen and fludioxonil is lower than that with the 7000E, the 7010C delivers a higher sensitivity at lower concentrations. This is shown in Figure 10C and can be critical for the analysis of these pesticides in the commodities with lower established MRLs.

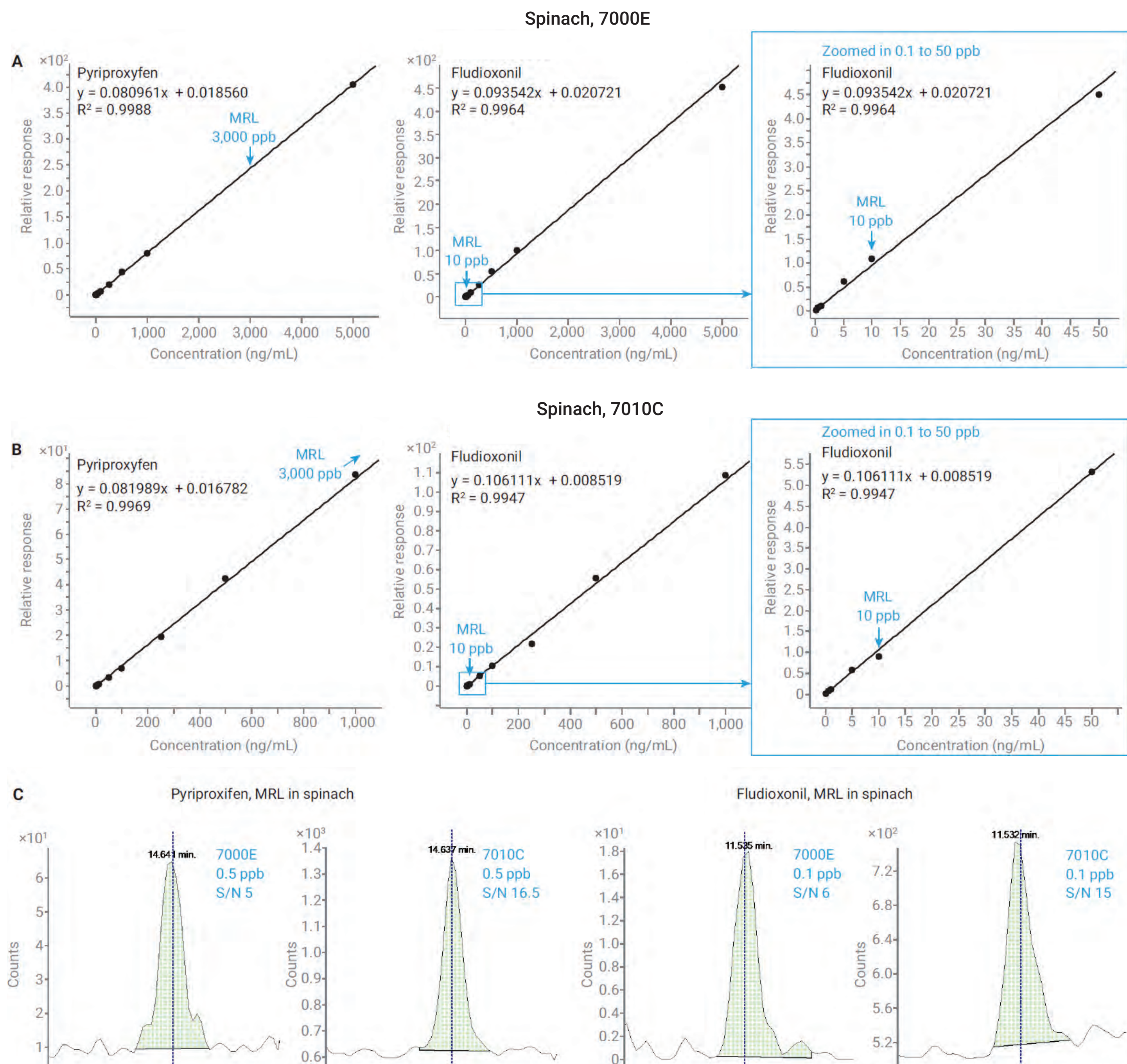


Figure 10. Matrix-matched calibration curves for pyriproxyfen and fludioxonil in spinach QuEChERS extracts with the 7000E GC/TQ (A) and with the 7010C GC/TQ (B); MRM chromatograms for pyriproxyfen and fludioxonil at 0.5 and 0.1 ppb in spinach QuEChERS extract analyzed with the 7000E and the 7010C GC/TQ (C).

Alternatively, samples with the MRLs above 1,000 ppb can be further diluted before the analysis with the 7010C GC/TQ. Superior sensitivity enabled with the HES allows for precise quantitation maintaining low LOQs even in the diluted sample. Additionally, injection of the dilutes samples increased maintenance-free operating time increased the number of injections that could be performed before the GC inlet liner needs replacement.

A summary in Figure 11 shows the calibration performance for the 203 pesticides that were analyzed in spinach, walnut, and cayenne pepper extracts with the 7000E and 7010C GC/TQ systems. The graph illustrates the number of compounds with the calibration correlation coefficient $R^2 > 0.99$, the calibration fit (linear or quadratic), and the calibration range.

As expected, considering the recommended loading for the HES not to exceed 1 ng per analyte, the upper calibration limit for the 7010C was lower when compared to the 7000E (1,000 ppb versus 5,000 ppb). However, the calibration range achieved with the 7010C was up to four orders of magnitude with a linear fit for most of the analyzed compounds. The 7010C GC/TQ equipped with the HES enables superior sensitivity yielding high S/N at low concentrations and allows for accurate quantitation at concentrations below 0.1 ppb. However, this was not required in this work as the MRLs for pesticides regulated in the commodities of interest did not require sub 0.1 ppb quantitation. Alternatively, samples with the MRLs above 1,000 ppb can be further diluted before the analysis with the 7010C GC/TQ. The HES enables maintaining high sensitivity at the LOQ level even in the dilutes sample.

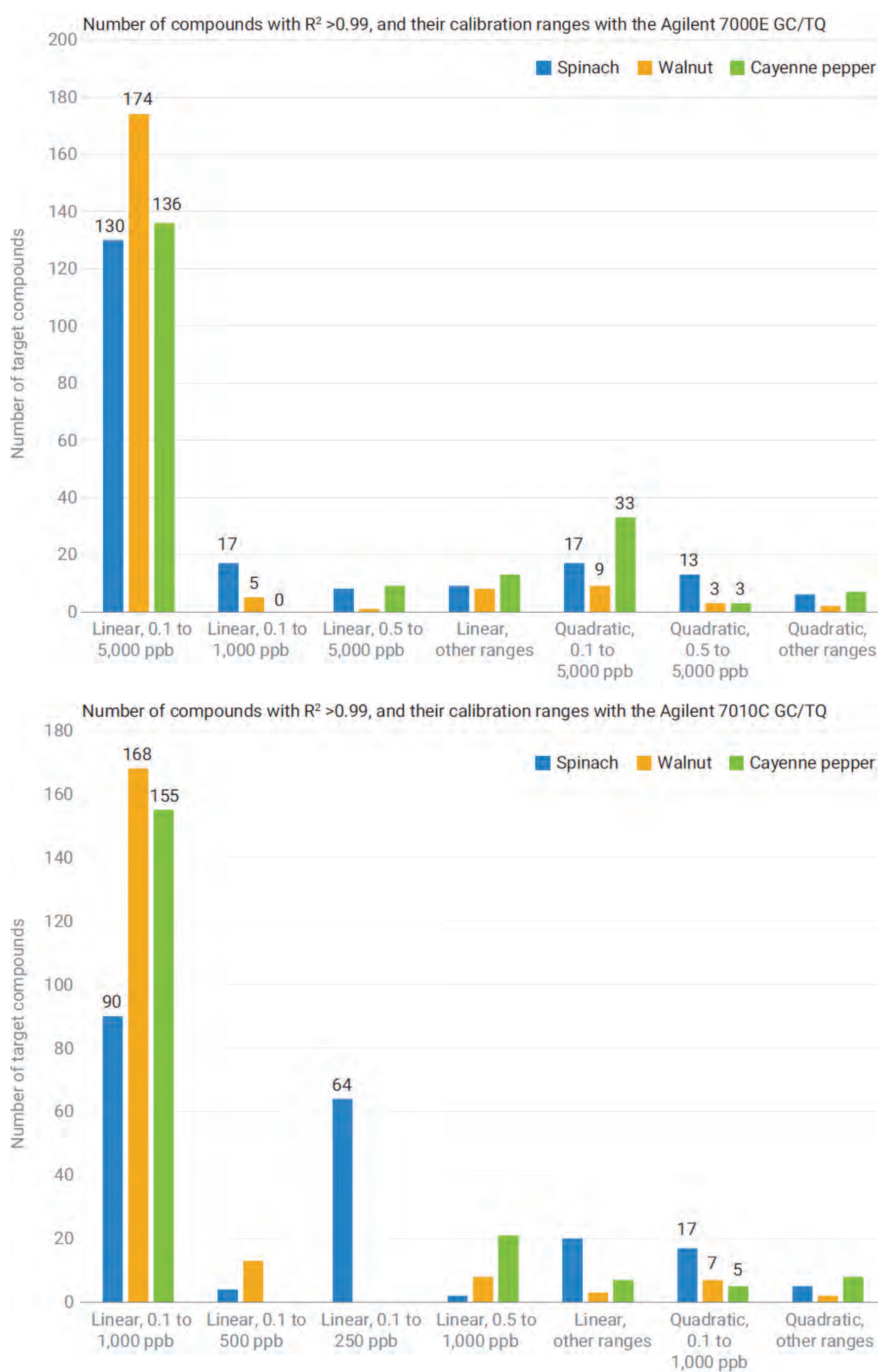


Figure 11. Calibration performance for the 203 pesticides with the 7000E and 7010C GC/TQ in spinach. The graph shows the number of compounds and their calibration ranges.

Conclusion

This application note described five best practices in sample preparation and Agilent 8890/7000E and 8890/7010C triple quadrupole GC/MS system analysis applied to 203 pesticides in challenging food matrices, including spinach, walnut, and cayenne pepper. These practices included:

- Simplified and improved sample preparation achieved with the novel and improved Agilent Captiva EMR pass-through clean up following the traditional Agilent QuEChERS extraction
- Evaluation of in-source loading of the matrix in full scan data acquisition mode
- Midcolumn backflushing
- Leak-free GC/triple quadrupole system enabled with the self-tightening collared column nuts and CFT gold-plated flexible metal ferrules
- Use of temperature-programmed multimode inlet with a 2 mm dimpled liner (no glass wool)

The resulting method allowed for excellent calibration performance over a wide dynamic range up to over four orders of magnitude. The calibration performance was as wide as 0.1 to 5,000 ppb and 0.1 to 1,000 for most of the compounds with the 7000E and the 7010C, respectively. The 7010C demonstrated superior sensitivity yielding a higher signal-to-noise ratio at lower concentrations. The wide dynamic ranges in combination with high sensitivity make the 7000E and the 7010C the ideal tools for analyzing pesticides at their MRLs in various commodities, including those with complex highly pigmented and oily matrices.

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Appendix 1

Compounds analyzed in this work and their observed retention times.

Name	Retenion Time (min)	Name	Retenion Time (min)	Name	Retention Time (min)
Allidochlor	4.893	Pyrimethanil	8.282	DCPA (Dacthal, Chlorthal-dimethyl)	10.062
Dichlorobenzonitrile, 2,6-	5.244	Diazinon	8.291	Fenson	10.201
Biphenyl	5.423	Fluchloralin	8.326	Diphenamid	10.288
Mevinphos, E-	5.597	Disulfoton	8.427	Bromophos	10.297
3,4-Dichloroaniline	5.708	Tefluthrin	8.431	Pirimiphos-ethyl	10.304
Pebulate	5.803	Terbacil	8.432	Isopropalin	10.358
Etridiazole	5.833	BHC-*delta*	8.504	Cyprodinil	10.407
cis-1,2,3,6-Tetrahydrophthalimide	5.966	Isazofos	8.527	MGK-264	10.443
N-(2,4-dimethylphenyl)formamide	5.973	Triallate	8.569	Isodrin	10.455
Methacrifos	6.055	Chlorothalonil	8.584	Metazachlor	10.532
Chloroneb	6.136	Endosulfan ether	8.857	Pendimethalin	10.535
2-Phenylphenol	6.246	Pentachloroaniline	8.913	Penconazole	10.562
Pentachlorobenzene	6.343	Propanil	8.942	Chlozolinate	10.584
Propachlor	6.888	Dimethachlor	8.996	Heptachlor exo-epoxide	10.621
Tecnazene	6.889	Acetochlor	9.093	Tolylfluanid	10.646
Diphenylamine	6.959	Vinclozolin	9.115	Allethrin	10.648
Cycloate	7.043	Transfluthrin	9.129	Fipronil	10.662
2,3,5,6-Tetrachloroaniline	7.059	Parathion-methyl	9.145	Chlorfenvinphos	10.676
Chlorpropham	7.102	Chlorpyrifos-methyl	9.146	Bromfenvinfos-methyl	10.683
Ethalfluralin	7.139	Tolclofos-methyl	9.233	Captan	10.732
Trifluralin	7.245	Alachlor	9.263	Triadimenol	10.746
Benfluralin	7.279	Propisochlor	9.333	Quinalphos	10.747
Sulfotep	7.376	Heptachlor	9.336	Triflumizole	10.77
Diallate I	7.481	Metalaxyl	9.337	Folpet	10.847
Phorate	7.498	Ronnel	9.396	Procymidone	10.858
BHC-*alpha* (benzene hexachloride)	7.636	Prodiamine	9.556	Chlorbenside	10.918
Hexachlorobenzene	7.768	Fenitrothion	9.596	Bromophos-ethyl	11.041
Dichloran	7.798	Pirimiphos-methyl	9.598	Chlordane-*trans*	11.043
Pentachloroanisole	7.823	Linuron	9.668	DDE-o,p'	11.09
Atrazine	7.885	Malathion	9.743	Paclobutrazol	11.106
Clomazone	7.982	Pentachlorothioanisole	9.758	Tetrachlorvinphos	11.169
BHC-beta	8.025	Dichlofluanid	9.764	Endosulfan I (*alpha* isomer)	11.273
Profluralin	8.117	Metolachlor	9.902	Chlordane-*cis*	11.305
Terbuthylazine	8.119	Anthraquinone	9.916	Flutriafol	11.322
BHC-gamma (Lindane, *gamma* HCH)	8.146	Fenthion	9.928	Fenamiphos	11.355
Terbufos	8.159	Aldrin	9.942	Chlorfenson	11.382
Propyzamide	8.175	Chlorpyrifos	9.964	Nonachlor, trans-	11.392
Pentachloronitrobenzene	8.219	Parathion	9.98	Bromfenvinfos	11.4
Fonofos	8.251	Triadimefon	10.011	Flutolanil	11.402
Pentachlorobenzonitrile	8.259	Dichlorobenzophenone, 4,4'-	10.033	Iodofenphos	11.479

Name	Retention Time (min)	Name	Retention Time (min)	Name	Retention Time (min)
Prothiofos	11.514	Carbophenothion	12.849	Phenothrin I	14.334
Fludioxonil	11.556	Carfentrazone-ethyl	12.851	Tetradifon	14.445
Profenofos	11.56	Methoxychlor olefin	12.865	Phosalone	14.61
Pretilachlor	11.592	Edifenphos	12.949	Azinphos-methyl	14.64
DDE-p,p'	11.637	Norflurazon	12.964	Pyriproxyfen	14.662
Tricyclazole	11.645	Lenacil	12.976	Leptophos	14.666
Oxadiazon	11.659	Endosulfan sulfate	13.04	Cyhalothrin (*Lambda*)	14.731
Dieldrin	11.73	DDT-p,p'	13.054	Mirex	14.898
Oxyfluorfen	11.737	Hexazinone	13.23	Acrinathrin	15.076
Myclobutanil	11.747	Methoxychlor, o,p'-	13.241	Fenarimol	15.121
DDD-o,p'	11.799	Tebuconazole	13.294	Pyrazophos	15.168
Flusilazole	11.8	Propargite	13.352	Azinphos-ethyl	15.252
Bupirimate	11.831	Piperonyl butoxide	13.404	Pyraclofos	15.303
Fluazifop-p-butyl	12.007	Resmethrin	13.44	Permethrin, (1R)-cis-	15.656
Nitrofen	12.023	Captafol	13.466	Permethrin, (1R)-*trans-*	15.772
Ethylan	12.063	Nitralin	13.563	Pyridaben	15.807
Chlorfenapyr	12.064	Iprodione	13.726	Fluquinconazole	15.895
Endrin	12.127	Tetramethrin I	13.836	Coumaphos	15.902
Chlorobenzilate	12.194	Pyridaphenthion	13.838	Prochloraz	15.958
Endosulfan II (beta isomer)	12.291	Endrin ketone	13.898	Cyfluthrin I	16.207
DDD-p,p'	12.383	Phosmet	13.931	Cypermethrin I	16.421
Ethion	12.453	Bromopropylate	13.952	Flucythrinate I	16.75
DDT-o,p'	12.457	EPN	13.955	Ethofenprox	16.829
Chlorthiophos	12.503	Bifenthrin	13.956	Fluridone	17.034
Nonachlor, cis-	12.508	Methoxychlor, p,p'-	14.062	Fenvalerate I	17.459
Endrin aldehyde	12.618	Fenpropathrin	14.077	Fluvalinate-tau I	17.646
Sulprofos	12.669	Tebufenpyrad	14.142	Deltamethrin	18.177
Triazophos	12.674				

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Application Note

Food Testing & Agriculture

60-Second Screening of Foods Using the Agilent QuickProbe GC/MS System

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Abstract

The Agilent QuickProbe GC/MS system, a direct insertion sampling device for GC/MS, was evaluated for the screening of nonextracted food samples. Foods analysis benefits from fast screening because it quickly identifies samples that are suspect and require further investigation.

Introduction

Typical GC/MS screening of foods and botanicals requires sample preparation such as QuEChERS or other liquid extraction methods. Using the QuickProbe system enables a simple and fast screening analysis that requires no sample preparation. The QuickProbe unit contains a short GC column, and is mounted on the top of the oven of either an Agilent 5975 or 5977 GC/MSD instrument. Sampling is accomplished by touching the sample with a glass probe and inserting the probe into an open atmosphere heated inlet. Ultra fast heating of the column in the presence of helium flow accomplishes the separation of sample components. Data acquisition and analysis is performed using Agilent MassHunter Workstation Acquisition and Unknowns Analysis software, and spectra are identified by searching against user or commercial libraries. Many food sample types have been studied including various oils, spice mixes, beverages, plant material, and flavorings. Samples may consist of either unprepped samples before extraction, as described here, or extracts resulting from the existing laboratory workflow.

Experimental

An Agilent 5977B single quadrupole mass spectrometer was coupled to an Agilent 7890B GC instrument equipped with a separate QuickProbe control unit (Figure 1). The QuickProbe system (G3971A) had an open inlet containing a specialty liner with frit (5190-5104), as shown in Figure 2, a 1.5 m × 0.25 mm, 0.1 µm DB-1HT column, and a 0.7 m × 0.18 mm, 0.18 µm DB1-MS column used as a restrictor into the mass spectrometer.

Helium was used as the carrier gas. The GC/MS system was autotuned. Round tip, glass sample probes (5190-5118) were obtained in touchless packaging (Figure 3), and were held using the QuickProbe holder (G3971-60200) shown in Figure 4, that works as the sample insertion device. Pocket tip probes (5190-5113) contain an indentation or "pocket" at the tip, and are useful for powders. Table 1 lists instrument conditions. Some variations in column temperature hold time and ramp rate were also used.

Figure 1. Agilent QuickProbe (G3971A) device mounted on an Agilent 5977 GC/MS system.

Figure 2. Specialty liner with frit (5190-5104).

Figure 3. Sample probes in touchless packaging (round tip, 5190-5118; pocket tip, 5190-5113).

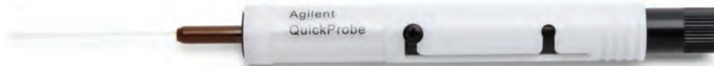

Figure 4. Probe holder shown in loading position with inserted probe on left side (G3971-60200).

Sampling was performed by first inserting a glass probe into the probe holder then, while in loading position (Figure 4), scraping the probe along the solid food or plant material. In a liquid sample, the tip of the probe was dipped into the liquid. Powdered or granular samples were loaded by rubbing the glass probe with sample or tapping the pocket tip probe into the sample. Sample introduction was performed by first retracting the glass probe into the holder. The start button on the QuickProbe unit and the plunger on the probe holder were simultaneously depressed to start the run and position the probe into the hottest part of the inlet. Insertion time was generally five seconds, but this could be varied as required. MassHunter Workstation Acquisition and Unknown Analysis software were used for data acquisition and processing. A minimum match factor of 60 was used for NIST library matches.

Results and discussion

Various food components were easily differentiated using the QuickProbe GC/MS system. The chromatograms resulted from analysis of nonextracted food samples. They demonstrate the power of chromatographic separation coupled with mass spectral deconvolution to screen highly complex samples and identify targets (Figures 5 to 9). NIST library match scores for each component are in parentheses. As a demonstration, several types of oils such as fish, sesame seed, and vegetable were differentiated using the GC/MS QuickProbe system.

Table 1. Instrument conditions.

QuickProbe and GC Conditions	
Inlet Temperature	250 °C (isothermal only)
Injection Mode	Split (the split is fixed at ~1:10)
Column Temperature	35 °C, hold for 6 seconds 4 °C/sec to 325 °C, hold for 0 seconds (or extended hold)
Run Time	Generally 40 to 60 seconds
Transfer Line Temperature	280 °C
MS Conditions	
Ion Source Temperature	280 °C
Quadrupole Temperature	150 °C
Ionization	EI mode
EMV Mode	Gain factor
Gain Factor	10 (should be lowest value required to detect peaks of interest; minimum is 0.05)
Solvent Delay	0 minutes
Scan Type	Scan (38 to 550 µ, 6,250 µ/sec)
Scans Per Second	9.7

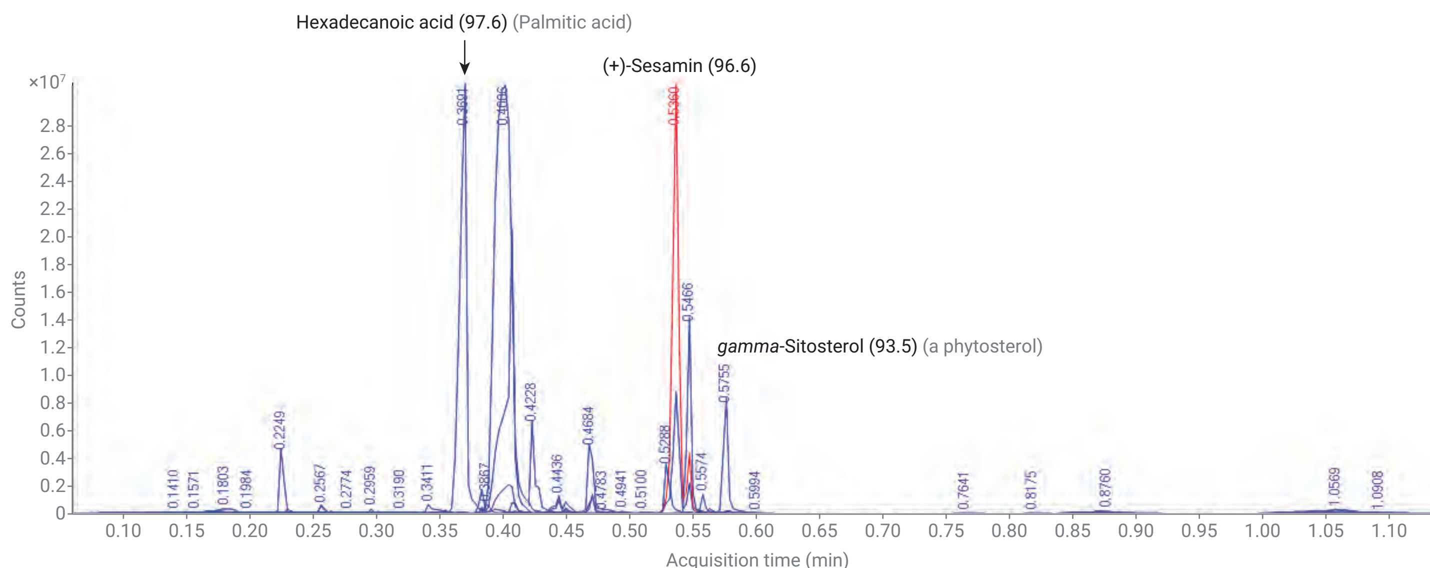

Figure 5. Sesame seed oil. The characteristic component sesamin is identified with a high library match score of 96.6.

This was due to the presence of characteristic components such as sesamin, in sesame seed oil (Figure 5), and cholesterol in fish oil (Figure 6). The profile for vegetable oil shown in Figure 7 shows a peak for 2,4-decadienal, which is formed upon oxidation and contributes to the characteristic aroma of fried foods.

Plant material was able to be screened for components by manually crushing a leaf around the glass probe. The characteristic compound umbellunone was found in California Bay Laurel leaf (Figure 8); this compound differentiates this species from the true bay leaf, or *Laurus nobilis*.[1] Native Americans used the California Bay Laurel leaves for various medicinal purposes due to their curative properties. This species is sometimes known as the "headache tree" because umbellunone can cause headaches in some sensitive individuals. Methyl eugenol was also determined to be a major constituent of the sample.

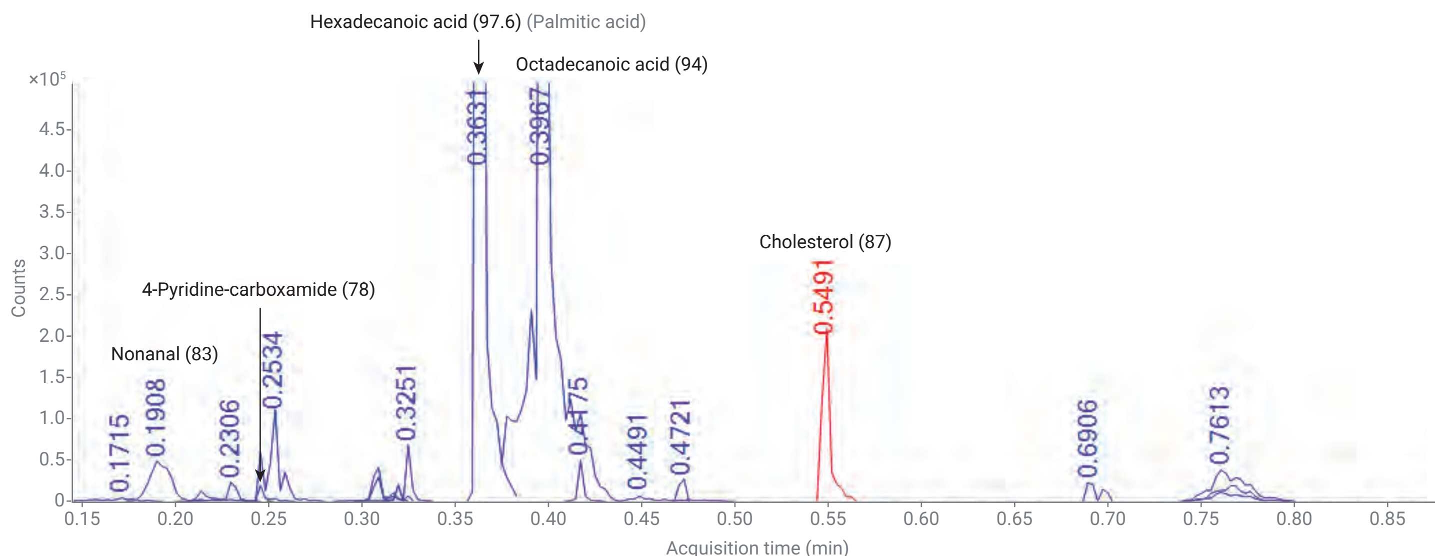

Figure 6. Commercial fish oil showing a peak for cholesterol.

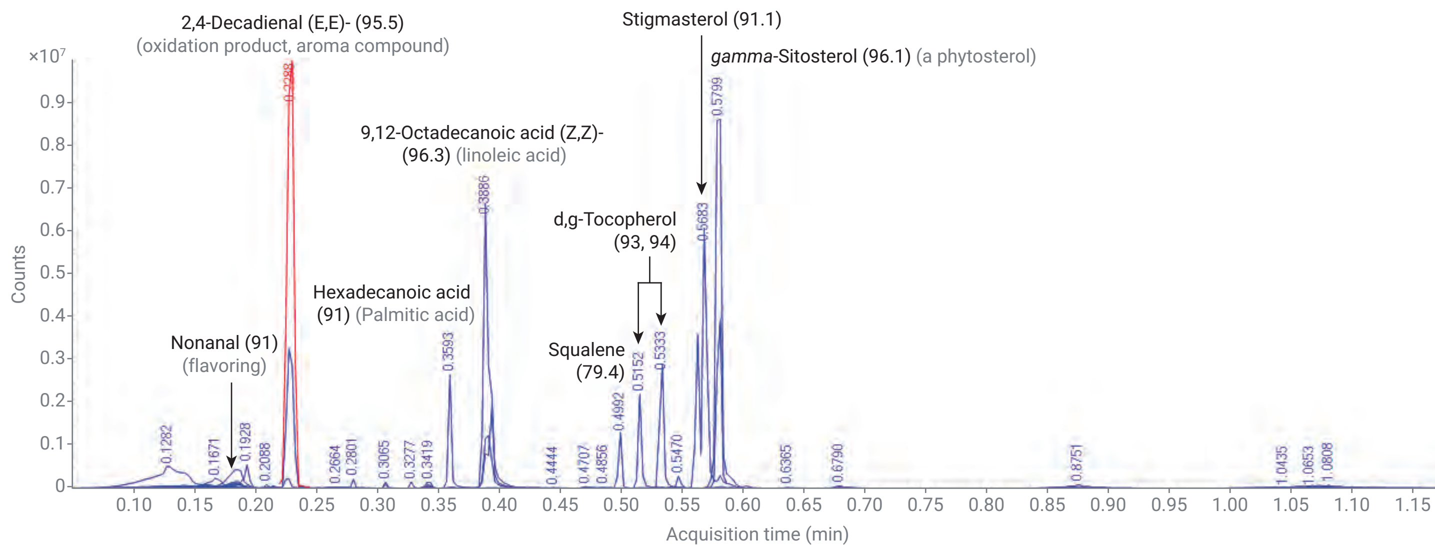

Figure 7. Vegetable oil profile (cottonseed).

Figure 9 shows a chromatogram for a peppery spice rub mix. The compound piperine, from black pepper, was determined in this sample along with *n*-isobutyl-2,4-decadienamide, which is found in herbs and spices. Vitamin E was also detected, and had a library match score of 81.

The QuickProbe GC/MS system successfully characterized several food samples in under one minute without the need for sample preparation. Diverse sample types such as liquids (oils), granular or whole food, and plant material were sampled using a round tip or pocket tip glass probe. Other means of sampling solid plant material (i.e., cannabis), using a thermal desorption technique, have been used with success, and are described elsewhere (Agilent publication 5994-1357EN).

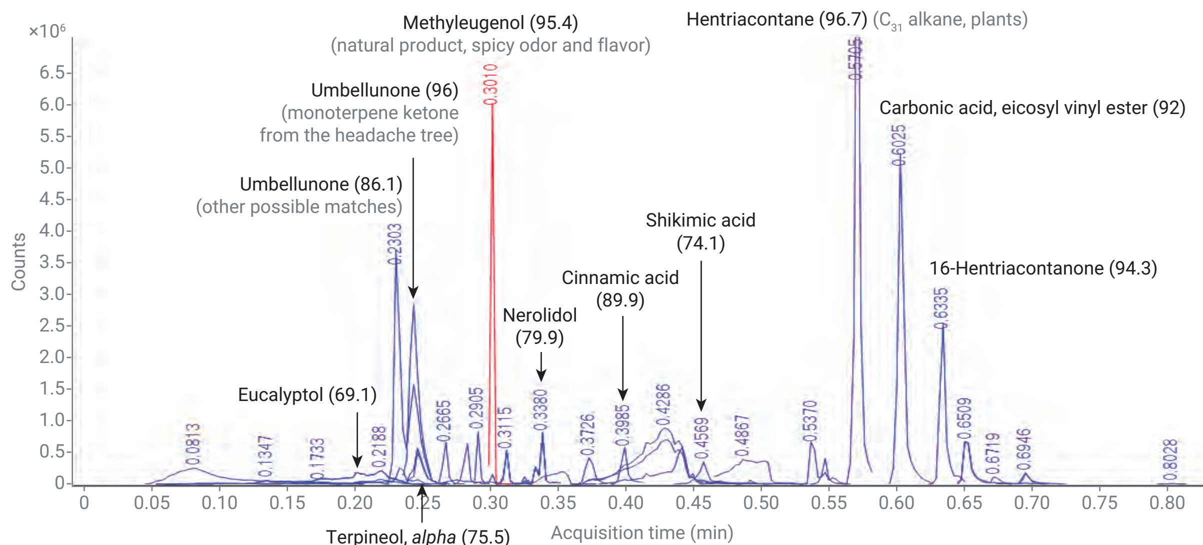

Figure 8. Leaf from the California Bay Laurel (headache tree).

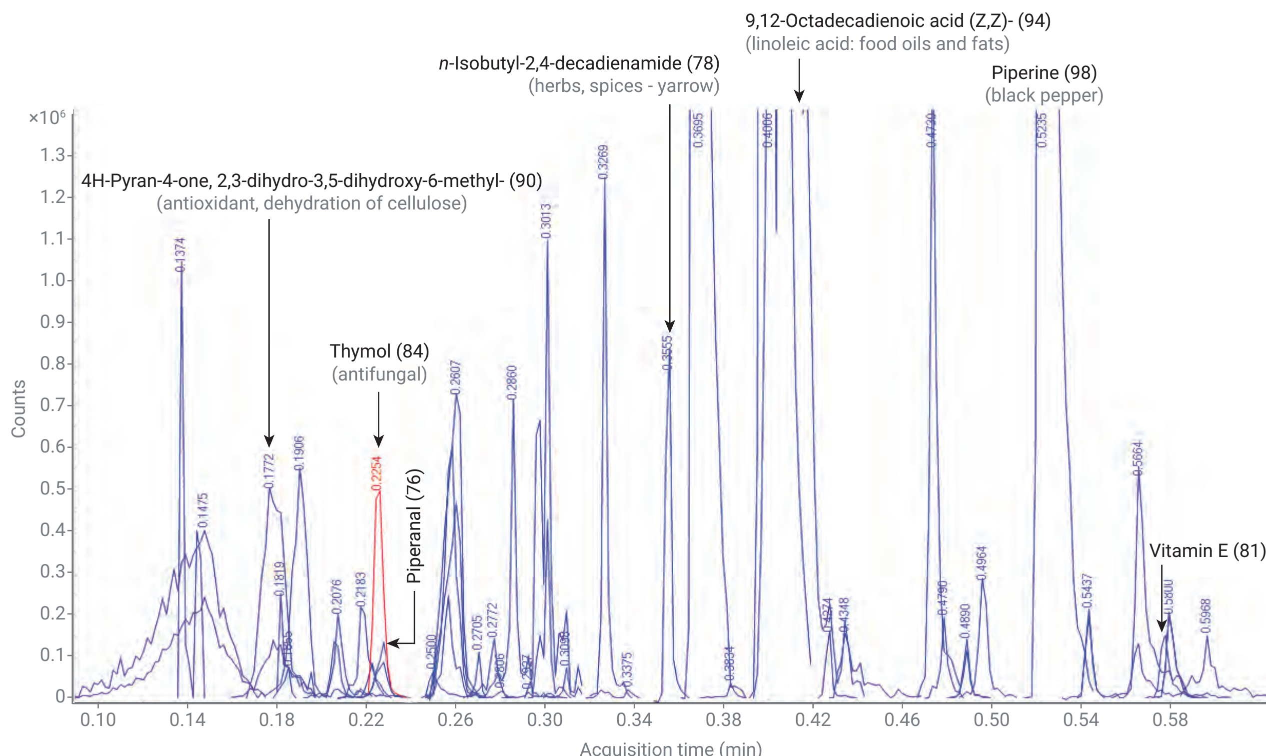

Figure 9. Spice rub mix with black pepper.

Conclusion

The power of the Agilent QuickProbe GC/MS system lies in its ability to quickly chromatograph complex foods and plants, without prior extraction, using a short GC column coupled to a mass spectrometer. Characteristic sample components were identified using Agilent Unknowns Analysis software with spectral match against the NIST library. Thus, a 60-second food screen is made possible using the QuickProbe GC/MS system.

Reference

1. Wang, M. *et al.* Application of GC/Q-TOFQ Combined with Advanced Data Mining and Chemometric Tools in the Characterization and Quality Control of Bay Leaves. *Planta Med* **2018** Sep, *84(14)*, 1045–1054. doi: 10.1055/a-0585-5987. Epub 2018 Mar 14.

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Application Note

Foods

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Determination of Heavy Metals and Trace Elements in Alternative Meats per EAM 4.7 Method for ICP-MS

Food safety analysis of plant-based protein foods and cell cultures using ICP-MS

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Introduction

People need to consume sufficient amounts of essential macronutrients—carbohydrates, fats, and protein—to support their body's energy needs. Protein is needed for growth, development, and repair of body tissues, and is especially important for building or maintaining muscles and for bone-health. Meat, poultry, fish, dairy products, and eggs are a major source of protein while plant-based sources include soya, beans, nuts, lentils, grains, cereals, fruit, and vegetables. Whether for ethical, dietary, health, or social reasons, there are increasing numbers of people globally who follow a vegan or vegetarian diet, or who are reducing their intake of animal-based foods. Concern about the impact of intensively farmed animals on the climate and natural resources may persuade even more people to limit the amount of meat in their diets (1).

The food industry is aware of the rise in popularity of meat-free foods and is actively investigating processes and products that can help meet the demand. The trend for meat-free foods can be seen by the ever-increasing selection of alternative protein products on supermarket shelves, and on the menus of fast-food outlets and restaurants. Some food companies are already selling products that are produced by cultivating meat-tissue from animal-origin cells grown in a bioreactor.

To ensure that non-meat based protein or cultured protein products are safe for human consumption, manufacturers must comply with Good Manufacturing Practice (GMP). Typically, GMP guidelines provide guidance for manufacturing, testing, and quality assurance of foods. Food safety analysis includes testing for chemicals, e.g., organic contaminants such as pesticide residues, and inorganic contaminants such as heavy metals, which are controlled in foodstuffs. In the United States (US), the Food and Drug Administration (FDA) regulates a wide range of foods and publishes analytical methods that laboratories should use to help ensure food safety. For example, FDA Elemental Analysis Manual (EAM) 4.7 is a comprehensive method that describes how to determine 12 elements in food digests (prepared using microwave assisted acid decomposition) by ICP-MS. EAM 4.7 also outlines a series of quality control (QC) tests to ensure that analysts can demonstrate instrument performance and data accuracy (*2*). Companies wanting to produce, import, or export cell-based alternative meats may need regulatory approval in each target market. However, it is likely that existing analytical testing of foods, such as EAM 4.7, can be applied to any newly developed cell-cultivated food products.

This study describes the use of the Agilent 7850 ICP-MS and Agilent SPS 4 autosampler for the analysis of 30 elements in different plant-based alternative meat samples and 29 elements in cell-culture solutions. Be was included in the plant-based protein analysis suite but was not an analyte of interest in the cell culture media study. The analytical method was adapted from a previous foods analysis study using the 7850 ICP-MS (*3*). The list of elements included all 12 heavy metal and trace elements specified in EAM 4.7: arsenic, cadmium, chromium, copper, lead, manganese, mercury, molybdenum, nickel, selenium, thallium, and zinc. In addition, the following trace and major elements were analyzed: aluminum, antimony, barium, beryllium, boron, calcium, cobalt, iron, magnesium, phosphorus, potassium, silver, sodium, strontium, sulfur, tin, titanium, and vanadium.

The quality of the data obtained for the elements analyzed was assessed through the measurement of four food certified reference materials (CRMs), a fortified method blank (FMB), and four fortified analytical portions (FAPs) of plant-based meat alternative foods. FAP refers to samples that are spiked before sample preparation. An FMB of the liquid cell media and FAPs of cell media, spent cell media, and conditioned spent cell media were also prepared and analyzed in this study.

Experimental

Calibration standards

The calibration standards were prepared in 2% nitric acid (HNO_3) and 0.5% hydrochloric acid (HCl). HCl is routinely added to samples for analysis using Agilent ICP-MS systems, as it ensures that chemically unstable elements such as Hg are retained in solution. Any Cl-based polyatomic overlaps formed are easily controlled using the standard helium (He) collision cell mode (*4*). Calibration standards were prepared from Agilent standard solutions including environmental calibration standard, p/n 5183-4688. Agilent single calibration standards were used for Hg (p/n 5190-8485), S (p/n 5190-8210), P (p/n 5190-8428, B (p/n 5190-8254), Ti (p/n 5190-8545), Sr (p/n 5190-8527), and Sn (p/n 5190-8543). Most trace elements were calibrated from 0.1 to 25 ppb. Cu, Zn, and Mn were calibrated up to 250 ppb. Hg was calibrated from 0.01 to 2.5 ppb. Mineral elements were calibrated from 5 to 25,000 ppb.

The internal standard (ISTD) solution containing 2 ppm ^{6}Li, Sc, Ge, Rh, Tb, and Bi (Agilent p/n 5188-6525) was prepared in 1% HNO_3, 0.5% HCl, and 10% isopropanol (IPA). Per the 4.7 method, IPA was added to the ISTD to ensure a consistent level of carbon in the solutions analyzed. This approach helps avoid the ionization enhancement that can affect As and Se sensitivities when variable levels of residual carbon are present in the samples after microwave digestion. The ISTD solution was added automatically online at a flow rate approximately 16 times lower than the sample flow.

Reference materials and samples

Four food matrix SRMs from National Institute of Standards and Technology (NIST, Gaithersburg, US) were used to validate the method. The SRMs included NIST 1577c Bovine Liver, NIST 1947 Lake Michigan Fish Tissue, NIST 1549a Whole Milk Powder, and NIST 1568b Rice Flour. The plant-based alternative meat samples (non-meat equivalents of fried chicken, beef burger, and minced beef) were bought in a supermarket in North Carolina, USA. A range of culture media liquid samples were obtained from a research project performed at UC Davis (*5*). The formulation of the cell culture media consisted of 40% Dulbecco's Modified Eagle Medium (DMEM), 40% Ham's F-10 Nutrient Mixture, 20% fetal bovine serum (FBS).

Standard and sample preparation

All the SRMs and plant-based alternative meat food samples were prepared as received (without homogenization or moisture removal) according to the digestion procedure outlined in the EAM 4.7 method. A MARS 6 closed-vessel microwave digestion system from CEM Corporation, USA was used. After accurately weighing the samples (approximately 0.5 g of food or SRM) into 75 mL PFA Xpress vessels, 8 mL of HNO_3 and 1 mL of H_2O_2 were added to the vessels. Duplicates of the samples, SRMs, and spiked samples (FAPs) were then digested in a single batch, using the heating program shown in Table 1. Each digestion batch can accommodate up to 40 varied food sample matrices, with a single program being used for all sample types. Following digestion, 0.5 mL concentrated HCl was added to the digests, followed by de-ionized water to a final weight of 100 g.

Table 1. Microwave digestion parameters.

Parameter	Setting
Power (W)	1800
Ramp Time (min)	25
Hold Time (min)	15
Temperature (°C)	200

Eight samples of the liquid cell culture media formulated for the cultivation of alternative meat samples at UC Davis were also analyzed in this study.

Samples 1 and 2 were from different batches of the same unused cell media formulation that had been incubated for different amounts of time. As a result, a slight difference in chemical composition was expected. Sample 1 was fresh media from the bottle, while sample 2 was analyzed after having been kept in the incubator for 21 days.

Samples 3, 4, and 5 were spent cell-culture media from the same batch, collected after the media had been used to grow primary embryonic chicken muscle precursor cells for 21 days. Spent media is the cell culture media that remains after the cells have been harvested and so contains unused nutrients and accumulated metabolites and waste products. Samples 3 to 5 were expected to be similar in composition, since they were "biological replicates", i.e. media used to incubate three separate cultures of the same type of cells. As a result, any differences in chemical composition between the three samples should be attributable to metabolic variation between the replicate cultures.

Samples 6, 7, and 8 were samples of the same batch of media used for samples 3, 4, and 5, but sampled after the cells had been growing in the media for 14, rather than 21, days. Samples 6, 7, and 8 were also biological replicates, used to grow three separate cultures of the same type of chicken embryo cells as were used for samples 3 to 5.

A 15 mg aliquot of each of the liquid cell culture media samples was diluted (rather than digested) in 15 mL of 2% HNO_3 and 0.5% HCl, before being analyzed directly by ICP-MS. Cell culture media is valuable, so the sample size was limited to 15 mg.

The analytical sequence of calibration standards, samples, and QC solutions is shown in Figure 1. The sample block was analyzed repeatedly with automatic insertion of the periodic QC block after every 10 samples.

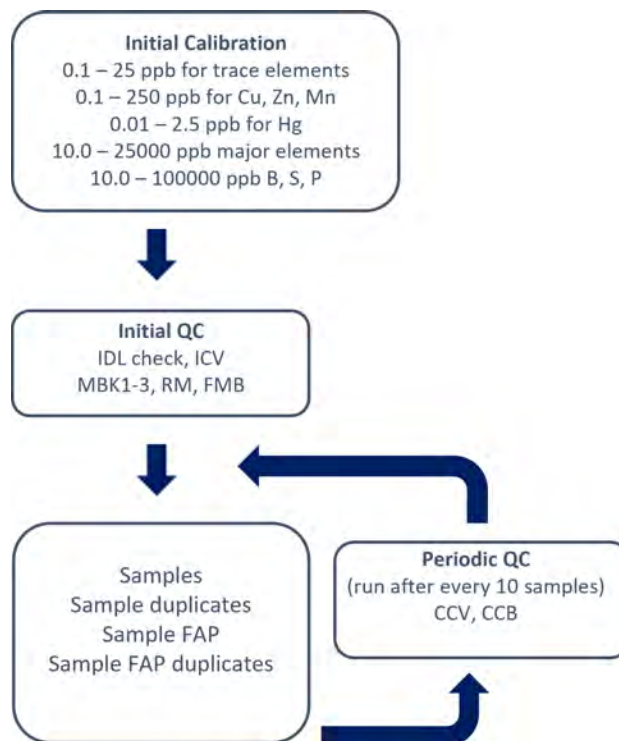

Figure 1. Analytical sequence.

Key: Instrument detection limit (IDL), initial calibration verification (ICV), method blank (MBK), reference material (RM), fortified method blank (FMB), fortified analytical portion (FAP), continuing calibration verification (CCV), continuing calibration blank (CCB).

Instrumentation

An Agilent 7850 ICP-MS, which includes the ORS[4] collision cell and UHMI aerosol dilution system, was used for the analysis. The standard ICP-MS sample introduction system was used, consisting of a MicroMist glass concentric nebulizer, temperature-controlled quartz spray chamber, and quartz torch with 2.5 mm id injector. A nickel-plated copper sampling cone was used, together with a nickel skimmer cone.

Based on previous experience of testing food digests (*3*), the preset plasma mode HMI-4 was selected, which applies an aerosol dilution factor of four times to the samples (*6*). When UHMI is selected, plasma settings are autotuned as appropriate for the matrix levels of the target sample types, as indicated by the shaded rows in Table 2. Other instrument operating conditions were optimized automatically using the ICP-MS MassHunter autotune function. All analytes were acquired in helium (He) mode (enhanced He mode for P, S, As, and Se). EAM 4.7 stipulates that an ICP-MS used for FDA regulated food analysis must be able to operate in helium mode with kinetic energy discrimination (KED). Reactive cell gases are not an acceptable alternative on single quadrupole ICP-MS, due to the risk of creating new spectral overlaps through the formation of reaction product ions. Operating the ORS[4] in He mode is the standard method used on Agilent ICP-MS systems, as it can reliably remove the typical polyatomic ion interferences on all common analytes (*4, 7*). Instrument operating conditions are listed in Table 2.

Table 2. Agilent 7850 ICP-MS operating conditions*.

ICP-MS Parameter	Setting
RF Power (W)	1600
Sampling Depth (mm)	10
Carrier Gas Flow (L/min)	0.80
Dilution (UHMI) Gas Flow (L/min)	0.15
Lens Tune	Autotune
Helium Cell Gas Flow (mL/min)	4.3 (10**)
Energy Discrimination (V)	5 (7**)

** Shaded parameters are defined in the method and HMI-4 plasma presets; all parameters were automatically optimized during start-up and autotuning. ** Enhanced He mode settings used for P, S, As, and Se.*

Results and discussion

Representative calibration curves are presented in Figure 2. The plots for Na, Mg, Mn, Cu, As, and Hg show excellent linearity across the calibrated range, with correlation coefficients of 0.9999 or better.

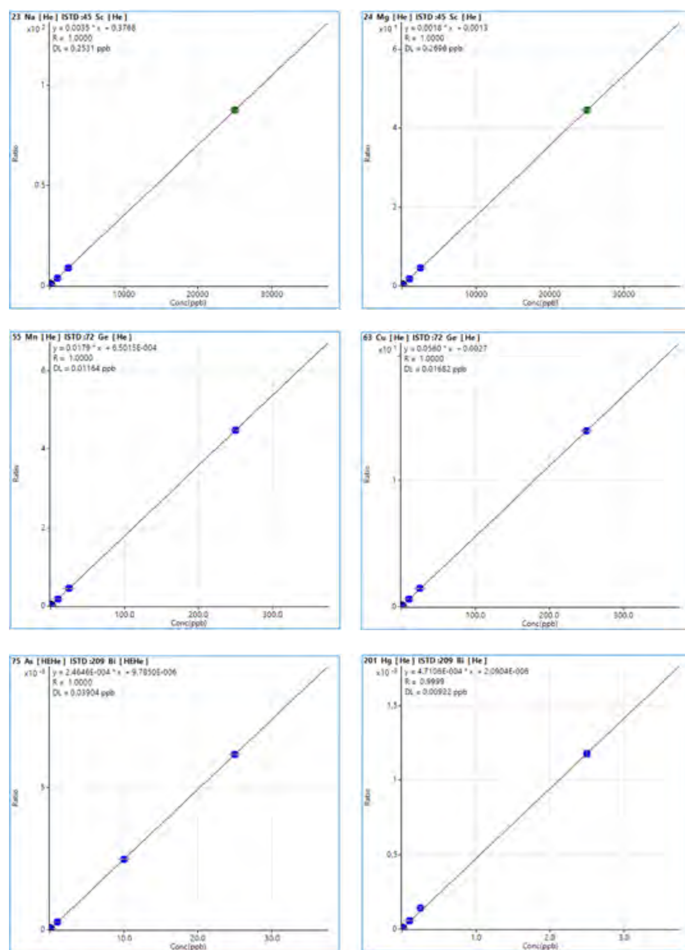

Figure 2. Representative calibration curves for major and trace elements.

Typical 7850 ICP-MS instrument detection limits (DLs) calculated from the ICP-MS MassHunter calibrations are shown in Table 3. The EAM method detection (LOD) and quantification limits (LOQ) – also shown in Table 3 – were calculated based on method blanks measured at the end of the run, n=10 (*8*). Data was acquired for 30 elements, including the 12 elements required by EAM 4.7, using He cell gas for all analytes.

Table 3. Agilent 7850 ICP-MS detection limits and EAM 4.7 nominal analytical limits, where provided.

Element	ICP-MS MassHunter		Calculated Based on EAM 4.7 Analytical Limits		EAM 4.7 Nominal Analytical Limits	
	DL (µg/kg)	BEC (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
9 Be	0.000	0.000	0.011	0.037	-	-
11 B	4.290	8.808	1.501	5.002	-	-
23 Na	7.410	275.1	7.505	25.02	-	-
24 Mg	0.140	0.384	0.141	0.471	-	-
27 Al	0.100	0.423	0.204	0.680	-	-
31 P	1.650	3.475	2.372	7.908	-	-
34 S	242.0	911.3	212.9	709.8	-	-
39 K	13.58	152.0	4.311	14.37	-	-
43 Ca	6.450	8.585	5.955	19.85	-	-
47 Ti	0.219	0.110	0.289	0.962	-	-
51 V	0.012	0.060	0.015	0.049	-	-
52 Cr	0.035	0.433	0.032	0.107	5.390	48.90
55 Mn	0.021	0.032	0.010	0.033	2.330	21.20
56 Fe	0.005	0.787	0.053	0.175	-	-
59 Co	0.001	0.002	0.001	0.003	-	-
60 Ni	0.024	0.024	0.006	0.020	6.380	58.00
63 Cu	0.006	0.055	0.018	0.060	6.020	54.70
66 Zn	0.159	1.003	0.116	0.387	37.40	340.0
75 As	0.029	0.043	0.004	0.014	1.270	11.60
78 Se	0.166	0.412	0.088	0.292	7.280	66.10
88 Sr	0.004	0.008	0.002	0.006	-	-
95 Mo	0.005	0.002	0.003	0.012	5.180	47.10
107 Ag	0.001	0.002	0.002	0.005	-	-
111 Cd	0.003	0.003	0.003	0.010	0.408	3.710
118 Sn	0.011	0.129	0.008	0.025	-	-
121 Sb	0.013	0.033	0.007	0.024	-	-
137 Ba	0.017	0.008	0.017	0.058	-	-
201 Hg	0.006	0.006	0.012	0.039	0.861	7.820
205 Tl	0.001	0.004	0.013	0.044	*0.281	*2.100
Pb**	0.002	0.024	0.001	0.005	1.200	10.90

*All elements were acquired in He mode (enhanced He for P, S, As, and Se). The Nominal Analytical Limits are given in EAM 4.7 and are based on method blanks measured during the single lab validation over one year; n = 143. *Based on a single lab validation (n = 27). **Pb was measured as the sum of the three most abundant isotopes, 206, 207, and 208.*

Verification of instrument calibration and sample digestion process

As part of the method quality control procedure specified in EAM 4.7, and to ensure the ongoing validity of the calibration, a CCV standard was analyzed five times during the analytical sequence. Most tested elements reported recoveries within the EAM acceptance criteria of ±10% of the actual concentration of the CCV (results not shown).

To verify the sample digestion process and the accuracy of the analytical method, two sets of the four NIST SRMs were analyzed in duplicate using the 7850 ICP-MS. As shown in Table 4, the mean concentrations were in good agreement with the certified concentrations, meeting the QC criteria requirements of the FDA EAM method of 80–120%. Since not all SRMs are certified for all analytes, blank cells indicate the absence of a certified or reference value.

Table 4. Mean measured concentrations of four NIST food-based SRMs using the Agilent 7850 ICP-MS. Mean calculated from triplicate sample digestion, each run in triplicate, n=9.

Element	NIST 1577c Bovine Liver					NIST 1947 Lake Michigan Fish Tissue				
	Conc Unit	Certified Conc	Mean Measured Conc	Recovery (%)*	QC Criteria (80–120%)**	Conc Unit	Certified Conc	Mean Measured Conc	Recovery (%)*	QC Criteria (80–120%)**
^{23}Na	mg/kg	2033	2039	100	Pass	-	-	-	-	-
^{24}Mg	mg/kg	620	614	99	Pass	-	-	-	-	-
^{31}P	mg/kg	11,750 R	12,189	104	Pass	-	-	-	-	-
^{34}S	mg/kg	7490	7541	101	Pass	-	-	-	-	-
^{39}K	mg/kg	10,230	10,195	100	Pass	-	-	-	-	-
^{43}Ca	mg/kg	131	115	88	Pass	-	-	-	-	-
^{51}V	µg/kg	8.17	8.52	104	Pass	-	-	-	-	-
^{52}Cr	µg/kg	53	57	107	Pass	-	-	-	-	-
^{55}Mn	mg/kg	10.46	10.18	97	Pass	mg/kg	0.076	0.071	93	Pass
^{56}Fe	mg/kg	197.94	199.86	101	Pass	mg/kg	3.79	3.38	89	Pass
^{59}Co	mg/kg	0.300	0.307	102	Pass	-	-	-	-	-
^{60}Ni	µg/kg	44.5	49.3	111	Pass	-	-	-	-	-
^{63}Cu	mg/kg	275.2	256.8	93	Pass	mg/kg	0.411	0.356	87	Pass
^{66}Zn	mg/kg	181.1	181.7	100	Pass	mg/kg	2.66	2.44	92	Pass
^{75}As	µg/kg	19.6	22.7	116	Pass	mg/kg	0.732	0.672	92	Pass
^{78}Se	mg/kg	2.031	2.182	107	Pass	mg/kg	0.475	0.426	90	Pass
^{88}Sr	µg/kg	95.3	96.8	102	Pass	-	-	-	-	-
^{95}Mo	mg/kg	3.30	3.49	106	Pass	-	-	-	-	-
^{107}Ag	µg/kg	5.9	6.1	104	Pass	-	-	-	-	-
^{111}Cd	µg/kg	97.0	98.4	101	Pass	-	-	-	-	-
^{121}Sb	µg/kg	3.13 R	3.74	120	Pass	-	-	-	-	-
^{201}Hg	µg/kg	5.36 R	5.93	111	Pass	mg/kg	0.254	0.274	108	Pass
Pb	µg/kg	62.8	63.6	101	Pass	-	-	-	-	-

Pb was measured as the sum of the three most abundant isotopes, 206, 207, and 208.

Table 4 continues on next page

Table 4 continued...

Element	NIST Whole Milk Powder SRM 1549a					NIST Rice Flour SRM 1568b				
	Conc Unit	Certified Conc	Mean Measured Conc	Recovery (%)*	QC Criteria (80–120%)**	Conc Unit	Certified Conc	Mean Measured Conc	Recovery (%)*	QC Criteria (80–120%)**
^{23}Na	mg/kg	3176	3648	115	Pass	-	-	-	-	-
^{24}Mg	mg/kg	892	1018	114	Pass	mg/kg	559	525	94	Pass
^{31}P	mg/kg	7600	8792	116	Pass	mg/kg	1530	1711	112	Pass
^{39}K	mg/kg	11920	13673	115	Pass	mg/kg	1282	1307	102	Pass
^{43}Ca	mg/kg	8810	10195	115	Pass	mg/kg	118.4	125.0	105	Pass
^{52}Cr	-	-	-	-	-	mg/kg	118.4	124.5	105	Pass
^{55}Mn	mg/kg	0.184	0.189	103	Pass	-	19.2	19.2	100	Pass
^{56}Fe	mg/kg	1.85 R	2.12	115	Pass	-	7.42	7.68	104	Pass
^{63}Cu	-	-	-	-	-	mg/kg	2.35	2.39	102	Pass
^{66}Zn	mg/kg	33.8	34.7	103	Pass	mg/kg	19.42	18.55	96	Pass
^{75}As	-	-	-	-	-	mg/kg	0.285	0.335	118	Pass
^{78}Se	mg/kg	0.242	0.288	119	Pass	mg/kg	0.365	0.425	116	Pass
^{111}Cd	-	-	-	-	-	mg/kg	0.0224	0.0201	90	Pass
^{201}Hg	-	-	-	-	-	µg/kg	5.91	6.00	107	Pass

*R - Reference mass fraction values. * FDA Elemental Analysis Manual (Section 3.4 Special Calculations) 3.4 Equation 20. ** FDA EAM 4.7 QC Criteria (80–120%) for NIST certified values.*

Matrix effects and spike recoveries

To test for nonspectral interferences (matrix effects), an FMB was prepared by spiking the blank at 1 µg/kg for most trace elements, 50 µg/kg for Al, Fe, Cu, Zn, and 4000 µg/kg for major elements including K, P, and S. The FMB was analyzed periodically throughout the entire analysis run. All recoveries were within the EAM 4.7 method acceptable % recovery range of 90–110%, as shown in Table 5.

A spike recovery (FAP) test was carried out to check the accuracy of the 7850 ICP-MS method for the analysis of the plant-based (meat-substitute) food products. Each sample was spiked with all elements at 1 or 50 µg/kg and measured using the 7850 ICP-MS. For samples that had naturally occurring elemental concentrations below 1 µg/kg, a 1 µg/kg spike is reported. For samples with higher naturally occurring concentrations, the 50 or 4000 µg/kg spike results are reported. The recoveries for all elements in the fortified plant-based beef-substitute food samples were within the EAM 4.7 method QC criteria of ±20%, as shown in Table 5.

Table 5. The mean recovery results are based on the analysis of replicate sample digests, each run in duplicate on the Agilent 7850 ICP-MS, n=2. The low spike concentration was 1 µg/kg and the high spike concentration was 50 or 4000 µg/kg.

		Method Blank			Plant-based "Minced Beef"		
	Conc Unit	Method Blank Conc	Recovery Low Spike (%)	Recovery High Spike (%)	Native Conc	Recovery Low Spike (%)	Recovery High Spike (%)
11 B	µg/kg	7.472	-	93	<LOD	-	111
23 Na	mg/kg	15.98	-	108	3650	-	*
24 Mg	mg/kg	<LOD	-	105	210.5	-	*
27 Al	µg/kg	0.448	106	101	2659	-	103
31 P	mg/kg	<LOD	-	96	2116	-	*
34 S	mg/kg	<LOD	-	**	1371	-	*
39 K	mg/kg	<LOD	-	105	2655	-	*
43 Ca	mg/kg	<LOD	-	102	1565	-	*
47 Ti	µg/kg	<LOD	102	91	152	-	103
51 V	µg/kg	<LOD	106	-	15.3	-	104
52 Cr	µg/kg	<LOD	106	-	89.2	-	103
55 Mn	µg/kg	<LOD	-	98	4085	-	104
56 Fe	mg/kg	0.859	-	106	33.16	-	*
59 Co	µg/kg	<LOD	105	-	35.2	-	104
60 Ni	µg/kg	<LOD	106	-	188	-	103
63 Cu	µg/kg	0.053	-	107	1615	-	105
66 Zn	mg/kg	<LOD	-	103	44.54	-	*
75 As	µg/kg	<LOD	104	-	16.3	-	109
78 Se	µg/kg	<LOD	104	-	78.0	101	98
88 Sr	µg/kg	<LOD	95	-	1686	-	92
95 Mo	µg/kg	<LOD	105	-	275	-	105
107 Ag	µg/kg	<LOD	-	102	1.481	-	92
111 Cd	µg/kg	<LOD	106	-	9.470	-	104
118 Sn	µg/kg	3.580	91	-	634	NA	
121 Sb	µg/kg	<LOD	104	-	<LOD	96	-
137 Ba	µg/kg	<LOD	103	-	391	-	100
201 Hg	µg/kg	<LOD	101	-	<LOD	111	-
205 Tl	µg/kg	<LOD	98	-	9.341	110	-
Pb***	µg/kg	<LOD	104	-	6.697	114	-

*All elements were acquired in He mode (enhanced He for P, S, As, and Se). *Spike level too low compared to native concentration, ** below calibration range. NA = not applicable, as not measured. ***Pb was measured as the sum of the three most abundant isotopes, 206, 207, and 208.*

Quantitative results for plant-based protein food samples

Quantitative results are given in Table 6 for three plant-based, alternative meat food samples. In addition to the 12 elements specified in EAM 4.7, data is provided for Be, B, Na, Mg, Al, P, S, K, Ca, Ti, V, Fe, Co, Sr, Ag, Sn, Sb, and Ba.

Table 6. Quantitative results (n=9) measured using the Agilent 7850 ICP-MS for three plant-based meat-alternative food samples.

	Conc Unit	Plant-based Fried "Fried Chicken"	Plant-based "Beef Burger"	Plant-based "Minced Beef"
9 Be	µg/kg	3.29 ± 2.64	3.51 ± 2.75	<LOD
11 B	µg/kg	2952 ± 204.6	2753 ± 153.5	<LOD
23 Na	mg/kg	10293 ± 350	2537 ± 48	3650 ± 231
24 Mg	mg/kg	281 ± 26360	627 ± 24679	210 ± 34
27 Al	µg/kg	65704 ± 28678	1291 ± 124.9	2659 ± 520.7
31 P	mg/kg	3291 ± 242784	1874 ± 76	2116 ± 312
34 S	mg/kg	24576 ± 434	1756 ± 68	1371 ± 237
39 K	mg/kg	1920± 91	4416 ± 86	2655 ± 161
43 Ca	mg/kg	169 ± 9	1025 ± 38	156 ± 22
47 Ti	µg/kg	63362 ± 17691	81.80 ± 11.98	152.4 ± 44.53
51 V	µg/kg	42.92 ± 11.86	8.21 ± 2.92	15.35 ± 1.43
52 Cr	µg/kg	190.7 ± 58.17	178.9 ± 8.71	89.19 ± 13.72
55 Mn	µg/kg	5610 ± 592.5	11450 ± 561.5	4085 ± 856.3
56 Fe	µg/kg	43416 ± 5195	33040 ± 410.0	33159 ± 6752
59 Co	µg/kg	583.5 ± 142.3	1085 ± 75.93	35.17 ± 4.59
60 Ni	µg/kg	581.8 ± 25.01	147.2 ± 19.20	188.5 ± 12.62
63 Cu	µg/kg	4201 ± 861.6	2295 ± 42.85	1615 ± 286.5
66 Zn	µg/kg	13990 ± 989.2	46159 ± 405.1	44540 ± 3098
75 As	µg/kg	30.39 ± 4.48	12.97 ± 3.21	16.35 ± 2.40
78 Se	µg/kg	82.22 ± 28.25	67.56 ± 11.85	78.04 ± 11.96
88 Sr	µg/kg	1272 ± 160.2	2467 ± 157.0	1686 ± 165.5
95 Mo	µg/kg	902.2 ± 121.9	856.1 ± 22.26	274.8 ± 45.87
107 Ag	µg/kg	14.10 ± 18.89	2.41 ± 0.76	1.48 ± 0.41
111 Cd	µg/kg	14.07 ± 1.34	9.91 ± 1.54	9.47 ± 1.28
118 Sn	µg/kg	684.7 ± 24.55	697.8 ± 26.94	634.2 ± 13.62
121 Sb	µg/kg	<LOD	<LOD	<LOD
137 Ba	µg/kg	861.2 ± 44.50	2691 ± 177.15	390.6 ± 80.28
201 Hg	µg/kg	<LOD	<LOD	<LOD
205 Tl	µg/kg	34.15 ± 29.97	7.15 ± 1.61	9.34 ± 0.80
Pb*	µg/kg	19.53 ± 0.89	21.48 ± 5.91	6.70 ± 1.10

*All elements were acquired in He mode (enhanced He for P, S, As, and Se). *Pb was measured as the sum of the three most abundant isotopes, 206, 207, and 208.*

Quantitative results and spike recoveries for cell-based samples

Method blank, quantitative results for liquid cell culture media, spent media, and conditioned spent media samples, and spike recovery data (FMB and FAP) are given in Tables 7, 8, and 9. Differences in the concentration of some elements measured in the same sample types (Table 8 and 9) were observed since the samples were from different batches of the cell culture media. The FBS in the media is a likely source of variation (for example, the high concentration of Pb in Sample 3), as it is a complex, mostly undefined, and variable product, which works well for cell culture. Research is underway to find a cost-effective way to replace FBS with plant-based ingredients for cultivated meat cell cultures.

To test for nonspectral interferences (matrix effects), two FMBs were prepared by spiking the blank at 10, 1000, or 4000 ppb. The 1000 ppb-level spikes were used for Na, Mg, Ca, and Fe and the 4000 ppb spikes for P, S, and K. The low-level spike was used for the remaining trace elements. The FMB was analyzed periodically throughout the entire sample run. All recoveries were within the EAM 4.7 method acceptable % recovery range of 90–110%, as shown in Table 7.

A spike recovery (FAP) test was carried out to check the accuracy of the 7850 ICP-MS method for the analysis of liquid cell culture media. Eight culture media samples were spiked with trace elements at 10 ppb and major elements at 1000 ppb. The recoveries for all elements in the cell culture media samples were within the EAM 4.7 method QC criteria of ±20%, as shown in Tables 7 to 9.

Table 7. Method blank, quantitative results (n=9), and spike recoveries for media liquid samples.

		Method Blank			Liquid Cell Culture Media			
					Sample 1		Sample 2	
	Conc Unit	Method Blank Conc	Recovery Low Spike (%)	Recovery High Spike (%)	Culture Media Liquid (No Cells)	Recovery (%)	Culture Media Liquid (No Cells)	Recovery (%)
11 B	µg/kg	7.472	-	93	<LOD	103	<LOD	96
23 Na	mg/kg	15.98	-	108	2152	*	9913	*
24 Mg	mg/kg	<LOD	-	105	11.63	104	36.34	104
27 Al	µg/kg	0.448	106	101	<LOD	113	<LOD	109
31 P	mg/kg	<LOD	-	96	32.77	104	137.42	106
34 S	mg/kg	<LOD	-	**	<LOD	101	<LOD	98
39 K	mg/kg	<LOD	-	105	166.8	99	691.5	80
43 Ca	mg/kg	<LOD	-	102	30.92	102	125.1	99
47 Ti	µg/kg	<LOD	102	-	<LOD	98	<LOD	102
51 V	µg/kg	<LOD	106	-	<LOD	102	<LOD	101
52 Cr	µg/kg	<LOD	106	-	<LOD	101	<LOD	100
55 Mn	µg/kg	<LOD	-	98	<LOD	103	<LOD	101
56 Fe	mg/kg	0.859	-	106	0.380	107	2.044	107
59 Co	mg/kg	<LOD	105	-	<LOD	101	0.065	101
60 Ni	µg/kg	<LOD	106	-	<LOD	102	<LOD	104
63 Cu	µg/kg	0.053	-	107	<LOD	105	0.104	105
66 Zn	µg/kg	<LOD	-	103	<LOD	104	1.265	112
75 As	µg/kg	<LOD	104	-	<LOD	101	<LOD	102
78 Se	µg/kg	<LOD	104	-	<LOD	99	<LOD	96
88 Sr	µg/kg	<LOD	95	-	<LOD	105	<LOD	106
95 Mo	mg/kg	<LOD	105	-	<LOD	102	0.011	103
107 Ag	µg/kg	<LOD	-	102	<LOD	81	<LOD	82
111 Cd	µg/kg	<LOD	106	-	<LOD	105	<LOD	106
118 Sn	µg/kg	3.580	91	-	<LOD	100	<LOD	103
121 Sb	µg/kg	<LOD	104	-	<LOD	106	<LOD	106
137 Ba	µg/kg	<LOD	103	-	<LOD	104	<LOD	104
201 Hg	µg/kg	<LOD	101	-	<LOD	115	<LOD	101
205 Tl	µg/kg	<LOD	98	-	<LOD	104	<LOD	105
Pb***	µg/kg	<LOD	104	-	<LOD	104	<LOD	105

*All elements were acquired in He mode (enhanced He for P, S, As, and Se). *Spike level too low compared to native concentration, **below calibration range. ***Pb was measured as the sum of the three most abundant isotopes, 206, 207, and 208.*

Table 8. Quantitative results (n=9) and spike recoveries for spent media liquid samples incubated for 21 days.

		Spent Media After Being Used to Culture Primary Embryonic Chicken Muscle Precursor Cells for 21 days					
	Conc Unit	Sample 6	Recovery (%)	Sample 7	Recovery (%)	Sample 8	Recovery (%)
11 B	µg/kg	<LOD	96	<LOD	103	<LOD	102
23 Na	mg/kg	1221	*	2228	*	6541	*
24 Mg	mg/kg	8.333	106	11.88	106	27.93	106
27 Al	µg/kg	<LOD	94	<LOD	111	<LOD	108
31 P	mg/kg	24.35	105	31.08	106	89.59	107
34 S	mg/kg	<LOD	100	<LOD	102	<LOD	104
39 K	mg/kg	102.5	82	154.1	104	446.7	108
43 Ca	mg/kg	48.99	108	25.09	105	79.14	108
47 Ti	µg/kg	<LOD	104	0.062	102	0.081	103
51 V	µg/kg	<LOD	102	<LOD	101	<LOD	102
52 Cr	µg/kg	<LOD	102	<LOD	103	<LOD	102
55 Mn	µg/kg	<LOD	104	0.014	103	<LOD	103
56 Fe	mg/kg	14.03	107	0.371	107	0.837	107
59 Co	mg/kg	<LOD	102	<LOD	101	<LOD	102
60 Ni	µg/kg	0.030	102	<LOD	102	<LOD	102
63 Cu	µg/kg	1.145	109	<LOD	106	<LOD	105
66 Zn	µg/kg	5.212	98	<LOD	105	0.423	105
75 As	µg/kg	3.724	100	6.749	100	6.463	100
78 Se	µg/kg	<LOD	98	<LOD	98	<LOD	99
88 Sr	µg/kg	<LOD	105	<LOD	104	<LOD	104
95 Mo	mg/kg	<LOD	102	<LOD	102	<LOD	103
107 Ag	µg/kg	<LOD	80	<LOD	80	<LOD	80
111 Cd	µg/kg	<LOD	104	<LOD	104	<LOD	104
118 Sn	µg/kg	<LOD	100	<LOD	99	<LOD	99
121 Sb	µg/kg	<LOD	104	<LOD	105	<LOD	105
137 Ba	µg/kg	<LOD	105	<LOD	104	<LOD	103
201 Hg	µg/kg	<LOD	117	<LOD	114	<LOD	119
205 Tl	µg/kg	3.813	104	4.235	104	6.944	104
Pb	µg/kg	228.7	100	0.058	104	<LOD	105

*All elements were acquired in He mode (enhanced He for P, S, As, and Se). *Spike level too low compared to native concentration. **Pb was measured as the sum of the three most abundant isotopes, 206, 207, and 208.*

Table 9. Quantitative results (n=9) and spike recoveries for conditioned media liquid samples incubated for 14 days.

		Spent Media After Being Used to Culture Primary Embryonic Chicken Muscle Precursor Cells for 14 days					
	Conc Unit	Sample 6	Recovery (%)	Sample 7	Recovery (%)	Sample 8	Recovery (%)
11 B	µg/kg	<LOD	103	<LOD	103	<LOD	104
23 Na	mg/kg	3457	*	1774	*	2378	*
24 Mg	mg/kg	18.08	107	9.834	106	14.39	106
27 Al	µg/kg	<LOD	114	<LOD	105	<LOD	109
31 P	mg/kg	46.33	109	23.92	106	39.31	107
34 S	mg/kg	<LOD	105	<LOD	103	<LOD	106
39 K	mg/kg	232.3	106	126.9	105	159.7	104
43 Ca	mg/kg	42.79	110	20.72	104	32.27	104
47 Ti	µg/kg	<LOD	103	<LOD	104	<LOD	105
51 V	µg/kg	<LOD	103	<LOD	102	<LOD	102
52 Cr	µg/kg	<LOD	102	<LOD	103	<LOD	101
55 Mn	µg/kg	<LOD	104	<LOD	103	<LOD	102
56 Fe	mg/kg	0.683	108	0.280	107	0.794	106
59 Co	mg/kg	<LOD	103	0.013	102	0.015	101
60 Ni	µg/kg	<LOD	104	<LOD	104	<LOD	104
63 Cu	µg/kg	0.046	107	0.039	107	0.033	106
66 Zn	µg/kg	0.404	110	<LOD	106	<LOD	107
75 As	µg/kg	<LOD	103	<LOD	101	<LOD	100
78 Se	µg/kg	<LOD	101	<LOD	98	<LOD	99
88 Sr	µg/kg	<LOD	107	<LOD	106	<LOD	105
95 Mo	mg/kg	<LOD	104	<LOD	104	<LOD	103
107 Ag	µg/kg	<LOD	82	<LOD	82	<LOD	81
111 Cd	µg/kg	<LOD	106	<LOD	105	<LOD	106
118 Sn	µg/kg	<LOD	103	<LOD	102	<LOD	99
121 Sb	µg/kg	<LOD	106	<LOD	106	<LOD	104
137 Ba	µg/kg	0.407	106	<LOD	104	<LOD	104
201 Hg	µg/kg	<LOD	110	<LOD	108	<LOD	111
205 Tl	µg/kg	3.117	106	2.220	105	2.659	105
Pb**	µg/kg	0.381	106	<LOD	105	<LOD	105

*All elements were acquired in He mode (enhanced He for P, S, As, and Se). *Spike level too low compared to native concentration. **Pb was measured as the sum of the three most abundant isotopes, 206, 207, and 208.*

ISTD recovery (%)

The analytical sequence outlined in Figure 1 was analyzed repeatedly over 48 hours. All the ISTD recovery plots were within ±20%, with no internal standard failures throughout the run, meeting the criteria specified in EAM 4.7 (Figure 3). The results demonstrate the robustness of the 7850 ICP-MS plasma and high matrix tolerance of the system with UHMI over long runs.

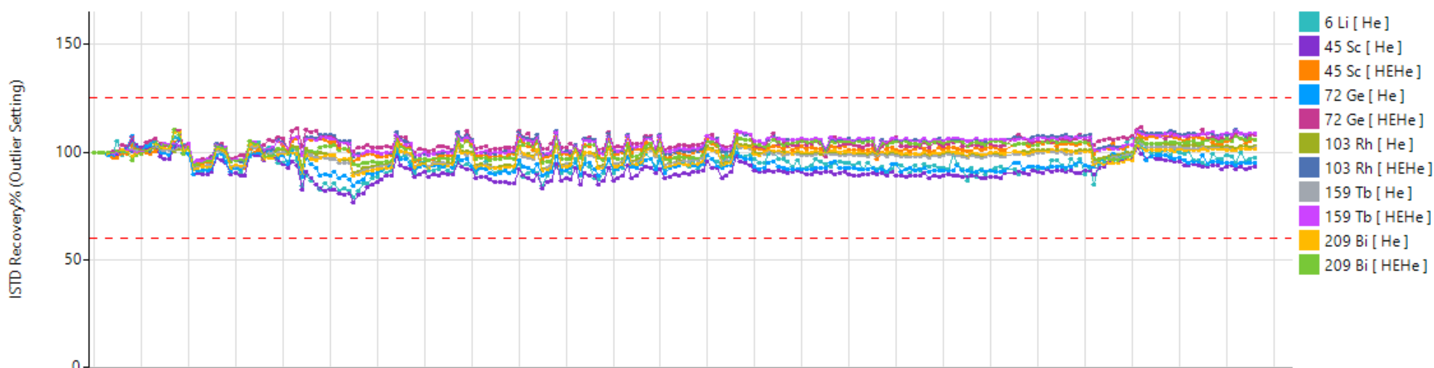

Figure 3. Stability of ISTD measurements over 48 hours. The ISTD recoveries have been normalized to the calibration blank for all samples.

IntelliQuant data

When an analyst develops a quantitative method using an ICP-MS MassHunter preset method, an IntelliQuant Quick Scan acquisition is predefined in the He mode tune step. No special setup or separate calibration is needed for IntelliQuant, simplifying the analysis. IntelliQuant automatically acquires full mass-spectrum data in every sample with only two seconds additional measurement time, allowing the analyst to quickly see which elements are present in the samples. Because IntelliQuant data is acquired in He collision cell mode, analytes are free from common polyatomic ion overlaps, ensuring the quality of the data.

In this study, IntelliQuant data was acquired for each plant-based food sample and SRM with the 7850 ICP-MS operating in He mode. The data can be displayed in a periodic table heat map view, as shown for the plant-based "minced beef" sample in Figure 4. The color intensity heat map shows the approximate concentration of up to 78 elements in each sample, with a darker color indicating a higher concentration of an element. The IntelliQuant data is a quick and simple way to get an overview of the elemental content of a sample and identify the presence of any unexpected elements.

Figure 4 shows that the plant-based "minced beef" sample contained a relatively high concentration of Rb. Rb wasn't calibrated as part of the quantitative study, so the natural isotope template feature of IntelliQuant was used to check the Quick Scan spectrum to confirm its identity. Figure 5 shows a good fit to the natural isotope template for Rb, confirming its presence in the sample.

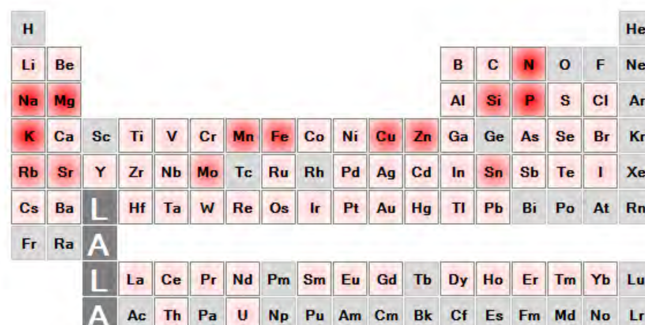

Figure 4. Periodic table heat map view of ICP-MS IntelliQuant data acquired for the plant-based "minced beef" sample.

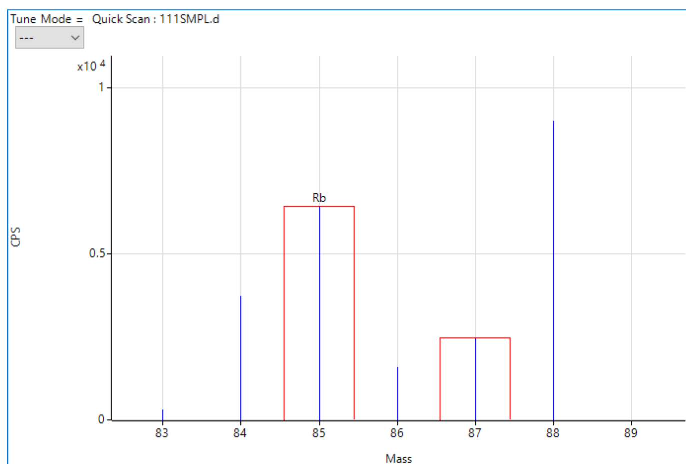

Figure 5. The unexpectedly high concentration of Rb in the plant-based minced beef sample was confirmed using the isotope template fit function in the IntelliQuant Quick Scan mass spectrum.

Conclusion

The Agilent 7850 ICP-MS was used to analyze 30 elements in a range of plant-based protein foods and 29 elements in a range of cell culture media. The analysis was done in accordance with US FDA EAM method 4.7 for food and related products and included the 12 elements specified in the 4.7 method. All the food samples were prepared in the same batch using a single microwave digestion method, while the cell media samples were simply diluted before analysis.

The 7850 ICP-MS method was predefined based on a previous EAM 4.7 food analysis batch, and the instrument was autotuned, saving development time. All elements were measured using a single data acquisition mode, with effective removal of polyatomic interferences ensured by operating the ORS[4] collision cell in He-KED mode. Also, as part of the quantitative method using He-KED mode, IntelliQuant data was acquired for each sample. The IntelliQuant data for the plant-based "minced beef" sample was displayed as a heat map of the periodic table, showing approximate concentration ranges for each of the measured elements. IntelliQuant's isotopic template was used to confirm the identity of uncalibrated elements such as rubidium in the plant-based "minced beef" sample.

The accuracy of the quantitative method was evaluated by analyzing four food-based SRMs and conducting a spike recovery test of the plant-based "beef" sample. Excellent recoveries were achieved for both tests, within EAM 4.7 method QC criteria of ±10% and ±20%, respectively. The 7850 ICP-MS exceeded the nominal detection limit requirements specified in the EAM method. Also, using the UHMI aerosol dilution technology, the 7850 showed excellent stability over a 48-hour ISTD recovery run, demonstrating the robustness of the method.

The same 7850 ICP-MS method was also used to analyze various liquid cell culture media samples and spiked samples. Good spike recovery data was achieved for eight samples, confirming the suitability of the method to support the development of cultured meat products—a growth market in the food industry.

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