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Current Trends in Food and Beverage Analysis eBook

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This e-book examines some of the current trends in food and beverage analysis from experienced industry experts and in-house specialists from Thermo Fisher Scientific

Analysis of a variety of matrices are discussed, from rice to beer, and each article features a different analytical technique.

In the first article, Dr. Antonio Signes-Pastor, Queens University Belfast and Dr. Simon Nelms, Thermo Fisher Scientific tell us about Applying ICP-MS to Speciation and Quantitative Analysis of Arsenic in Foodstuffs and Beverages. From them we learn about the recent EU regulations regarding arsenic in rice based foodstuffs, the latest developments in arsenic speciation using IC-ICP-MS and the performance of ICP-MS for total arsenic measurement in a selection of food samples.

In the second article, Dr. Elena Ciceri, Thermo Fisher Scientific, discusses the Direct Analysis of Food and Beverages using SPME-GC-MS/MS, a technique that uses no sample cleanup, is automated and highly specific. Dr. Ciceri explains how to optimize and validate a simple, inexpensive, and rapid method to confirm and identify biologically active flavorings using automated headspace solid-phase microextraction coupled with GC-MS/MS.

The third article is focussed on Prebiotic Ingredient Characterizations using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD). Here, Dr. Parul Angrish and Dr. Manali Aggrawal both from Thermo Fisher Scientific, explain the key structural features of prebiotics and outline the best technique for the analysis of prebiotics in routine monitoring or research applications.

In the final article, Aaron McLeod the Director at Hartwick College Center for Craft Food and Beverage, USA, educates us on new approaches for automation of malt chemistries and potential benefits and cost-efficiencies of discrete analysis methods.

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EXECUTIVE OVERVIEW

Applying ICP-MS to Speciation and Quantitative Analysis of Arsenic in Foodstuffs and Beverages

by Antonio Signes-Pastor,[1] Manus Carey,[1] Andy Meharg[1] and Simon Nelms,[2]

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In this summary, the latest developments in arsenic speciation using IC-ICP-MS will be discussed together with an overview of total arsenic analysis using ICP-MS for a selection of food and beverage samples.

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Introduction

Arsenic is a ubiquitous element present in the environment as both inorganic and organic species with different oxidation states. Inorganic arsenic is mainly found as arsenite (AsIII) and arsenate (AsV), which has been classified as a Group 1 carcinogen by the International Agency for Cancer Research. Other detrimental health effects have also been attributed to inorganic arsenic exposure such as neurological, cardiovascular, respiratory and metabolomics diseases, and early life exposure has a marked effect throughout the lifespan even at low to moderate levels. In general, organic arsenic is considered non-toxic and comprises several compounds such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide, tetramethylarsonium, arsenocholine, arsenobetaine, aresenosugars amongst others.

Seafood products contain high concentration of total arsenic, most of which is organic arsenic especially arsenobetaine, which is a non-toxic and excreted unchanged in urine. Other organic species found in seafood, such as arsenosugars and arsenolipids, are metabolized predominantly to DMA before being excreted. In contrast, rice contains DMA - a much higher inorganic arsenic compared with other cereals such as wheat, barley and other foods in general and the inorganic arsenic level in rice bran is usually commercialized as super food are extremely high and can reach concentrations greater than 1 ppm / 1 mg/kg. The question is why does rice have such a high content of inorganic arsenic compared with other foodstuffs? Rice accumulates higher inorganic arsenic because of the nature of the plant and to the anaerobic paddy field flooded conditions, which renders inorganic arsenic highly available for plant uptake.

High concentrations of inorganic arsenic have also been found in rice-based products such as rice milk, baby rice, rice cereals, rice cakes, soups etc. and the positive correlation between rice content and the inorganic arsenic concentration has been reported. Rice is the world's major dietary staple and accounts for more than 50% of the inorganic arsenic consumed. The levels of inorganic arsenic in rice are such that they are expected to give rise to excess cancer rates in high rice consumers.

The first analytical technique for arsenic analysis was developed back in 1836, the Marsh test used in forensic analysis to test for arsenic poisoning. A more recent technique to determine total arsenic uses hydride generation atomic spectroscopy. This is a robust technique and can be used for arsenic speciation when coupled with chromatographic systems. However, the most effective analytical technology for arsenic speciation, because of its high selectivity and is chromatography coupled to inductively coupled-mass spectroscopy (ICP-MS).

Regulation

The European Union withdrew the provisional weekly tolerable intake of 15 µg/kg body weight established by the Joint FAO/WHO Expert Committee on Food Additives in 2009. This followed reports that lung, bladder and skin cancer, as well as a range of other adverse effects were observed at inorganic arsenic exposure lower than these benchmarks. Consequently, dose lower confidence limit values between 0.3 and 8 µg/kg body weight were established for cancers of the lung, skin and bladder, as well as skin lesions.

In order to reduce exposure, the EU has formulated regulations on the maximum levels of inorganic arsenic in rice, that were written into EU law on 3 January, 2016. The application of analytical techniques, such as chromatography coupled with ICP-MS for arsenic speciation in rice and rice-based products is crucial in order to ensure compliance to the regulations. The new EU regulations establish four maximum levels of total inorganic arsenic, including for arsenite and arsenate. The maximum level of 0.2 µg/kg has been established for non-parboiled milled rice (polished or white rice), and the level of 0.25 µg/kg has been established for parboiled rice and husked rice. The level of 0.30 µg/kg has been established for rice waffles, rice wafers, rice crackers and rice cakes. Finally, the most restricted maximum level of 0.1 µg/kg has been established for rice destined for the production of food for infants and young children, because compared with adults, young children less than four years of age are more sensitive to the adverse health effects of inorganic arsenic. Additionally, young children have intakes of food two to threefold higher on a body weight basis when compared with adults.

The US Food and Drug Administration (FDA) has also recently proposed the maximum level of 0.1 µg/kg for infant rice cereals.

Developing Optimal Protocols for Arsenic Speciation in Rice

When applying chromatography ICP-MS techniques for arsenic speciation it is important to develop optimal protocols that provide effective extraction of the arsenic species from the rice and other foodstuff samples, good species separation, low limits of detection (LODs) and inclusion of authentic standards and CRMs for quality control.

The protocol developed for arsenic speciation begins with powdered rice samples weighed accurately to 0.1 g into 50 mL polypropylene centrifuge tubes to which 10 mL of 1% concentrated nitric acid is added and allowed to sit overnight. Samples are microwave digested where temperature is first increased to 55 °C in 5 minutes and then held for a further 10 minutes. Temperature is then increased to 75 °C in 5 minutes and held for 10 minutes. Finally, temperature is increased to 95 °C in 5 minutes and held for 30 minutes. The digestate is cooled at room temperature and centrifuged at 4500 g. Finally, a 1 mL aliquot is transferred to a 2 mL polypropylene vial and 10 µL of analytical-grade hydrogen peroxide is added to convert any arsenite to arsenate.

Addition of hydrogen peroxide before analysis with chromatography ICP-MS is a very important step because it is total inorganic arsenic that is of interest and errors related to arsenic species separation needs to be avoided. This may occur because when using anion exchange chromatography on an HPLC system, arsenite elutes at the solvent front potentially co-eluting with cation arsenic species and when using ion chromatography, arsenite and DMA have similar elution times.

Species Separation: Figure 1 shows two examples of arsenic speciation using anion exchange chromatography on HPLC-ICP-MS without addition of hydrogen peroxide. The American brown rice sample shows that arsenite, arsenic III and DMA have similar proportions. The second chromatogram shows a long grain sample with a higher concentration of arsenite, but also containing DMA and arsenate. A good separation of arsenite and DMA is obtained with this technique, but the shape of the peaks is not well defined, especially at low concentration of arsenic species.

Figure 2 shows another example of anion exchange chromatography ICP-MS analysis. The chromatogram of the rice sample does not show any arsenite peak because it is oxidized to arsenate with the hydrogen peroxide. However, an unknown peak at the solvent front was found that could potentially co-elute with arsenite if hydrogen peroxide is not added, which would cause an over estimation of the arsenite concentration.

To identify the unknown peak eluted at the solvent front, cation exchange chromatography ICP-MS analysis was performed. We found that the unknown peak co-elutes with tetramethylarsonium (tetra). The other cation arsenic species, such as arsenocholine and trimethylamine oxide, had significantly different retention times. Therefore, it was possible to certify that the unknown peak was tetramethylarsonium and not arsenite. When using anion chromatography ICP-MS the DMA and the arsenite peak co-elute with similar retention times near to 200 seconds. However, we can see very well defined peaks – better than those obtained with anion exchange chromatography on HPLC -ICP-MS. When overlapping the arsenite and DMA standard chromatograms the co-elution of these two arsenic species is more evident and shows that it is not possible to integrate the peaks (Figure 3).

Figure 1

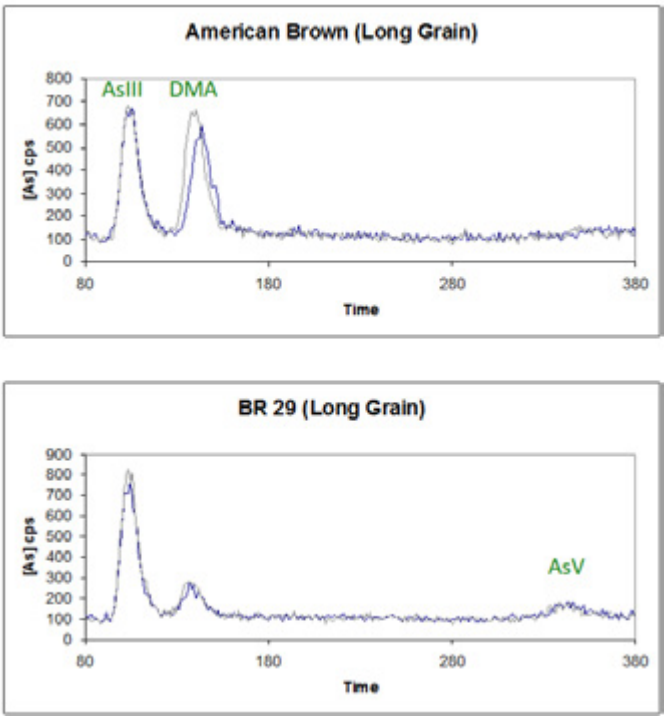


Figure 2

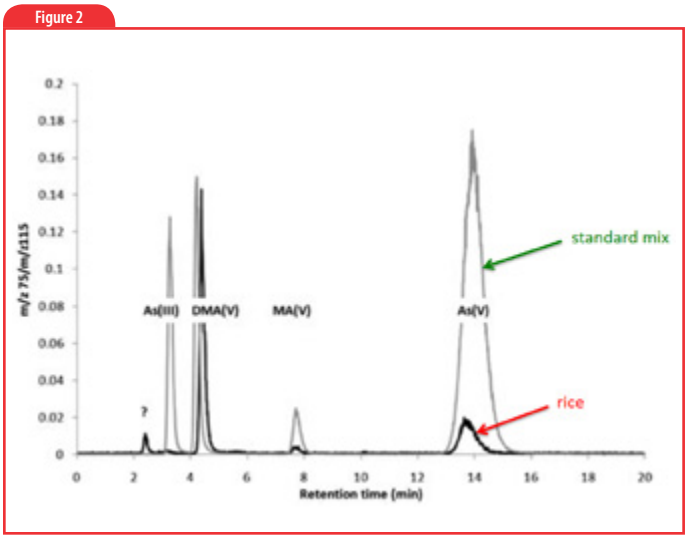


Figure 3

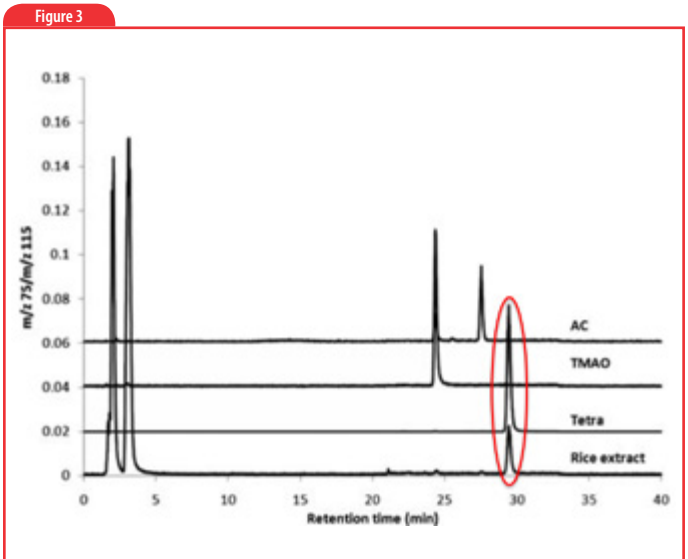


Figure 4

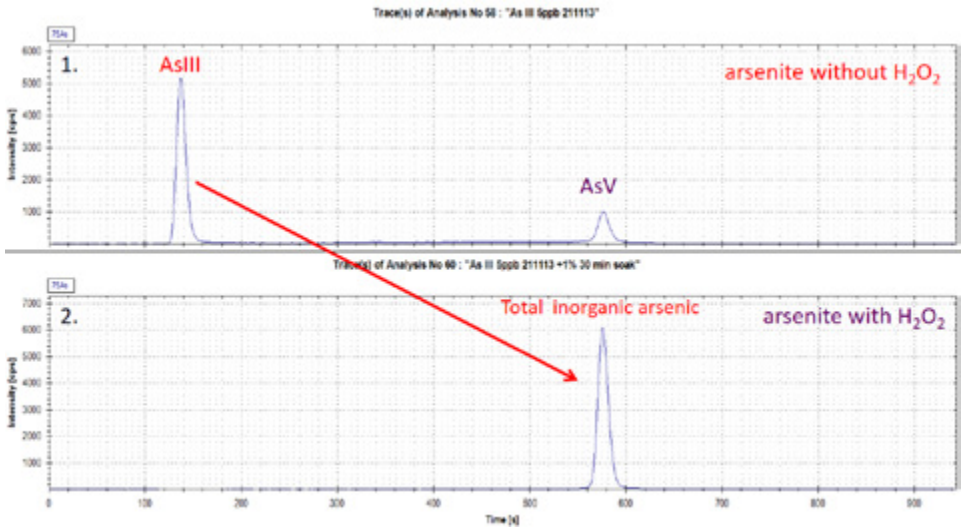


Figure 5

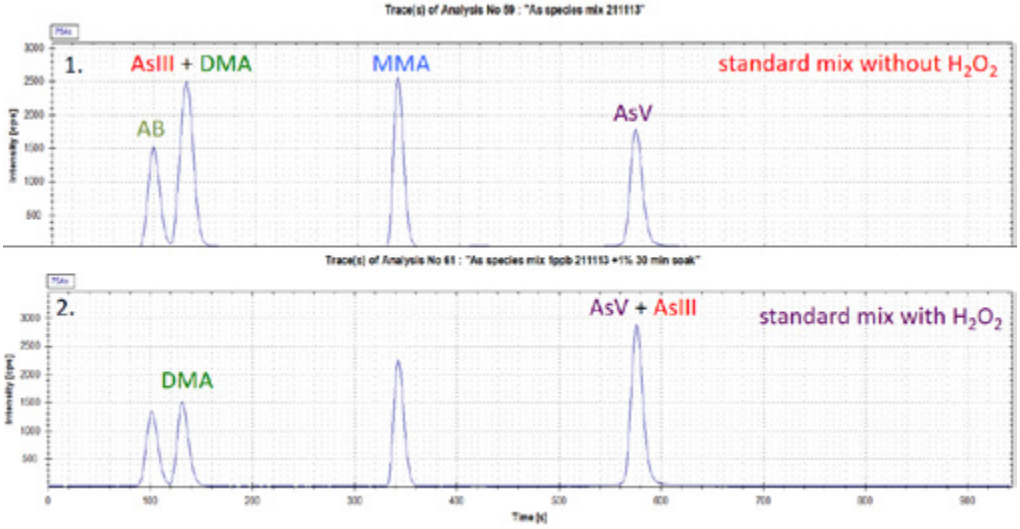
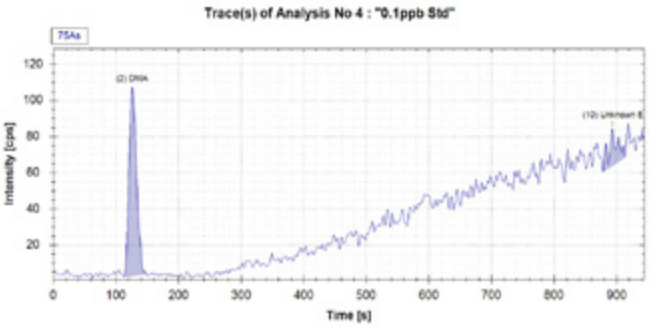


Figure 4 shows an arsenic, arsenite III and arsenate arsenic V standard chromatogram obtained with ion chromatography ICP-MS when hydrogen peroxide was not added. There is a clear separation between these two inorganic arsenic species in the first chromatogram. When adding hydrogen peroxide all the arsenite is oxidized to arsenate, which represents the total inorganic arsenic shown in the second chromatogram.

Figure 5 shows a standard mix chromatogram obtained with ion chromatography ICP-MS. The first chromatogram shows the absence of hydrogen peroxide and arsenite and DMA co-elute. The second chromatogram shows what happens when hydrogen peroxide is added, and all the arsenite, arsenic III is oxidized to arsenate. However, the other arsenic species were not affected by the addition of hydrogen peroxide and remained the same. When overlapping the chromatograms, the effect of adding hydrogen peroxide to a standard mix analyte by ion chromatography ICP-MS. All the arsenite is oxidized to arsenate while the other species are not affected.

Limits of Detection: Figure 6 shows a standard DMA of 0.1 µg/L or 100 ppb chromatogram obtained with ion chromatography ICP-MS. Despite the very low concentration this analytical technique is able to generate a very well defined peak, which highlights the sensitivity detection capabilities of the ion chromatography ICP-MS. The limit of detection (LOD) is calculated as the mean of the blank concentrations plus three times the standard deviation

Figure 6

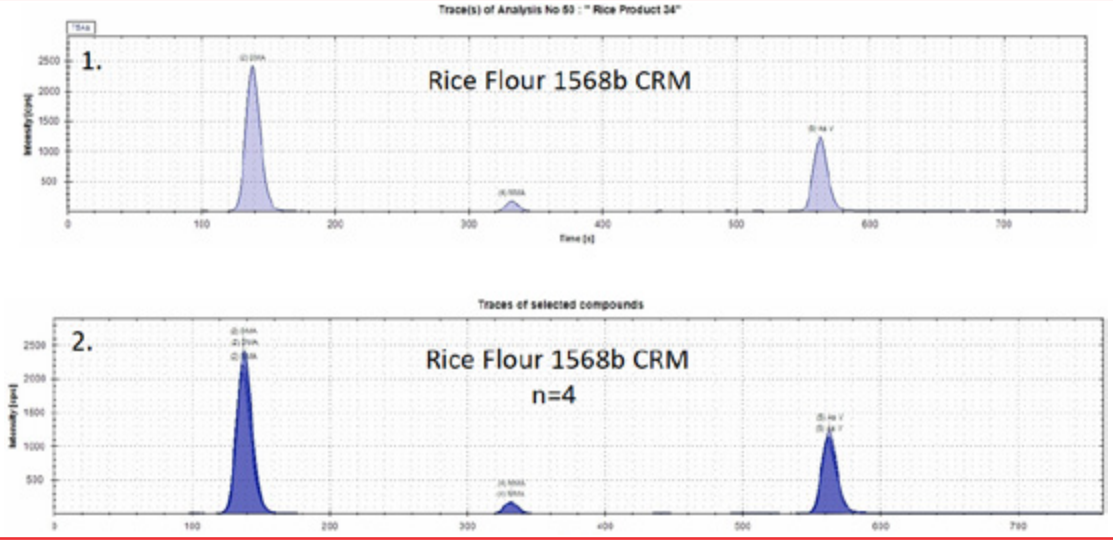


of the blank concentrations multiplied by the dilution factor.

Here an example of LOD calculated from DMA that we have obtained recently. The LOD was 2.8 µg/kg that equates to an instrumental LOD of 0.03 µg/L. As we can see this shows more than enough sensitivity for both DMA and inorganic arsenic detection in rice.

Authentic Standards: For each batch of samples for arsenic speciation with ion chromatography ICP-MS an authentic standard mix was included to identify and verify retention times and it usually included arsenobetaine, DMA, Tetra, MMA and arsenate.

Figure 7



Certified Reference Materials: Also included were several replicate samples of certified reference material. The rice CRMs that were used are the rice flours and the reference material 1568b commercialized by the National Institute of Standards & Technology. In Figure 7, the first chromatogram is an example of the rice flour 1568b CRM analysis by IC-ICP-MS. The three arsenic species with concentration certified were clearly identified. The second chromatogram shows the overlap of four replicate samples of rice flour 1568b CRM included in a batch of 80 rice-based samples, which took several hours to run. In addition to the three arsenic species with concentration certified DMA, MMA and inorganic arsenic it was observed that IC-ICP-MS was very stable throughout the analysis as the four CRM chromatograms match perfectly.

Matrices: Note that although a protocol has been described for arsenic speciation in rice this can be used for other matrices such as plant materials, soil solution, animal and human tissues, urine etc.

RICENIC Study

This study has determined inorganic arsenic in rice-based products widely consumed by infants and young children. Samples of commercial baby rice, rice cereals and rice crackers belonging to the most popular commercial brands or manufacturers were purchased from 36 food shops in the United Kingdom.

We analysed 29 baby rice samples, 53 rice cereals and 97 rice crackers. Nearly half of the database had levels of inorganic arsenic over 100 ppb, which is the maximum level established for rice destined for the production of food for infants and young children. About 60% of the baby rice and rice crackers exceeded the maximum level of 100 ppb and, therefore, would be illegal. In contrast, only 20% of rice-based cereals had higher inorganic arsenic than 100 ppb - this would not be legal because their packaging did not indicate they are specifically for infants and young children. The new regulation does not specify a tier maximum inorganic arsenic level for them. Furthermore, the EU regulation does not contemplate that young children consume boiled rice and rice-based products that are not labelled as being destined for them, which regularly contains higher inorganic arsenic than 100 ppb and contribute to increasing children's exposure to these toxic elements.

Organic products are usually associated with a healthy, more nutritious option that is increasing production of organic baby foods. We analysed baby rice, rice cereals and rice crackers and products under organic and non-organic standards. We found that organic rice-based products contain significantly higher inorganic arsenic than those produced under non-organic standards. This is because organic products usually include whole grain, which contains much higher inorganic arsenic than polished white rice. Indeed, most of the inorganic arsenic in rice grain is concentrated in the surface layers, which are removed during the polishing process. Therefore, it is advisable to use white rice versus brown rice to feed infants and young children in order to decrease exposure and health risks.

Rice from the Iberian peninsula represents about 36% of the total rice production in Europe. In this study, we explore the geographical variation of inorgonic arsenic in paddy field samples and commercial rice from this region. Samples of soil, rice shoots, grain were collected from the main rice production regions in Spain and Portugal. Total arsenic in soil solution was analysed using direct ICP-MS. Arsenic speciation in rice grain and shoots was analysed using IC-ICP-MS.

Although paddy field soil contained low to moderate total arsenic concentration, rice shoots contain a median of 2.7 µg/kg and about 26% of all commercial rice analysed had a concentration of inorganic arsenic of greater than 100 ppb. We identified the regions where rice contains lower concentrations of inorganic arsenic, which should be collected along with an adequate processing methodology to produce rice-based products for infants and young children in order to keep the inorganic arsenic as low as possible and below the limit established in all rice-based products destined and consumed by infants and young children.

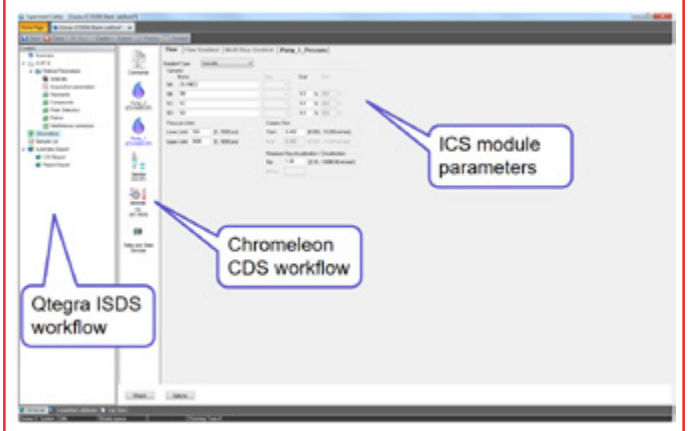
What is EU 2015/1006?

EU 2015/1006 is an amendment to the Commission Regulation 1881/2006, which covers setting maximum levels for certain contaminants in foodstuffs. This regulation includes lead, cadmium, mercury and tin so the new regulation is the addition of inorganic arsenic to this list of elements. It has been developed because inorganic arsenic has been shown to cause cancer (lung, bladder and skin cancer in particular), at exposure levels lower than previously thought. High consumers of rice are mostly exposed to inorganic arsenic through their dietary exposure. Dietary exposure for children less than three years old from rice-based foods is estimated to be about 2-3 times greater than that of adults so it's a serious concern especially for young children.

What levels of inorganic arsenic need to be measured and in what samples? In particular, we're looking at non-parboiled rice (polished or white rice), parboiled rice and husked rice and things such as rice waffles, rice wafers, rice crackers and so on so anything that could be ingested in dietary form of rice products so particularly important for children and babies. So the question is if arsenic is present in your food, how much of it is actually inorganic arsenic? If you just measure total arsenic it could give you a false answer, which could mean that food could be wasted that is actually safe to consume. So as well as health implications there are also economic implications.

Figure 8

Figure 9



Instrumentation

So how can you separate inorganic arsenic such as (As (III) and AS (V)) from other arsenic species in the sample? We have shown some of the applications using IC already and we can apply speciation using chromatography techniques coupled to ICP-MS.

Figure 8 shows a couple of systems we can use for this analysis, being the Thermo Scientific Dionex ICS-5000 system for separation and Thermo Scientific iCAP RQ system for detection. In terms of software we ideally want a system that can run the IC or HPLC with the ICP-MS in an integrated way allowing unattended collecting data. The way we do this is to use the Qtegra intelligent scientific data solution software. This works initially by creating a lab book, which is configured to contain the method information, the IC method, any QA/QC protocol information, the sample list, results, report, and audit trail all in one lab book.

In terms of the method set up Figure 9 shows the workflow on the left-hand side going through the parameters that need to be implemented for the analysis, we have the Chromeleon workflow system for editing, in our case, the ion chromatography method parameters and we have the module parameters for the ICS system displayed there. These are easy to interact with and change for a single lab book environment. We can configure the autosampler and we can configure the sample list. We can change conditions including chromatographic duration on a sample-by-sample basis, sample vial location, injection volume and so on. We also have manual control features for priming the pump, for example, or controlling the autosampler. The system then allows us to go on to process the data within the lab book environment once the data has been collected, with peak integration routines built into the system so that we can calculate peak areas with various smoothing routines.

Subsequently after picking off each individual peak from the peak search routine in the software, we can generate the calibration and generate all the sample data ready for exporting and processing and reporting (Figure 10).

Figure 10

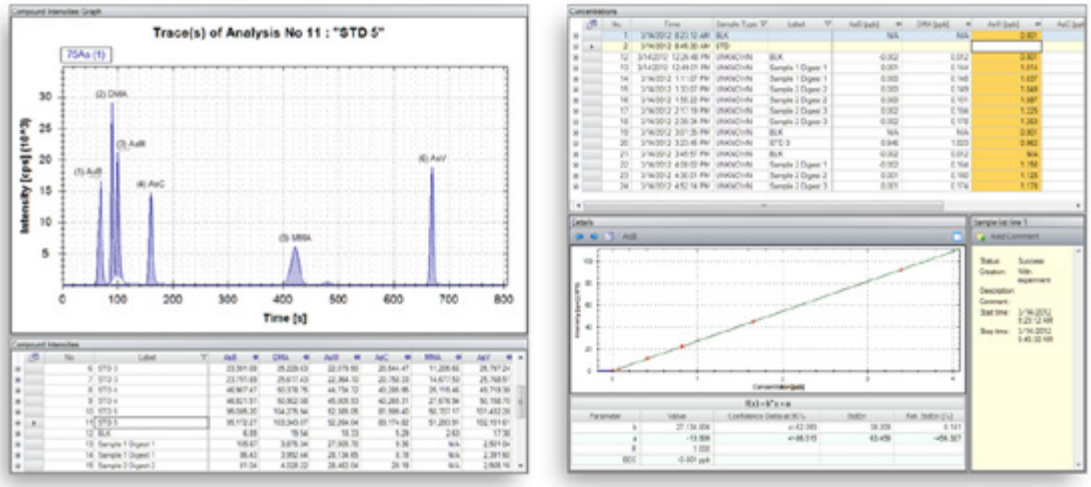
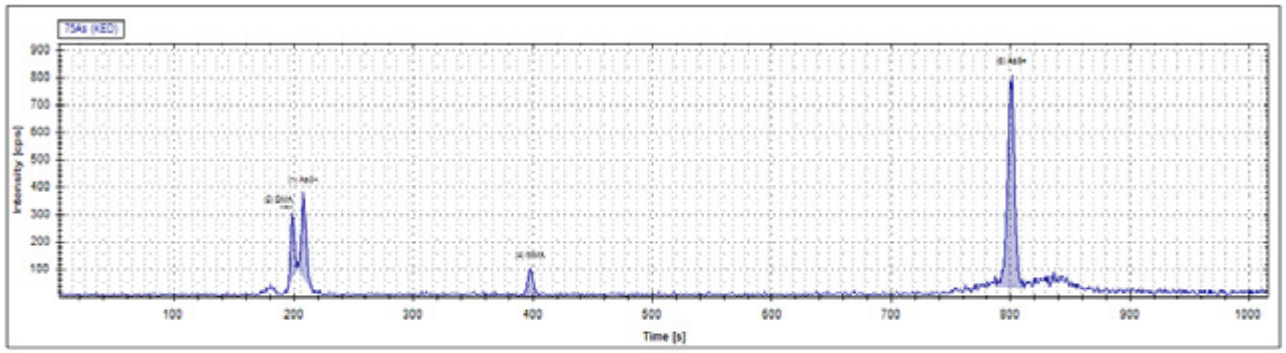
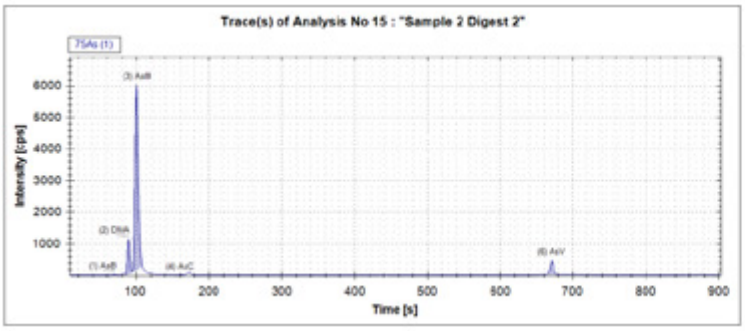


Figure 11



Sample i.d.	DMA [ng g^{-1}]	As (III) [ng g^{-1}]	MMA [ng g^{-1}]	As(V) [ng g^{-1}]	Sum [ng g^{-1}]	Total As [ng g^{-1}]
Juice 2	Not detected	0.5 ± 0.01	Not detected	0.8 ± 0.01	1.3	1.7 ± 0.05
Juice 3	0.4 ± 0.05	0.3 ± 0.01	0.1 ± 0.05	0.7 ± 0.01	1.5	1.8 ± 0.05

Figure 12



Species distribution %	DMA	As III	As V	Sum
Sample 1	12%	82%	6%	100%
	12%	82%	6%	100%
	12%	81%	7%	100%
Sample 2	12%	83%	5%	100%
	12%	83%	5%	100%
	12%	82%	6%	100%
Sample 3	10%	84%	6%	100%
	10%	87%	3%	100%
	11%	86%	3%	100%

Application Examples

The first example is arsenic speciation in apple juice. This has come about because high levels of arsenic have been reported in apple juice in the US in recent years. Analysis by the FDA has shown concentrations in the range of 2-6 ppb in apple juices measured. The important thing is to distinguish between the different forms of arsenic because we know that inorganic As (III) and AS (V) are particularly toxic whereas organic forms such as arsenobetaine are considered to be less toxic or not toxic. The US EPA specifies that total arsenic is considered to be at a level less than 10 ng/g for drinking water. The FDA are reviewing the data in the light of recent scares and are considering setting a guidance level for inorganic arsenic in apple juice.

In this work IC-ICP-MS was used to determine the concentration of six arsenic species: the two inorganic species As (III) and As (V) and the four organic species listed here: arsenobetaine, arsenocholine, monomethylarsonic acid (MMA) and dimethylarsinic (DMA).

For the work, three different apple juices were analysed for total arsenic. Two of them were positive in terms of the concentration of arsenic detected. The types of samples analysed were a retail brand, a supermarket discount brand and a regional manufacturer. For sample preparation, 1 mL juice was diluted in 2 mL of 2% nitric acid plus 7 mL deionised water so effectively a ten times dilution for the speciation work. Juice 2 and 3, where the arsenic levels were detected - the results are shown in Figure 11. We can see As (III) at a level of about 0.5 mg/g and As(V) at around about 0.7 / 0.8 mg/g in the two samples – so a detectable concentration. The total arsenic in those samples was around the 2 ppb 2 mg/g concentration. This method enabled low detection limits in the region of 0.001 and up to 0.01 ng/g in the different species and a detection level for total arsenic was down to 0.005 ng/g so very very low – way lower than the required legislative level. Just as a reminder, the current EPA maximum contaminant level is 10 ng/g for drinking water.

The second application example is arsenic in organic brown rice syrup. Organic brown rice syrup is commonly used as a sweetener for toddler formulas and cereals. It is considered to be a healthier alternative to fructose containing corn syrups and the marketing tends to be directed to the organic food market for customers who are particularly aware of healthy living. There is an onus on that group of individuals who like to consider that they are heating healthy so the detection of arsenic in the food is obviously a major consideration. It's also used in cereal bars or energy products for endurance athletes as they may contain OBRS as a major ingredient in some cases.

For this analysis, three different samples were sourced in Germany. Total arsenic determination was carried out after closed vessel microwave digestion and you can see the sample preparation displayed there: 0.5 g sample digested in half concentrated nitric acid for about 10 minutes at 180 °C. The sample was diluted subsequently and then further dilution was performed prior to measurement to bring down the acid concentration for the ICP-MS system. All the samples here contained over 100 µg/kg arsenic. Subsequent to the total arsenic analysis, speciation analysis was then conducted for all these samples.

For species extraction we used an open vessel extraction approach- for the exact details of the method see Huang et al., ***J. Anal. At. Spectrom.***, 25(2010), 800-802. After extraction we performed centrifugation and filtration and then a bit of further dilution to bring it ready for analysis by the IC system. The most abundant species detected was As (III) but As (V) was also present. The most abundant organic arsenic species was dimethylarsinic acid. Arsenobetaine was also detected. In this method, the extraction efficiency was determined to be around 75% (Figure 12).

For the third application we examined total arsenic quantification rice flour and chicken reference materials. We also performed multi-element determination with arsenic included in the elemental suite. The samples were prepared by closed vessel microwave digestion. In this case, 0.5 g of sample was digested using a mixture of nitric and hydrocholoric acids. Reference materials were analysed 5 times each during an extended run of 80 other digested food samples to test analysis repeatability.

For the analysis for the rice flour sample, total arsenic was found at a level of around 52 ng/g against a certified level of 49 ng/g so the accuracy was acceptable. For the chicken reference material, the result was 115 ng/g against a certified level of 109 ng/g. For the rice flour analysis, we found a precision of approximately 1.4 % RSD, so good repeatability of the analysis of the sample results throughout the analysis. For the chicken reference materials, a precision of approximately 1% was found, so again good robustness and repeatability.

Summary

We have developed methods based on hyphenation of IC with ICP-MS developed for speciation analysis of arsenic at trace levels in food samples: organic brown rice, syrup and apple juice in particular. We achieved low method detection limits primarily because of the high sensitivity of ICP-MS as a detection system. The narrow chromatographic peaks achieved with the IC system improves the detection in sensitivity as well for species analysis. Low flow-rates also reduce sample- and mobile-phase consumption using the IC approach. We can run through the system at typically 1 mL/min with the mobile phase. The method was also developed for total arsenic quantification in food samples using direct ICP-MS. This method was a single collision cell analysis mode using pure helium gas with kinetic energy discrimination for arsenic and a range of other elements. It demonstrated accurate and precise results with good internal standard robustness during the run so a very straightforward method for food analysis, multi-element analysis as well as arsenic as part of the suite. We can say that legislation for arsenic levels in foodstuffs is being established as we move forward and that is reflected in the development of last year's EU 2015/1006 regulation.

About the Authors

Dr. Antonio Signes is a Marie Curie Post-Doctoral Research Fellow at the Institute for Global Food Security – QUB. His research focuses on arsenic soil-plant transfer and distribution in the food chain, and human exposure. The elevated level of inorganic arsenic, a non-threshold human carcinogen, in rice compared to other foodstuff is of particular interest. He is currently working on the RICENIC project funded by FP7-PEOPLE-2013-IEF, which aims to provide an accurate risk assessment of inorganic arsenic exposure for infants and young children in the European Union and devise feasible strategies to reduce inorganic arsenic burden in rice-based products based on agricultural and manufacturing practices. The usage of state-of-the-art analytical techniques, such as IC-ICP-MS, to carry out arsenic speciation in different biological matrices is crucial in his research. Dr. Antonio Signes has published several publications in the area food science, analytical chemistry, environment, and agricultural sciences.

Simon Nelms is the European Regional Marketing Manager for Thermo Fisher Scientific's trace elemental analysis products. He has been part of the Thermo Fisher Scientific team for almost 20 years, since starting as an ICP-MS applications specialist. His instrumentation experience covers the whole range of trace elemental analysis applications, from food analysis to environmental sample testing. Simon is now responsible for marketing AA, ICP-OES and ICP-MS products in Europe for the food, environmental, pharmaceutical and clinical sectors. He holds a BSc in Analytical Chemistry and a PhD in research involving ICP-MS method development.

ADDITIONAL RESOURCES

Meet the Authors

Read a short interview with the presenters of this webinar.

Poster

A Guide to Trace Elemental Speciation

Application Note

Determination of Inorganic Arsenic in Rice using IC-ICP-MS

Application Note

IC-ICP-MS speciation analysis of As in apple juice using the Thermo Scientific iCAP Q ICP-MS

Application Notebook

Trace Elemental Species Separation and Detection

Brochure

Thermo Scientific iCAP RQ ICP-MS

Journal Article

Inorganic arsenic in rice-based products for infants and young children

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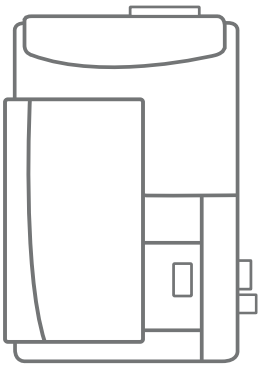

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EXECUTIVE OVERVIEW

Direct Analysis of Food and Beverages using SPME-GC-MS/MS No Cleanup, Automated and Highly Specific

by Elena Ciceri (Thermo Fisher Scientific, Italy)

There is a large number of flavoring substances, which occur in nature and are known to contain undesirable active components. These compounds are called biologically active flavorings. They are used to improve or modify the organoleptic properties of foods. They either alter or enhance the flavors of natural food, or they add flavor to food products that do not have the desired flavor.

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This article will illustrate a GC-MS method developed for the quantitative determination of artificial flavoring in food and beverages.

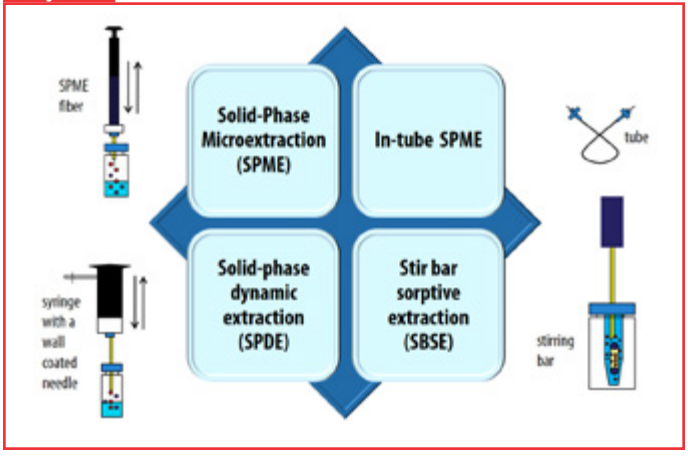
Bioactive Flavorings

These compounds are used to improve or modify organoleptic properties of foods, such as altering or enhancing the flavors of natural food (meats and vegetables), or creating flavor for food products that do not have the desired flavors (candies and other snacks).

There are a large number of flavoring substances that occur naturally and are known to contain undesirable active components. These compounds are called biologically active flavorings. Occasionally, some of these compounds exhibit toxicity to human health and in some instances can even be carcinogenic. Consequently, they are officially regulated to limit the use of the most critical compounds.

How are these compounds treated in the legislation? In 2008 a new regulation was introduced by the EU, which contained two lists; the first containing 15 flavoring substances that are banned from direct food addition in their chemically pure forms, and a second list of 10 compounds that are permitted in food and beverages to stipulated levels, but only where they are naturally occurring. In the US some of these compounds are also regulated, including β-asarone, safrole, thujone and coumarin, where usage is restricted or completely banned.

Figure 1



Examples of naturally occurring biologically active flavorings include estragole, safrole and methyl eugenol (e.g., in fennel, anise, nutmeg, coriander, cinnamon, basil), coumarin (e.g., in Cassia leaf, cinnamon leaf, bark, peppermint oils, vanilla grass, lavender, chicory, tonka bean), pulegone (e.g., in peppermint oil, oregano, beans and teas), thujone (e.g., in wormwood, junipers and sage), β-asarone (e.g., in asarum) and menthofuran (e.g., in peppermint oils).

Sorptive Extraction Techniques

The purpose of this study was to develop a method for the determination of aromatic flavoring in food and beverages. Preparation of the sample is the most time-consuming step having also a big influence on the complete analysis, especially regarding the extraction of analyte and cleanup of extract. As our lab is focused on the development of an easy, fast and reliable method we decided to use a modern extraction technique to facilitate this step. GC-MS was the technique of choice for the analysis of these volatile compounds. As an extraction technique we decided to employ solid phase microextraction (SPME), a solvent extraction technique where no cleanup is necessary.

So what is SPME? This technique belongs to the family of sorptive extraction techniques that are based on the distribution equilibrium of analytes between the sample matrix and a non-miscible liquid phase. A very common option here is polydimethylsiloxane coated on a solid support, such as a fiber. Analytes are extracted from the matrix into the non-miscible extracting phase on the support material (usually a fiber). SPME is commonly used in conjunction with GC, but LC is also possible and used quite often. These sorpive techniques are divided into four groups (Figure 1) – the principles of these techniques is the same, but there are small differences.

SPME is a solvent-free sample preparation technique that uses a fused-silica fiber coated with a prepared stationary phase attached to a modified micro syringe. In-tube SPME uses an alternative to a coated fiber, being an internal coated capillary through which the sample flows. The trapped

Figure 2

Thermo Scientific™ TriPlus RSH™ Autosampler: SPME

Thermo Scientific™ TRACE™ 1310 Gas Chromatograph

Thermo Scientific™ TSQ™ 8000 EVO Triple Quadrupole GC-MS/MS

Thermo Scientific™ TraceFinder™ Software

Auto SRM to develop transitions

analytes are then desorbed and eluted by a solvent. This technique was developed because of the difficulty of interfacing SPME with LC systems. Solid phase dynamic extraction (SPDE) is a recent variant of dynamic in-tube. Stir bar sorptive extraction (SBSE) was developed to overcome the limited extraction capacity of the SPME fiber. A glass stir bar is coated with a thin bonded sorbent layer, usually polydimethylsiloxane, to give a large surface area of stationary phase leading to a higher phase ratio and a better recovery of the sample. Removal of analyte from the bar is achieved either by GC-thermal desorption or elution with an LC solvent. We decided to use SPME in this method optimization.

The principle of SPME is based on a single partition step between sample and sorbent phase. The sorbent phase is often coated on some solid support, such as a fiber. The increasing popularity of this technique is the result of reduced time and increased cost-effectiveness.

SPME was developed by Pawliszyn and co-workers in 1990, and is a solvent-free sample preparation technique using a fused silica fiber coated with a stationary phase attached to a modified microsyringe. The technique enables isolation of volatile and semi-volatile analytes, and only very small amounts of sample are required. It can also be applied to a wide range of matrices. SPME is performed simply by placing the sample into a headspace vial, adding a small amount of water and salt, depending on the method, closing the vial, placing the vial in the autosampler tray of the instrument and starting the sequences. The vial is transferred into a heated oven for incubation to allow the analytes to equilibrate between the liquid and the gas phases. Then, the SPME fiber is inserted into the headspace of the vial leading to analyte enrichment.

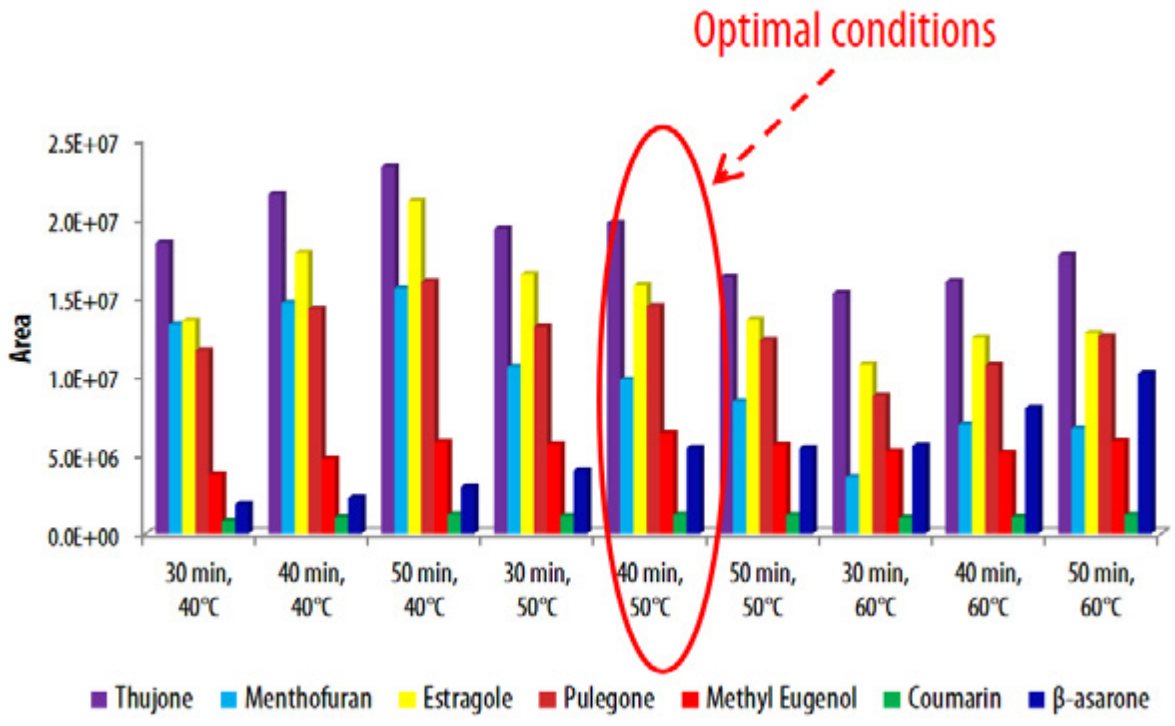
How does it work? The standard liquid syringe tool of the autosampler is replaced by a dedicated SPME holder tool. This tool first takes a vial from the tray and places it into the heating oven. Then, the fiber coated with stationary phase is immersed in the headspace of the vial and the fiber itself is ejected from its sheath. During the extraction period compounds are adsorbed onto the fiber – this stage occurs during heating and shaking of the vial. When the extraction is complete, the fiber is inserted into the hot injection port for desorption of the analytes and injection into the column. This takes a couple of minutes – then the analysis begins. Between analyses the fiber is cleaned at high temperature in the fiber station or in the second unused injection port.

SPME is now probably the most widely used method for food and beverage analysis, offering many benefits over existing headspace techniques. It is used for specific analysis of water (pesticides, PCBs etc.), alcoholic drinks (flavors, volatile patterns etc.), milk and cheese (flavors), juice and soft drinks (phthalates) and honey and preserves (naphthalene, volatile profiles etc.).

For this study we used the Thermo Scientific™ TriPlus RSH™ with SPME as the autosampler, connected to a Thermo Scientific™ TRACE™ 1310 GC coupled to a Thermo Scientific™ TSQ™ 8000 EVO Triple Quadrupole GC-MS/MS (Figure 2). Thermo Scientific™ TraceFinder™ was used for both qualitative and quantitative analysis and the Auto SRM functionality for automatically setting and optimizing the transitions.

One of the goals of the method development was to conform to the official regulations for BAF analysis. This meant that for this method we

Figure 3



needed to include all compounds present in the regulated method list. However, it was not possible to include all the regulated compounds as some were not volatile, and dedicated derivatization was required for them to be suitable for GC-MS analysis. Finally, we worked with a group of seven compounds for which we optimized the SPME parameters:

Choosing the fiber: Because of different polarities of the compounds selection was not straightforward. We chose three types of fiber most suitable for the target compounds and used very commonly in the literature – PDMS, DVB/CAR/PDMS and CAR/PDMS. After some testing we settled on the PDMS fiber, which is suitable for non-polar, high-molecular weight compounds and from our analysis this offered the best recoveries with low carryover.

Vial shaking: A continuous shaking was found to be beneficial for extraction of our compound.

Extraction time and temperature: Temperatures were tested from 40-70 °C, and times from 30-50 mins. As can be seen in Figure 3, 40 mins at 50 °C was found to give optimal conditions for thujone, menthofuran, estagole and pulegone. For β-asarone, greater temperature and longer equilibration is better; however, 40 minutes at 50 °C was considered an acceptable compromise. For coumarin and methyleugenol maximum recovery was achieved under the selected conditions with no improvement at higher temperature and longer extraction times.

Incubation and desorption time of fibre: 2.5, 5 and 10 minutes were tested for both incubation and desorption. For incubation, no difference was found between the times so 5 minutes was chosen because of good results for coumarin and β-asarone, the most problematic compounds. For desorption there was no significant differences in the times. However, 5 minutes was chosen because of good results for coumarin and β-asarone.

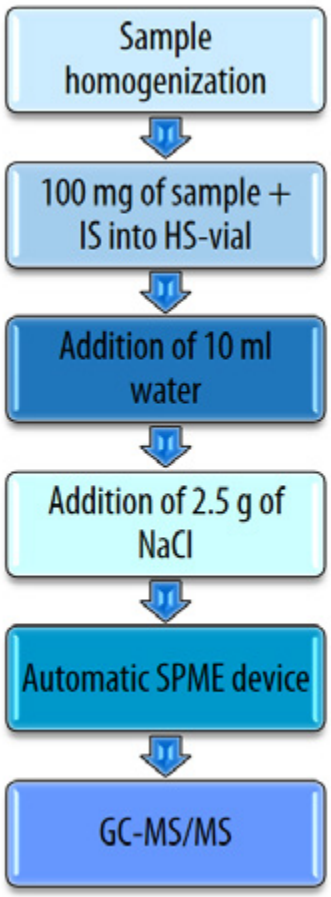
Addition of salt: The sensitivity of the headspace method can be improved by adding soluble salts, such as NaCl, $NaHCO_3$, K_2CO_3 or $(NH_4)_2SO_4$. Addition of the salt reduces the solubility of the analyte in the liquid phase increasing the probability of it in the headspace. 0.2-3.0 g of NaCl were tested and the optimal amount was found to be 2.5 g.

Amount of sample: For sensitivity reasons 100 mg of sample was considered. For liquid samples 100 mg was diluted in 10 mL of water. For solid samples we compared two methods of sample preparation. The first was to weight 100 mg of sample and leave alone in the vial; the second was also to weight 100 mg of sample but to dilute in 10 mL of water. For all but thujone, coumain and β-asarone the best results were obtained with the second process – so this was subsequently chosen as the method choice.

Effect of ethanol and sugar: Three different drinks were analysed under different analytical conditions. 1, 2 and 5 mL of sample were diluted in 10 mL of water to provide different concentrations of ethanol and sugar. The effect of these different concentrations was evaluated by looking at an internal standard (dicyclohexylmethanol). There were substantial and variable effects, resulting from the sugar and ethanol content, on the peak area of the internal standard. To minimize these unpredictable effects the method was optimized by using smaller amount of sample. Therefore, the effect of sugar and ethanol can be proportionally decreased – in this way, the effect can be negated by dilution and the method can handle the unknown sample composition without the need to optimize and validate for each sample matrix.

Sample preparation: Figure 4 shows the sample preparation process used for all samples.

Figure 4



Analytical Method

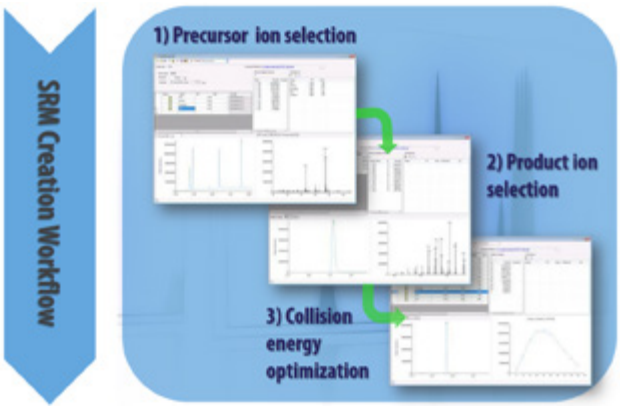
Three different matrices were tested: liquid, semi-solid and solid. The target analytes were thujone, menthofuran, estragole, pulegone, methyleugenol, coumarin and β-asarone, and the two internal standards, d4-coumarin and dicyclohexylmethanol.

Regarding the instrumental method setup one important aspect was to identify the best MS/MS transition for all the target analytes with optimization of the collision energy. To achieve this we used the AutoSRM functionality of the software. This function can facilitate the method development process with a full automated optimization of the method parameters. The optimized parameters were tracked in a clear and simple way. AutoSRM is comprised of a three-step process –highlighted in Figure 5 – step 1 is precursor ion selection, step 2 is product ion selection and step three is collision energy optimization.

The purpose of step one is to select the precursor ions – the programme starts from a full scan analysis and peaks are identified with a library search. By clicking on each peak a list of the most intense ions is shown, and these are selected to be included in a working list and used for the second injection – the product ion scan. Once the precursor ions have been selected the AutoSRM software automatically starts the scan acquisition of the product ion at three different collision energies. Users are not required to set any method or sequence to do this – AutoSRM takes care of it. Together with the chromatographic peak and the product ion spectra the software returns a table with the most intense product ion masses that can be selected.

The final step of the AutoSRM setup is the collision energy optimization. Now that you have selected your precursor and product masses AutoSRM

Figure 5



will acquire the transitions at multiple collision energies. Because of the fast scanning capability of the TSQ 8000 Evo GC-MS system three transitions per compound, each at 10 unit collision energies, can be analysed from a single injection. This will give you a well-defined maximum for your collision energy. Once complete, AutoSRM allows the TSQ 8000 Evo instrument method to be ready for routine analysis. Moreover, the transition and the retention time can be exported into the compound database linked to the method automatically in Thermo Scientific TraceFinder software.

Validation and Method Performance

After developing the method we performed in-house validation. We were focused on confirming the specificity, linearity, precision, LOQ/LOD, accuracy and robustness. The method has been optimized and validated for three different food categories: liquid (such as alcoholic/non-alcoholic beverages); semi-solid processed foods (such as soup); and solid food (such as muesli and bakery products). The method performance was established by spiking the blank matrix because no certified material was available on the market. Two spiked levels (low and high) were prepared with six replicates. A seven-point calibration curve was setup and measured at the beginning and end of the sequences. The linearity range was from 0.01–2.00 mg/kg for six flavorings and 0.10–2.00 mg/kg for coumarin. In all instances the correlation coefficient linearity function was at or above 0.99. Specificity is confirmed based on the percent of the transition ion, quantifier and qualifier of the correct retention time for the respective flavoring standard. Moreover, the measured peak area is short of the quantifier and qualifier ion were in close accordance with the ionization of the standard. Table 1 shows the precision of the method, expressed as RSD%. The SPME technique can deliver very good repeatability in the results.

Sample Analysis

Finally, the optimized method was applied to 40 samples from the global market. The samples were divided into three categories: liquid (4 spirits, 5 herbal and peppermint liqueurs, 5 flavoured milks and 3 energy drinks); semi-solid (5 canned soups and 10 sauces), and solid (5 herbal teas and 3 breakfast cereals). Figure 6 shows an example chromatogram of the analysis of peppermint liqueur. This shows the comparison of one sample measured with both full scan (top trace) and SRM mode (middle trace). The lower trace shows the standard for comparison.

Figure 7 shows a chromatogram of a standard mix liquid matrix. The level of the target compound is around the LOQ. You can see the individual mass traces which were obtained in SRM mode.

Table 1

Analyte	Relative standard deviation (RSD %)							
	level 1	level 2	liquid matrix		semi-solid matrix		solid matrix	
	mg/kg	mg/kg	level 1	level 2	level 1	level 2	level 1	level 2
Thujone	0.1	1	6	5	8	5	17	17
Menthofuran	0.1	1	14	5	2	15	19	21
Estragole	0.1	1	6	4	9	8	17	13
Pulegone	0.1	1	3	6	7	5	8	16
Methyleugenol	0.1	1	7	6	3	4	9	12
Coumarin	1	10	13	2	13	3	13	7
β-asarone	0.1	1	8	11	6	5	4	10

Figure 6

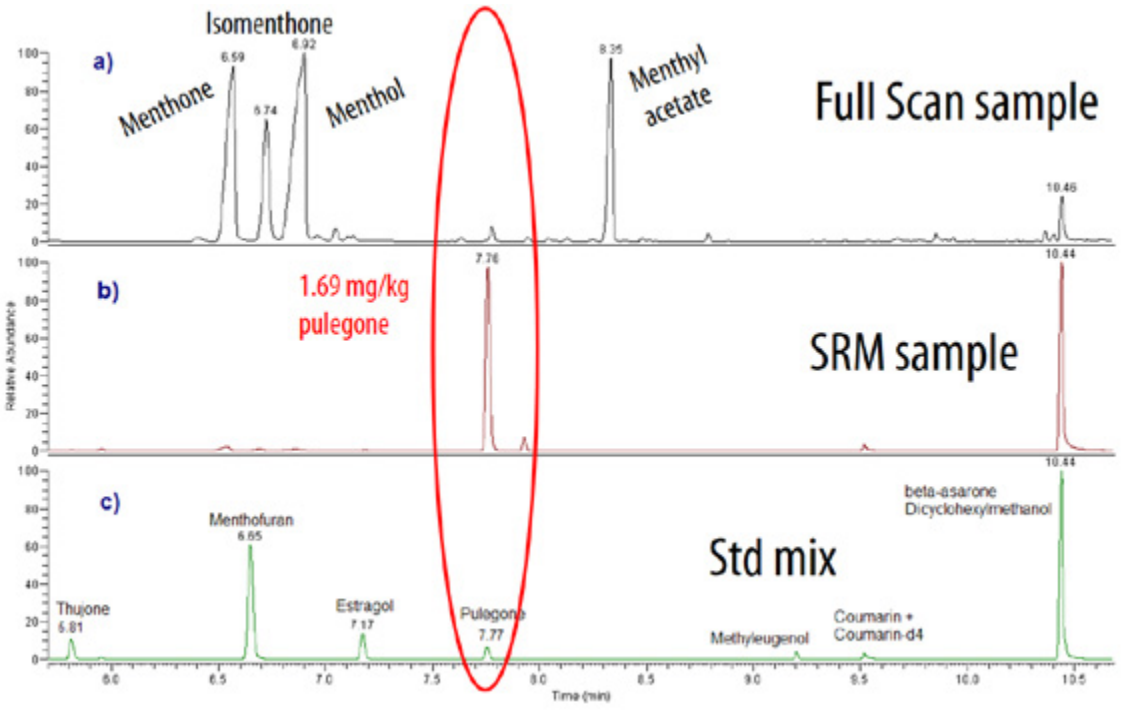
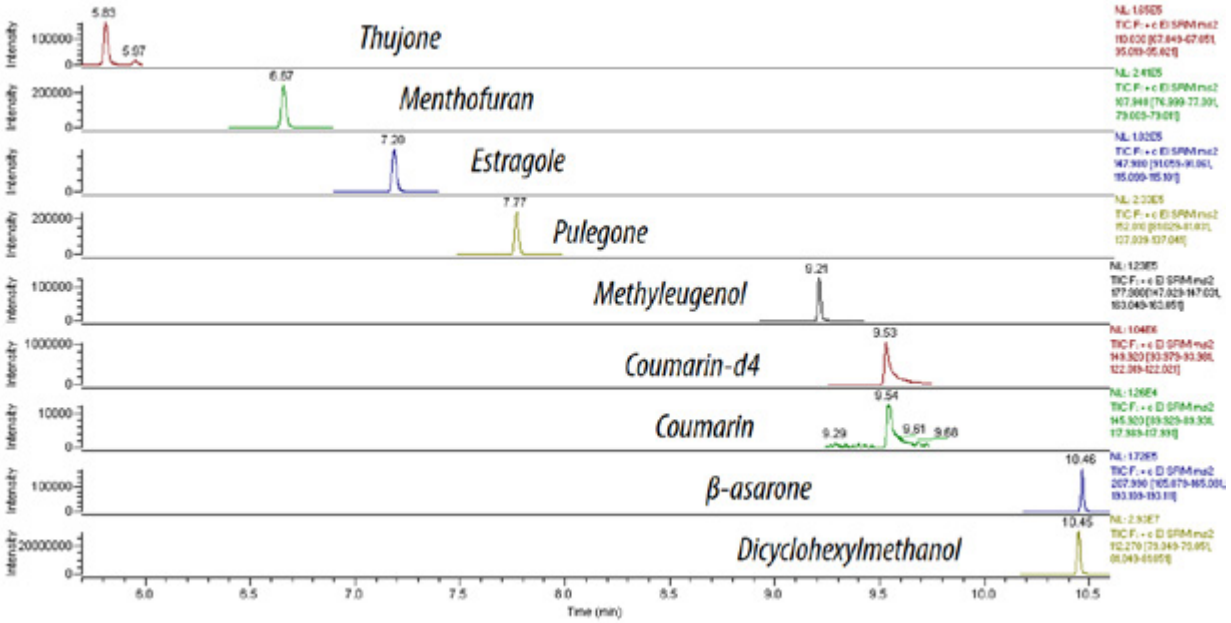


Figure 7



Conclusion

This validated method is capable of determining low levels of all the seven biologically active flavouring substances. The benefits of this method and instrumentation include that it can be used for all food types (liquid, semi-solid and solid), it is suitable as an official method for regulatory purposes (European Regulation 1334/2008), it has very high sensitivity and selectivity (and the use of MS/MS for detection guarantees a high level of confidence in identification), and the robust automated SPME shows very good repeatability.

About the Author

Elena Ciceri joined Thermo Fisher Scientific in 2007 and has been actively involved in various roles at the organisation with a focus on research and development, technical support, marketing and application support for gas chromatography and mass spectrometry systems. Currently she is product and sales specialist expert for gas chromatography and mass spectrometry systems for Europe. She is the author of several application notes and technical notes, which range from determination of contaminants in environmental samples to validation/authentication of food and beverage samples. She has vast expertise in troubleshooting skills and solving customer's analytical challenges. Elena Ciceri received her PhD in Chemistry from the University of Insubria, Italy.

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EXECUTIVE OVERVIEW

HPAE-PAD for Prebiotic Ingredient Characterizations

by Dr Parul Angrish & Dr Manali Aggrawal (both Thermo Fisher Scientific, USA)

High-performance anion exchange chromatography at high pH coupled with pulsed amperometric detection (HPAE-PAD) is one of the most employed techniques for carbohydrate determination used in routine monitoring or research applications. The compatibility of electrochemical detection with gradient elution allows mixtures of simple sugars, and oligo- and polysaccharides to be separated with high resolution in a single run. This technique has great impact on the analysis of oligo- and polysaccharides, including prebiotics.

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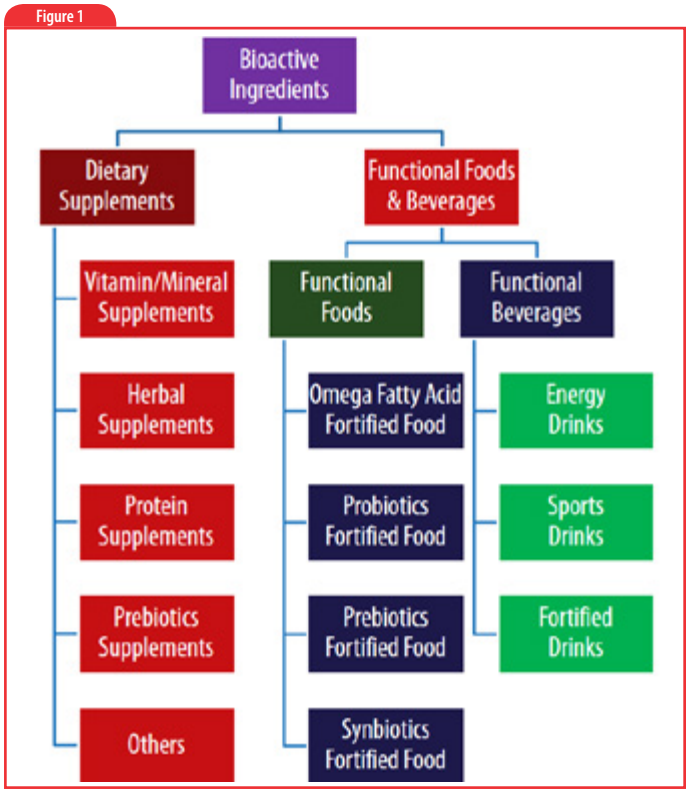
What are Prebiotics and Why are they Important?

Figure 1 shows the broad spectrum of compounds that can be classified under the term "bioactive ingredients". The bioactive ingredients market is growing rapidly as consumers increasingly turn towards healthy foods that fulfil the requirement of supplying the body with essential nutrients. With increasing education, consumers understand the types of food and beverages that help in maintaining a balanced diet, and manufacturers are taking advantage of this by offering convenience to the consumers and providing them with healthy nutrients in the form of food and beverages instead of supplements. Bioactive ingredients can be broadly categorized into two sections – dietary supplements and functional foods.

Dietary supplements contain ingredients that supplement the diet by adding more nutritional value. They include one or any combination of substances including minerals, amino acids, vitamins, and herbs. Dietary supplements are available in various forms including powders, pills, tablets, liquids, or gel capsules, and in general are consumed to add nutrients as well as reduce the risk of diseases.

Figure 1



Functional foods, as opposed to ordinary food, include components or ingredients that provide a specific medical or physiological benefit, in addition to the primary nutritional benefits. Functional foods, in general, need to meet the following requirements:

- they should be in a naturally occurring form, not in the form of a tablet, capsule, or powder
- they should be a part of the daily diet
- they should regulate biological processes to prevent or control diseases

A functional beverage is a non-alcoholic drink made with non-traditional ingredients such as herbs, minerals, vitamins, amino acids, or raw fruits/vegetables. They provide specific health benefits such as improving joint mobility, boosting the function of the immune system or heart, increasing energy and satiety, or creating a sense of well-being.

Prebiotics are typically non-digestible fiber compounds that pass through the upper gastrointestinal (GI) tract and stimulate the growth or activity of advantageous bacteria in the large intestine by acting as substrate (nutrient) for them. A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the GI microflora, which translates into benefits upon host well-being and health. Examples of such prebiotics ingredients include fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulins, and mannooligosaccharides (MOS).

Recently, researchers have revealed several direct and indirect beneficial effects of including prebiotics in food. These include improving calcium absorption, immune system effectiveness, reducing bowel acidity, and reduction of colorectal cancer risk, inflammatory bowel disease (Crohn's disease or ulcerative colitis), hypertension, and defecation frequency. Examples of consumer products that contain prebiotics include infant formula milk, yogurts and cereals.

There have been several studies both ***in-vitro*** and ***in-vivo*** underpinning the beneficial effects of prebiotics. Franco-Robles and Lopez [1] outlined the main prebiotic effects of fructans, including decreasing blood glucose (FOS, inulin), improved lipid metabolism (FOS, GOS, inulin and agavins), and improved mineral uptake (inulin, FOS, agavins).

Many foods are naturally rich in prebiotics (e.g., tomatoes, artichokes, onions, garlic, berries etc.), but there are also several commercial processes used to produce prebiotic ingredients. All these methods require the complete identification of desired oligosaccharides for batch-to-batch commercial production and also downstream development of new derivatives. As such, it is critical to understand the technologies available for complete analysis of these prebiotics.

The US prebiotic ingredients market is expected to experience significant growth over coming years as prebiotics are increasingly incorporated into different kinds of functional foods and dietary supplements. To this end, manufacturers are expected to increase the production of prebiotics ingredients needed to meet the consumer demand for prebiotics-based products.

As a consequence, the need to develop sensitive and reliable analytical methods for the characterization of such prebiotic-based products is of critical importance.

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD) has strong capabilities for complete prebiotic ingredient profiling (e.g., inulins, FOS, GOS, MOS), and is in-line with the prebiotic ingredient forecasts for intended use. For example, HPAE-PAD offers the capabilities for screening inulins for comprehensive characterization, which is one of the dominant prebiotic ingredients.

What is HPAE-PAD?

HPAE-PAD refers to a technique in which non-derivatized carbohydrates, ranging from simple sugars to complex carbohydrates, are analysed by high-performance anion-exchange (HPAE) chromatography coupled with pulsed amperometric detection (PAD).

HPAE chromatography is used to separate analytes that can be ionized under high pH conditions. Carbohydrates typically have pK_as in the range of 12–13. Once pH rises above the pK_a of the carbohydrate, these carbohydrates become ionized in solution – more specifically, they become oxyanions in solution and are then separated. These separations require hydroxide-based eluents, and for hybrid or complex carbohydrates, separations are accelerated and improved by using sodium acetate gradients in sodium hydroxide. The analytes can separated using the Thermo Fisher Scientific CarboPac column portfolio developed specifically for carbohydrates, ranging from monosaccharides to disaccharides, oligosaccharides and polysaccharides.

With the development of highly cross-linked, ethylvinyl benzene-divinyl benzene pellicular resins that have broad pH stability (0 to 14), separations at high pH conditions are feasible. The CarboPac columns' nonporous resins have small anion-exchange microbeads carrying the anion-exchange functional groups that are permanently attached electrostatically to a larger cation-exchange resin particle.

The nonporous nature of the resin minimizes band-broadening and imparts highly effective separation of a wide variety of carbohydrates, including branched oligosaccharides.

Pulsed Ampermometric Detection: Once separated, these underivatized analytes can be detected on a Au working electrode (WE) at high pH using pulsed amperometric detection (PAD). PAD applies a series of potentials (a waveform) to the WE and the carbohydrate is detected by its oxidation potential. The waveform is applied at a frequency of 2 Hz, resulting in oxidizing and reducing conditions on the electrode surface, which in turn causes oxidation of analytes bound to the WE surface.

Why HPAE-PAD for Carbohydrate Analysis?

Pulsed amperometry detects only those compounds that contain functional groups that become oxidized at the detection voltage employed. Consequently, the detection is sensitive and highly selective for electroactive species, because many potentially interfering species cannot be oxidized or reduced, and are not detected.

Neutral or cationic sample components in the matrix elute in, or close to, the void volume of the column. Therefore, even if such species are oxidizable, they do not usually interfere with analysis of the carbohydrate components of interest.

Key advantages of HPAE-PAD include:

- direct quantification of non-derivatized carbohydrates at low-picomole levels with minimal sample preparation and cleanup
- highly selective separations at high pH using a strong anion-exchange stationary phase

Figure 2

Thermo Scientific™ Dionex™ ICS-5000$^+$ HPIC™

Figure 2 shows a cross section of the Thermo Scientific™ Dionex™ ICS-5000$^+$ HPIC™ System, which is capable of operating up to 5000 psi. It allows sensitive and selective analysis of oligosaccharides at capillary, microbore or standard flow-rates or any combination in a dual system.

HPAE-PAD at high pH is one of the most useful techniques for carbohydrate compositional analyses for routine monitoring or research applications. The compatibility of electrochemical detection with gradient elution, coupled with the high selectivity of the anion-exchange stationary phases, allows mixtures of simple sugars and, oligo- and polysaccharides to be separated with high resolution in a single run. In terms of prebiotics there is interest in the complete ingredient characterization. In the food industry there is a significant increase in the demand for reproducible, fast and simple methods to profile these oligosaccharides and homologous sugar series such as inulins, MOS, FOS etc., either to monitor their quality

and conformity to the stringent labelling requirements, and even for checking for adulteration. Most HPLC approaches proposed for this application are limited by insufficient specificity and high detection limits, whereas HPAE-PAD has great impact on the analysis of oligosaccharides and polysaccharides, including prebiotics.

HPAE-PAD provides valuable insights for the selective determination of FOS and inulins present in prebiotic food ingredients to determine their degree of polymerization (DP) and isomers. Determination of DP of a particular prebiotic ingredient is critical towards its intended use as it influences some intrinsic properties such as activity, digestibility, caloric value, sweetening power and water-binding capacity.

So, HPAE-PAD fits perfectly into the value chain from suppliers to end users as a strong tool for the determination of DP and isomerism.

Fructooligosaccharides (FOS)

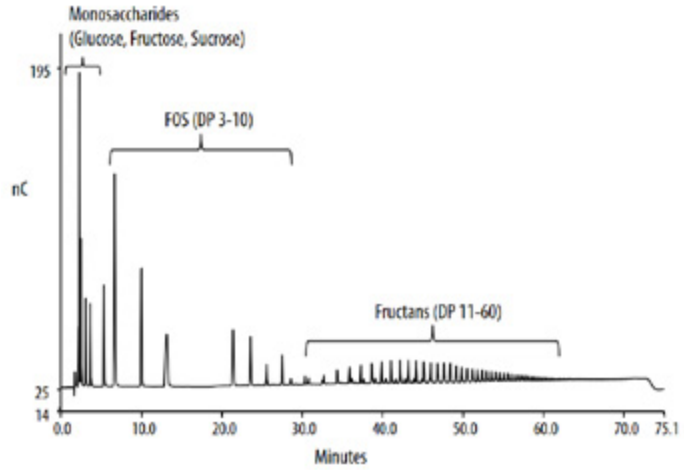
Both inulin and FOS are fructans that differ in DP. Inulin is a linear, highly polymerized fructan of DP 11–60, consisting of a linear chain of fructose with β-(2 ⟶ 1) linkages and a terminal glucose unit. Inulins are a group of naturally occurring polysaccharides produced by many types of plants, but can also be produced industrially (most often extracted from chicory root and dahlia tuber).

FOS has a DP of 2–9 and is produced from inulin by controlled hydrolysis. FOS is produced from inulin by controlled hydrolysis.

Depending upon the source, harvest time, and processing conditions, inulins and FOS can have different DPs and molecular weights; therefore, it is very important to analysis the complete profile of these prebiotics, and this can be achieved by HPAE-PAD.

HPAE-PAD enables complete, single-step separation of neutral and charged oligo- and polysaccharides differing by branch, linkage, and positional isomerism. HPAE-PAD is one of the most employed analytical techniques for the characterization of these molecules, and represents a powerful tool to evaluate changes in chain length distribution of FOS and inulin fermented by bifidobacteria, providing significant information about their prebiotic capabilities.

Figure 3



Column:	Thermo Scientific™ Dionex™ CarboPac™ PA2000+ Guard Analytical Column
Eluent:	-5 min: 100 mM NaOH/20 mM NaOAc, 0 -15 min: 100 mM NaOH/20 mM NaOAc, 15-70 min 100 mM NaOH/450 mM NaOAc, 70-70.1 min: 100 mM NaOH/20 mM NaOAc, 70.1-75 min: 100 mM NaOH/20 mM NaOAc, curve 5
Temp.	30 °C
Flow rate:	0.5 mL/min
Inj Volume:	10 µL
Detection:	PAD (Au) Disposable Waveform A (TN21)

Figure 4

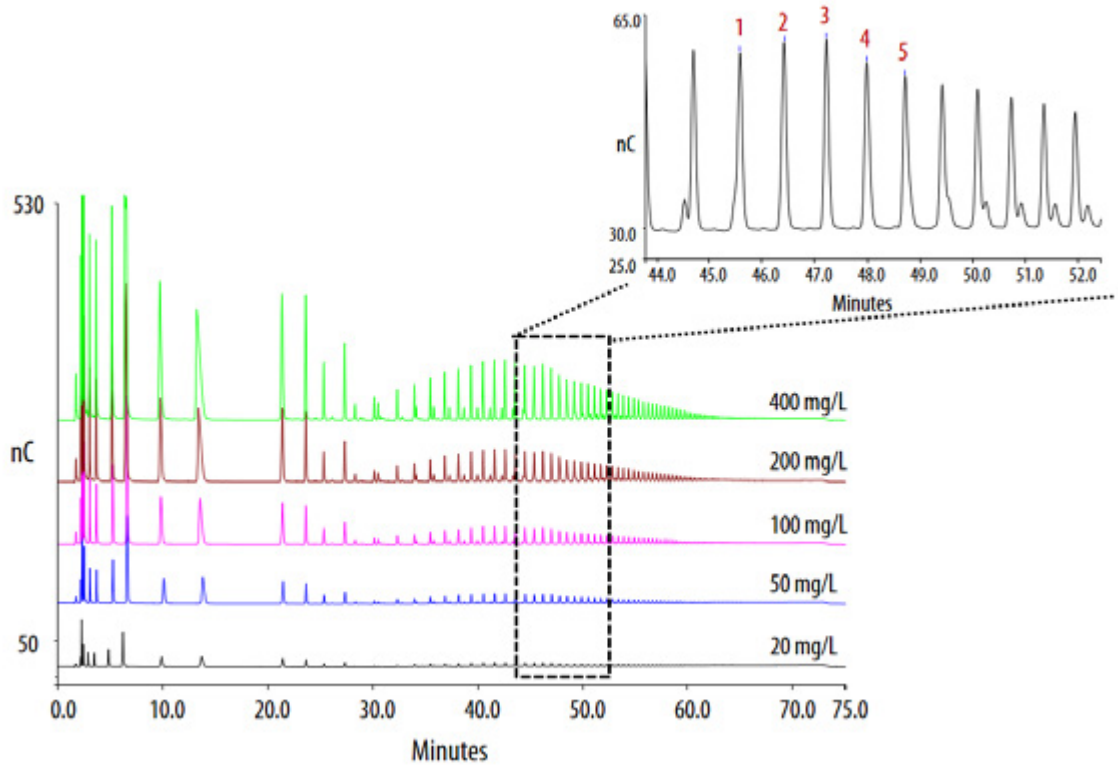


Figure 3 shows the chromatographic profile of inulin-FOS sample illustrating the separation of low molecular weight monosaccharides from high molecular weight FOS and fructans. Inulin-FOS is a prebiotic dietary supplement that helps in promoting probiotic bacterial growth. Good resolution and reproducible retention times of FOS and inulins up to DP 60 based on separation in a single run was obtained using a gradient profile shown in the conditions section of the figure. The separation was designed to be completed in 75 minutes, with an initial isocratic separation of glucose, sucrose and fructose, before employing a linear gradient of sodium acetate to separate fructans with a DP ranging from 3-60. The assignment of chromatographic peaks with DP higher than 3 is based on a generally accepted assumption that retention times of the homologous series of carbohydrates increases as DP increases, and thus each successive peak represents fructan with one fructose more than the previous peak.

In order to construct a calibration curve two prebiotic products were selected, inulin-FOS and chicory inulin, to prepare five working standards. Figure 4 shows the HPAE-PAD profile of inulin-FOS at five different concentrations ranging from 20-400 mg/L. Five peaks were chosen as markers, such that none were closely eluting. The concentration of these markers was determined by two methods – the first method was based on the %DP, and in the second method the markers are assigned the same five concentrations and the calibration curve is plotted for all five markers – all demonstrated excellent linearity.

Method accuracy was evaluated by measuring recoveries in spiked inulin-FOS samples. As noted earlier, there are no true standards available for these oligosaccharide samples. Thus samples and standards were interchanged to achieve this.

Samples were spiked with standards at a level that was 50–100% of the amount determined in the original sample. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The recovery percentages are within the range of 98–110%.

Galactooligosaccharides (GOS)

GOS are primarily composed of galactose, and often terminate with a glucose residue at the reducing end. These are enzymatically produced by transgalactosylation reactions of lactose catalyzed by β-galactosidases and give rise to galactose (Gal) oligomers with a terminal glucose with different glycosidic linkages and DPs. Depending on the enzymatic source used for their synthesis, the chemical structure of these oligosaccharides varies and, consequently, their effect on gut microflora can change. Thus, it is very important to analyse their complete profiles.

Figure 5 shows the chromatographic profile of GOS samples, showing the separation of small sugars and oligosaccharides ranging from DP4-13. For the Bimuno GOS sample we observe 13 peaks in this 45-minute separation window and we have identified them as DP1-13. The DP 1 and DP 2 peaks were identified based on the chromatogram of a mixture of glucose, galactose and lactose, and DP 3 was identified by the chromatogram of maltotriose. The assignment of peaks higher than DP3 was based on genuinely accepted assumption for the retention times of the homologous series of carbohydrates, where each successive peak has one more galactose than the previous peak.

In order to construct a calibration curve Bimuno GOS was used to prepare working standards and Figure 6 shows chromatograms of Bimuno GOS samples at five different concentrations. The calibration of GOS is based on individually calibrating each group of major DP fractions. The peaks for DP1-3 were not chosen because of the other interfering peaks present. Table 2 shows the calibration parameters of DP4 through DP13.

Accuracy was evaluated by measuring Bimuno GOS recoveries in an infant formula sample. GOS recoveries were determined based on the calibrations using Bimuno GOS, and two spiked concentration levels in the infant formula samples were examined (Figure 7). The recoveries of Bimuno GOS in infant formula were in the range of 90-110%.

Figure 6

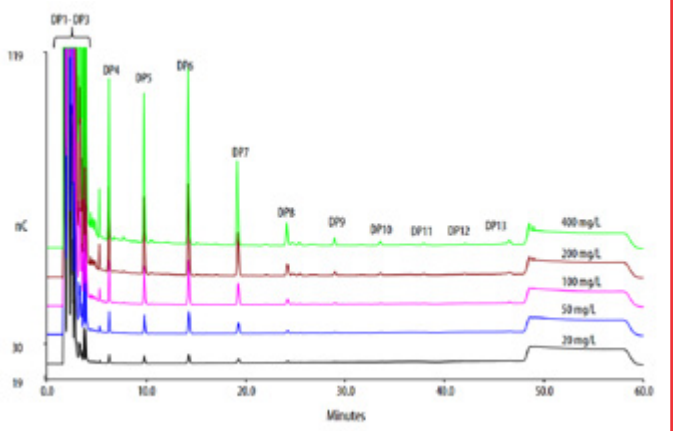
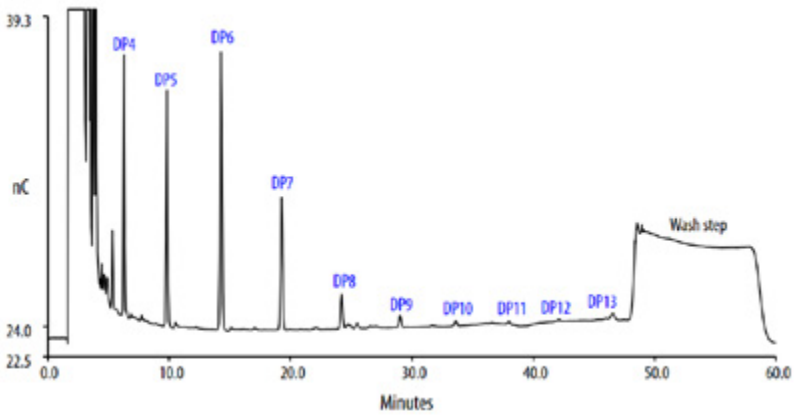
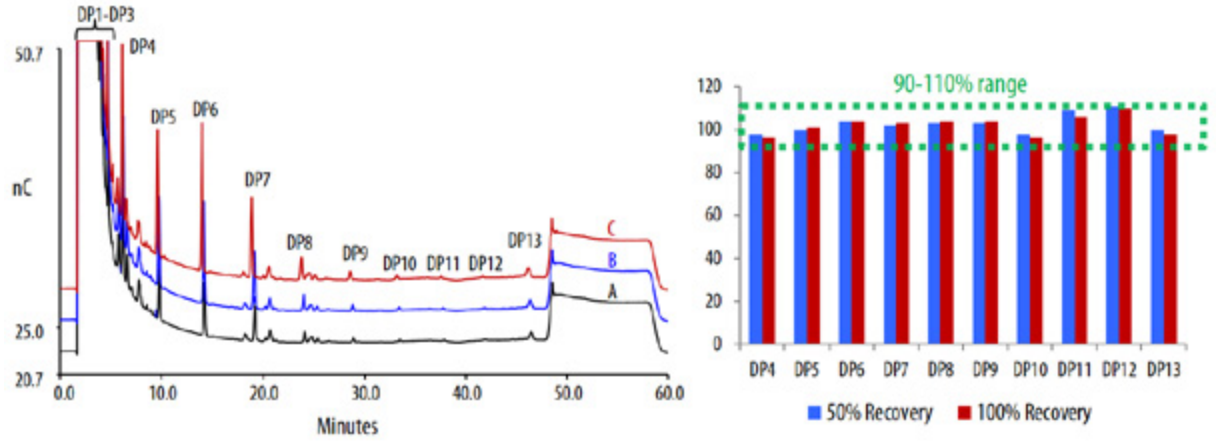


Figure 5



Column:	Dionex CarboPac PA200+ Guard Analytical Column
Eluent:	-5 -0 min: 100 mM NaOH/50 mM NaOAc, 0 -45 min: 100 mM NaOH/150 mM NaOAc, 45-55 min 100 mM NaOH/500 mM NaOAc, 55-60 min: 100 mM NaOH/50 mM NaOAc, curve 5
Temp.	30 °C
Flow rate:	0.5 mL/min
Inj Volume:	10 µL
Detection:	PAD(Au), Waveform A(TN 21)

Figure 7

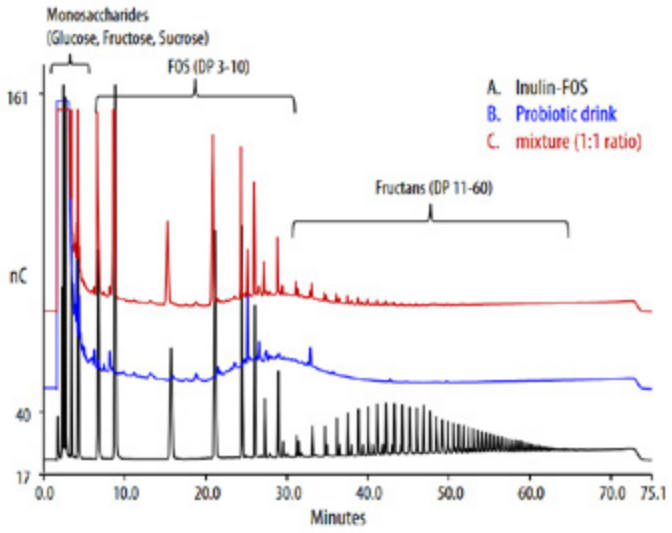


A) Unspiked Infant formula

B) Spiked 1 Infant formula(with 50% Bimuno-GOS)

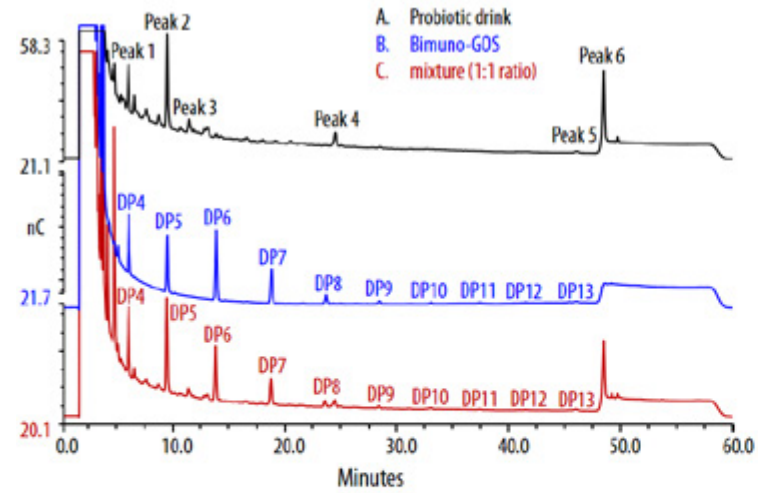
C) Spiked 2 Infant formula(with 100% Bimuno-GOS)

Figure 8



Column:	Dionex CarboPac PA200+ Guard Analytical Column
Eluent:	-5 min: 100 mM NaOH/20 mM NaOAc, 0 -15 min: 100 mM NaOH/20 mM NaOAc, 15-70 min 100 mM NaOH/450 mM NaOAc, 70-70.1 min: 100 mM NaOH/20 mM NaOAc, 70.1-75 min: 100 mM NaOH/20 mM NaOAc, curve 5
Temp.	30 °C
Flow rate:	0.5 mL/min
Inj Volume:	10 µL
Detection:	PAD (Au) Disposable Waveform A (TN21)

Figure 9



Column:	Dionex CarboPac PA200+ Guard Analytical Column
Eluent:	-5 -0 min: 100 mM NaOH/50 mM NaOAc 0 -45 min: 100 mM NaOH/150 mM NaOAc 45-55 min 100 mM NaOH/500 mM NaOAc 55-60 min: 100 mM NaOH/50 mM NaOAc curve 5
Temp.	30 °C
Flow rate:	0.5 mL/min
Inj Volume:	10 µL
Detection	PAD(Au), Waveform A(TN 21)

DP (mg/g) →	DP4	DP5	DP6	DP7	DP8	DP9	DP10	DP11	DP12	DP13
Probiotic drink	19.7	61.0	2.38	n.a.	n.a.	1.69	n.a.	n.a.	n.a.	1.84
Bimuno GOS	18.5	23.4	34.9	19.0	5.46	2.01	1.05	0.70	0.43	0.93
1:1 mix of Probiotic and GOS	27.5	54.6	35.1	18.3	5.90	2.03	1.00	0.68	0.39	2.19

Effects of Probiotics on Prebiotics

Probiotics are live bacteria and yeasts that are good for human health, especially digestive health (e.g., yogurt). Addition of probiotics to prebiotic foods has been demonstrated to have various benefits – often referred to as symbiotic effects. Prebiotics promote the growth of the probiotic organism by providing the specific substrate for its fermentation. Therefore, it is very important to study these interactions to understand the best combinations of prebiotics and probiotics – a very powerful tool to understand this is HPAE-PAD.

Figure 8 shows an example of the chromatographic profile following the addition of a probiotic to FOS. A probiotic drink was used in this study (made by fermenting skimmed milk with a special strain of the bacterium ***Lactobacillus casei*** Shirota). HPAE-PAD chromatographic profiles allowed the comparison of prebiotic profile with and without incubation with probiotic. The figure shows the results of this experiment, and demonstrates that the degradation of higher DP fructans is greater than the smaller FOS DP (≤ 10).

As shown in Figure 9, six major peaks were observed in the chromatographic profile. Because the probiotic drink has other carbohydrate components, such as corn dextrin, the five other major peaks may be carbohydrates other than GOS. The lower table lists the concentrations of the DPs calculated based on the individual calibration curves of the DP fractions of the Bimuno GOS. With the exception of DP8, the concentrations for all the DPs decreased, indicating degradation of Bimuno GOS following the addition of probiotics.

Summary

The HPAE-PAD method enables complete oligosaccharide profiling of fructans/FOS/GOS and determination of fructan/FOS/GOS content in prebiotic samples. This method demonstrates good precision and accuracy. The recoveries of fructans/GOS in samples were in the range of 90–110%. HPAE-PAD profiling is a powerful tool to evaluate the interactions of probiotics and prebiotics

Reference

[1] E. Franco-Robles and M. G. López, "Implication of Fructans in Health: Immunomodulatory and Antioxidant Mechanisms", The Scientific World Journal, vol., 2015, 2015.

About the Authors

Dr Parul Angrish is a product manager in the Ion Chromatography and Sample Preparation business unit at Thermo Fisher Scientific Inc. Her current interests focuses on expanding the carbohydrate applications in adjacent markets using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD). As a biotechnology professional, she has strong expertise in molecular biology reagents and chromatography instruments.

Dr Manali Aggrawal is currently a Senior Applications Chemist in the Ion Chromatography and Sample Preparation business unit at Thermo Fisher Scientific Inc. She is the author of several customer facing high-quality Application Notes and Technical Notes, which range from determination of contaminants in environmental samples to validation/authentication of food and beverage samples. Her current interests are in developing/validating methods for sugar analysis in various food samples using HPAE-PAD.

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Characterization of Prebiotics Using High-Performance Anion Exchange Chromatography (HPAEC) at High pH Coupled with Pulsed Electrochemical Detection (PED)

HPAC-PED is effective in the analysis of oligo- & polysaccharides, as well as prebiotics which can affect the composition and activity GI microflora.

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EXECUTIVE OVERVIEW

Automated Malt Analysis using Discrete Analyzers

by Aaron MacLeod (Director of the Center for Craft Food and Beverage at Hartwick College, USA)

Discrete analyzer technology offers faster, reproducible results with less sample and reagent use. All necessary analysis steps are automated. Routine malt analysis methods have been adapted for this technology including α-amylase, β-glucan, α-amino nitrogen and diastatic power measurement.

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Understanding Malt Analysis

Malt analysis is performed primarily in commercial malt houses around the globe as part of their process and product quality control. It is also used by research laboratories and plant breeders for screening germ plasm and looking at the effects of grain and malting quality on brewing. It is very useful for comparing different malts as well as trying to predict performance in the breweries. Consequently, brewers tend to look at malt analysis certificates of analysis or specification sheets in order to understand how a malt may perform in the brewery as well as looking for any problems that might arise as far as functional problems in the brewery. In addition, they are used as trade specifications so a brewer will have a set specification on raw materials that meet their requirements; for example, color or extract or enzyme levels that form a part of their recipe.

There are over a dozen parameters that constitute malt analysis and in this article we will be covering four of these specifically. All of this analysis gives us specific information about the quality of the malt in a few different categories. The first of these is endosperm modification – one of the goals of malting is to break down the endosperm cell walls that surround the starch and one of the best indicators for the progress of this is residual β-glucan in the worts. While the endosperm is being modified, protein is being modified, which can be monitored by measuring the amount of free amino acids produced in the wort. The third is enzymatic potential, which is very important for the brewery and is the result of malt enzymes that degrade starches in the mash to fermentable sugars – the two main indicators of enzymatic potential are diastatic power and α-amylase.

Standard Methods

Malt analysis is performed according to standard methods within the industry. There are three main groups responsible for these: the American Society of Brewing Chemists (ASBC) produce methods most followed by brewers in North America; The European Brewing Convention has official methods that are followed by brewers in Europe, South America and globally; and in Germany there are a group of methods called the MEBAK. While there are some standard processes throughout the three different method groups there are also differences between them.

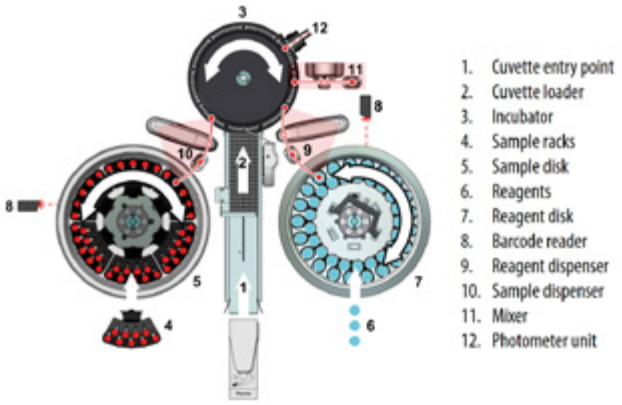
Chemistry Automation

Automation is employed in most malt testing facilities, primarily for the higher throughput. In a commercial malt house, where they are testing several different samples and blends and shipment samples and generating certificates, there may be approximately 100 samples per day. The official methods are all based on traditional wet chemistry and while they are inexpensive to perform as they don't require any advanced instrumentation, they are very labor intensive limiting the total number of samples that can be processed in a day manually. They are also much more prone to operator error so the training of technicians to perform these methods requires a lot of effort and standardization to get good results when performing chemistry manually.

Automation was introduced decades ago into this analysis to achieve a higher throughput and improve the precision of the assays. Traditionally in the malting industry, continuous flow analyzers have been the mainstay of chemistry automation. These can either be flow-injection instruments or segmented-flow depending on the type of chemistry that is being automated. These analyzers have a lot of benefits: the two main ones being high throughput capabilities and automation of complex chemistries. Some of the drawbacks are that they use a lot of reagent and the modules that come with the systems are customized for specific chemistries, meaning

Figure 1

Figure 2



that if you buy an instrument with four specific chemistries it can't do anything else, without purchasing additional modules.

Another class of automation solution is the discrete analyzer, which is what will be covered in this article. These are also very high throughput instruments, but they work using a different principle. While a continuous-flow analyser works by injecting sample successively into a continuously flowing stream of reagent, discrete analyzers use discrete vessels (cuvettes), in which every reaction proceeds on its own and there is a lot of automated liquid handling. Consequently, discrete analyzers are very high throughput and flexible because many different types of chemistries can be setup on them. One drawback of the discrete analyzers is that they tend to have more limited reaction conditions so, for example, it may not be possible to heat to high temperatures, or perform distillation or separation or some other aspect so there is sometimes a need to modify procedures to get them to work.

Thermo Scientific™ Gallery™ Plus System

Figure 1 shows the Thermo Scientific™ Gallery™ Plus system. A smaller instrument, the Gallery, is also available. It is essentially a robot liquid handling system with incubation and photometric measurement. Figure 2 illustrates the discrete analysis process in the Gallery system. There are dispensing pipettes that dispense fixed amounts of reagents and samples into individual cuvettes. Reagents can be added in a specific order to an incubation chamber and then onto photometric detection. This allows several additions and dilutions as necessary, and the tests can be programmed in advance.

Measurements are made using a light source and filter, and a wide range of filters are available down into the near UV region. The pathlength of the cuvettes is a standard 1cm which is very useful.

The workflow for these instruments is also very easy to use. Typically, you put your reagents and samples onto the instrument after they are prepared. Calibrations are run and stored within the instrument and can be updated as frequently as necessary. A sample schedule is then set up to run the samples and the robot pipetting takes case of the rest.

High Molecular Weight β-Glucan

We are now going to look at the four chemistries that were set up on the Gallery instrument. The first of these is high molecular weight β-glucan. This is a measure of residual cell wall components in the wort. We are looking for low levels of β-glucan in the wort, which indicate that a good amount of the endosperm wall has been degraded during the malting process. Brewers are looking for <120 ppm to ensure good filtration performance. High levels of β-glucan in the wort can lead to problems with wort separation and runoff or beer filtration in the brewery. The standard measurement that the ASBC methods are based on use a fluorescent dye binding with a dye called calcofluor. There are several dyes that are known to bind with β-glucan to produce a reaction. The Gallery instrument uses a novel photometric dye binding method and the molecular weight range of sensitivity is similar to what we see with the traditional fluorescent dye method. This is important if we want to achieve a result similar to the standard method.

The range is linear over a very large range of solution concentrations (Figure 3) and this is very useful because it means that the dilution can be done on-board the instrument for high level samples and that the results will come out well using dilution because of the linear absorbance range. One area in which the Gallery instrument excels is precision liquid handling. The amounts of reagent being used are very small (<200 µL) and the pipetting is very accurate, so what we end up with is very good method repeatability both within and between runs, giving coefficients of variation of 1% or better, which is excellent.

We also compared samples of different malts with a range of modification levels using both the Gallery instruments and a segmented flow instrument. Figure 4 demonstrates the good correlation between the two sets of results. There was a slightly high bias, but this can easily be corrected for.

Figure 3

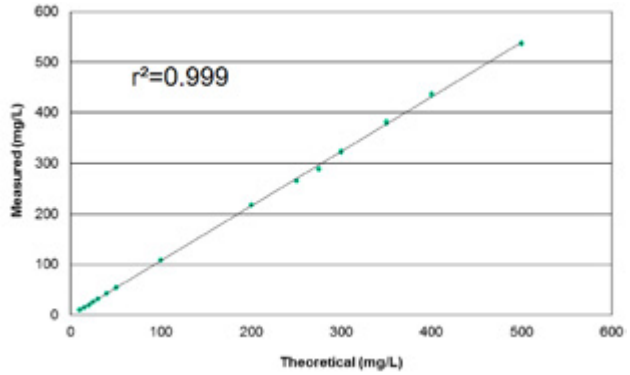


Figure 4

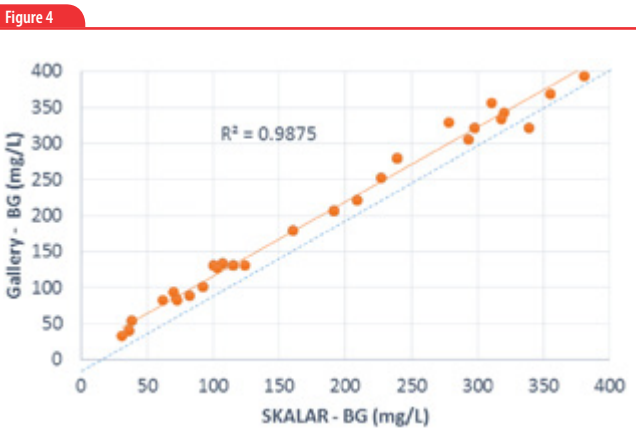


Figure 5

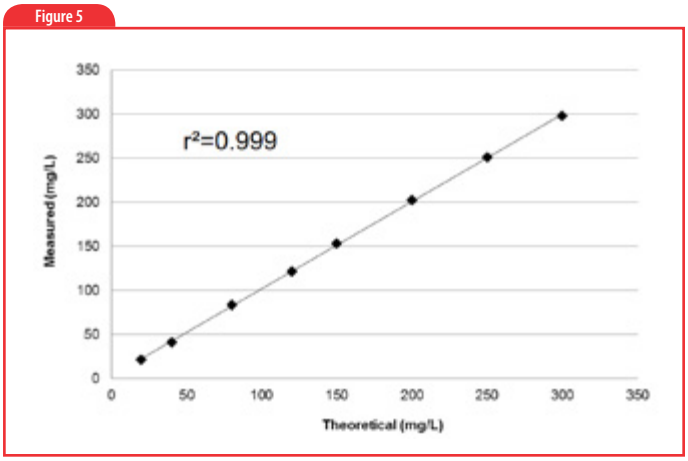
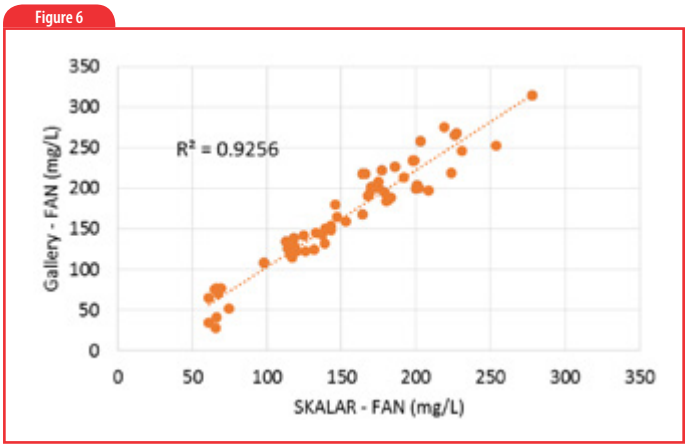


Figure 6



Free Amino Nitrogen

Moving onto considering free amino nitrogen (FAN), which is another important wort quality parameter because amino acids are required as a source of nutrition for yeast during fermentation. Adequate levels of FAN are required in a fermenting wort to achieve the required level of attenuation. However, low levels of FAN are necessary in the finished beer as that promotes long-term stability. There are different specifications for FAN between all malt (>150 mg/L) and adjunct brewing (>200 mg/L) and so it is an issue that brewers are looking at closely. It can also be looked at in the beer itself.

The standard method for FAN determination is based on spectrophotometric detection using ninhydrin reagent to form Ruhemann's purple absorbance at 570 nm. This reaction requires heating to 95 °C, which is not something that the Gallery instrument is capable of doing. Therefore, a different chemistry is implemented, which is based on the o-Phthaldialdehyde (OPA) reaction – this is a very commonly used chemistry in the wine, juice and related industries. In this instance, the reagents form an adduct with the alpha-amino nitrogen in the sample and the results are measured in the near UV range. Again, there is very good linearity over a large concentration range that would normally be encountered in the laboratory (Figure 5).

To measure method repeatability data for this work, there were three different samples with three different levels of FAN with multiple samples being run on the instrument (30 replicates). Coefficients of variance within and between runs were found to be 2-3%, which is again excellent.

Figure 6 shows a method comparison between the standard segmented flow and Gallery methods. The correlation is not quite as high as shown for β-glucan but here we are looking at two different methods of detecting the amino acids. When it comes to forming the reaction products, each amino acid has a slightly different relative response factor with the two different reagents so it is difficult to get exactly the same results, but even so the two sets of results are very comparable over the range that is important for malting and brewing.

Diastatic Power

Diastatic power represents the total activity of starch-degrading enzymes, and is primarily related to β-amylase activity that produces fermentable sugars. Diastatic power is higher when grain protein content increases, and is very much influenced by variety. Adjunct brewers need more diastatic power in their malts to convert starch from corn or other grains, and all-malt brewers tend to want less diastatic power.

Starch in malted barley is made up of two different sorts – amylose (linear chain) and amylopectin branched form). β-amylase is an exo-acting enzyme that breaks off maltose units (two glucose units) from the non-reducing end to create fermentable sugars. This forms the basis for the standard method of measuring diastatic power that is allowing an extract of the malt to digest a buffered starch solution for a fixed amount of time at a fixed temperature. This produces reducing sugars in proportion to the activity of the malt. Once the reducing sugars are produced potassium ferricyanide solution is used to measure the reducing power, and this is generally automated in a segmented-flow instrument.

Most reducing sugar assays require heating at high temperatures. As this is not possible with the Gallery instrument, a different method approach had to be used. This novel approach involves the same digestion of starch with an extract of the malt, as with the standard method. As maltose is primarily created we further hydrolyze this with an α-glucosidase to produce D-glucose. The formation of the glucose is proportional to the activity of the enzyme. The D-glucose is easily measured using hexokinase and glucose-6-phosphate dehydrogenase reagents, and these reactions can all be

Figure 7

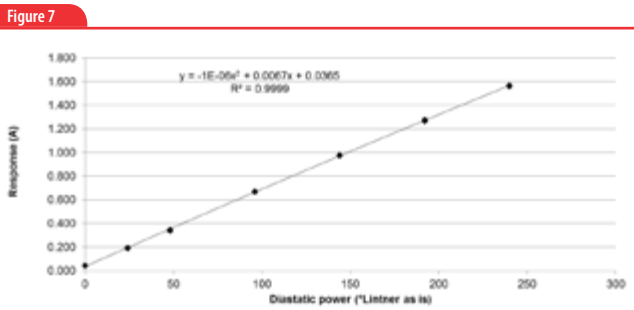


Figure 8

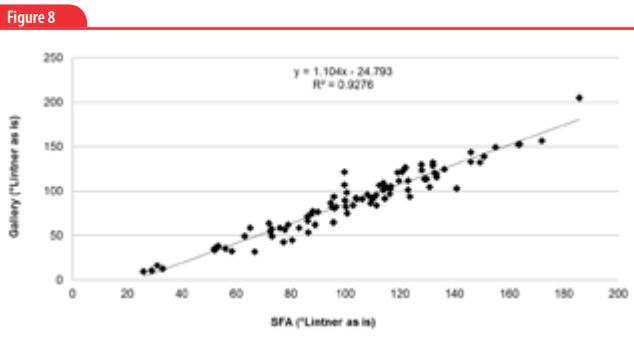


Figure 9

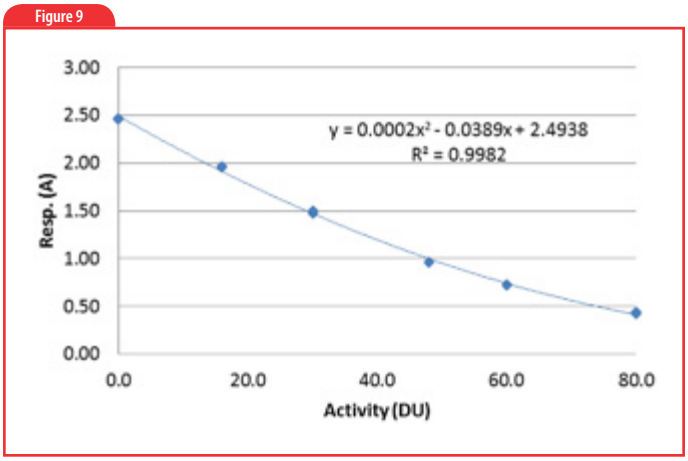
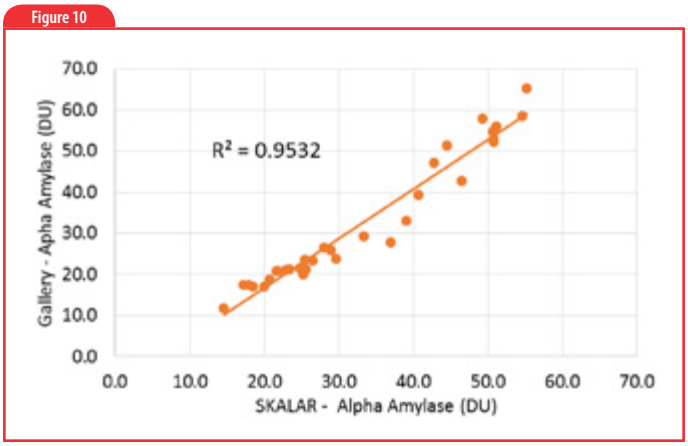


Figure 10



performed at 37 °C with a photometric endpoint measurement of 340 nm. We have thus taken a very difficult assay to automate and come up with an alternative chemistry.

A calibration curve is created using a crude malt extract, and Figure 7 shows a very good linear response over a large range of absorbances. The curve is fit second order but you can see that the result is linear.

Method repeatability with the Gallery instrument was very good. This is primarily the result of the excellent liquid handling of the instrument, as well as tight control of the temperature and other conditions within the incubator.

A side-by-side method comparison, where the instrument was operated on the same set of malt extracts, compared with the results from the segmented-flow system (Figure 8) was made. Over a large range the correlation was found to be very good between the two methods.

α-Amylase

The second enzyme of importance is α-amylase, which is related to rapid degradation of starch during matching or conversion, and is produce during the malting process so is heavily influenced by the malting conditions and the level of modification. Specifications are generally in the region of 50 D.U. for most all-malt brewers, but distillers require much higher levels of α-amylase.

α-Amylase is endo-acting so it breaks starch into smaller dextrins, which forms the basis of its analysis by the classic starch iodine reaction. In this instance, an extract of the malt is allowed to react with a dextrinized starch substrate and the de-colorization reaction with iodine is monitored over time. The higher the α-amylase activity the less iodine color you'll get after a fixed amount of time.

As this is a colorization reaction, the calibration curve goes in the opposite direction (Figure 9) - this is a second order curve but still shows a good dynamic range so quite a bit of sensitivity.

In checking the method repeatability, again we saw very low coefficients of variation of less than 2% for ranges commonly encountered in the laboratory. The side-by-side comparison also yielded a very good result between the Gallery instrument and the segmented-flow system (Figure 10).

Conclusion

While segmented-low systems have worked successfully and have served the industry well there is a lot of value in the use of these discrete instruments. The Gallery Discrete Analyzer is a rapid and precise instrument for chemistry automation. The adapted methods for malt analysis used with this instrument have shown to produce results that are comparable to the standard reference methods, and point to the discrete analyser being a game changer in the brewing industry in terms of automation. In addition, the instrument is easy to use and offers a very high level of precision.

About the Author

Aaron MacLeod has been involved with malting and brewing quality testing and research for over 10 years and is currently the Director of the Center for Craft Food and Beverage at Hartwick College. From 2005 to 2015 Aaron was a chemist in the Canadian Grain Commission's Grain Research Laboratory where he was responsible for providing quality assurance for malting barley grown in western Canada and conducted research on factors affecting malting barley quality and quality measurement methods. Aaron is also a member of the Technical Committee of the American Society of Brewing Chemists. He has published numerous articles in peer-reviewed journals, and presented at both domestic and international scientific meetings and conferences on topics related to malting and brewing science and quality testing methods.

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