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Miniature Mass Spectrometry

The benefits of thinking small

Cover Story

Features

Regulars

Miniature Mass Spectrometry and On-line Analysis of Flow Chemistry Research

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Flow chemistry is a developing practice in chemical research laboratories and has improved the efficiencies of some research techniques such as screening reactions at hyperthermal conditions and generating and using hazardous and toxic reagents. Although in its infancy, flow chemistry has progressed to the extent that total syntheses of natural products have been demonstrated.[1–8] This article will discuss the introduction and benefits of miniature mass spectrometry (MS) and how it can be combined with a preparative flow chemistry system to optimize reaction conditions, monitor reactive intermediates and highlight competing reaction pathways.

Photo Credit: Gregor Schuster/Getty Images

Flow chemistry offers some clear benefits over a volume-fixed batch chemistry approach. When optimized, it is capable of pumping reagent reservoirs together through a series of reactor zones (cooled, heated, microwave irradiated, electrochemical or photochemical cells) to provide a continuous stream of product. The product stream can then either be worked up in traditional ways, or purified, and processed further downstream.

Industrial companies involved with the research and development of novel molecules with biological activity also recognize the benefits associated with this approach and have invested in both commercial flow equipment and the development of in-house technologies.

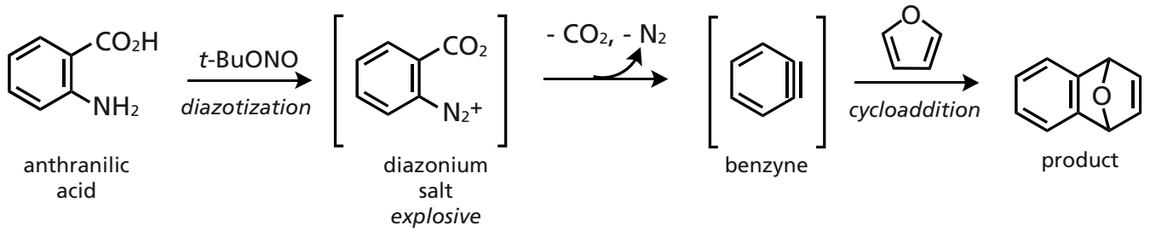
There are numerous benefits from using flow chemistry, with safety being one of the main advantages.

In research aimed at the discovery of molecules with bioactivity, bulk preparation of dangerous and toxic intermediates is preferably avoided. However, this issue is often encountered with chemical processing in batch when scale-up is required to achieve bulk quantities of material.

In flow chemistry scale is proportional to time rather than volume. The accumulation of hazardous material can be avoided by allowing the machines to pump for longer. Provided an optimized protocol is in place, only a small volume of hazardous yet reactive intermediate is generated and quenched at any point in time.

The experiment discussed in this article highlights how preparative flow chemistry and a miniature electrospray ionization mass spectrometer (ESI–MS) can be linked together. Traditional mass spectrometers can be used in this application; however, they are not often considered because of their size and bulk. Miniature mass spectrometers eliminate this issue, enabling them to be effectively coupled to the flow chemistry

Figure 1: Reactions leading to the formation of 1,4-endoxide-1,4-dihydronaphthalene.[9]



platform in the same laboratory and even the same fume cupboard.

A miniature mass spectrometer simplifies a typically disjointed practice. If a make-up pump and static splitter are added it is possible to take real-time samples and observe hazardous intermediates without the dangers associated with off-line manipulations.

Miniature Mass Spectrometry

Recent advances in techniques and materials that have emerged from research in micro-electro-mechanical systems (MEMS) technology have enabled the construction of chip-based mass spectrometers.

Laboratories can benefit from the use of miniature mass spectrometers because they overcome issues of bench space availability, noise and vibration, heat generation and laborious workflows. Specifically in the field of flow chemistry, on-line mass spectrometry (MS) provides real-time detailed snapshots of the materials discharged by a flow chemistry reactor system.

Experimental

A miniature ESI–MS system (Microsaic 3500 MiD) was used to demonstrate the continuous flow diazotization of anthranilic acid to generate an explosive diazonium salt intermediate. Under the right conditions this would eliminate nitrogen and carbon dioxide to generate benzyne that could then be trapped in a cycloaddition reaction.

Despite the efficiency of generating and reacting benzynes in this way, the practice has largely moved to a much milder (but far less economic) method since catastrophic explosions have been reported of the diazonium salt intermediate. When monitoring what is happening in the reaction, the scientist needs to wait for the experiment to run its course, make a sample and then transport it to a neighbouring room or floor that houses the analytical equipment. Once the data is acquired, it can then be analysed, reaction parameters can be amended and the process repeated in an effort to optimize the reaction conditions.

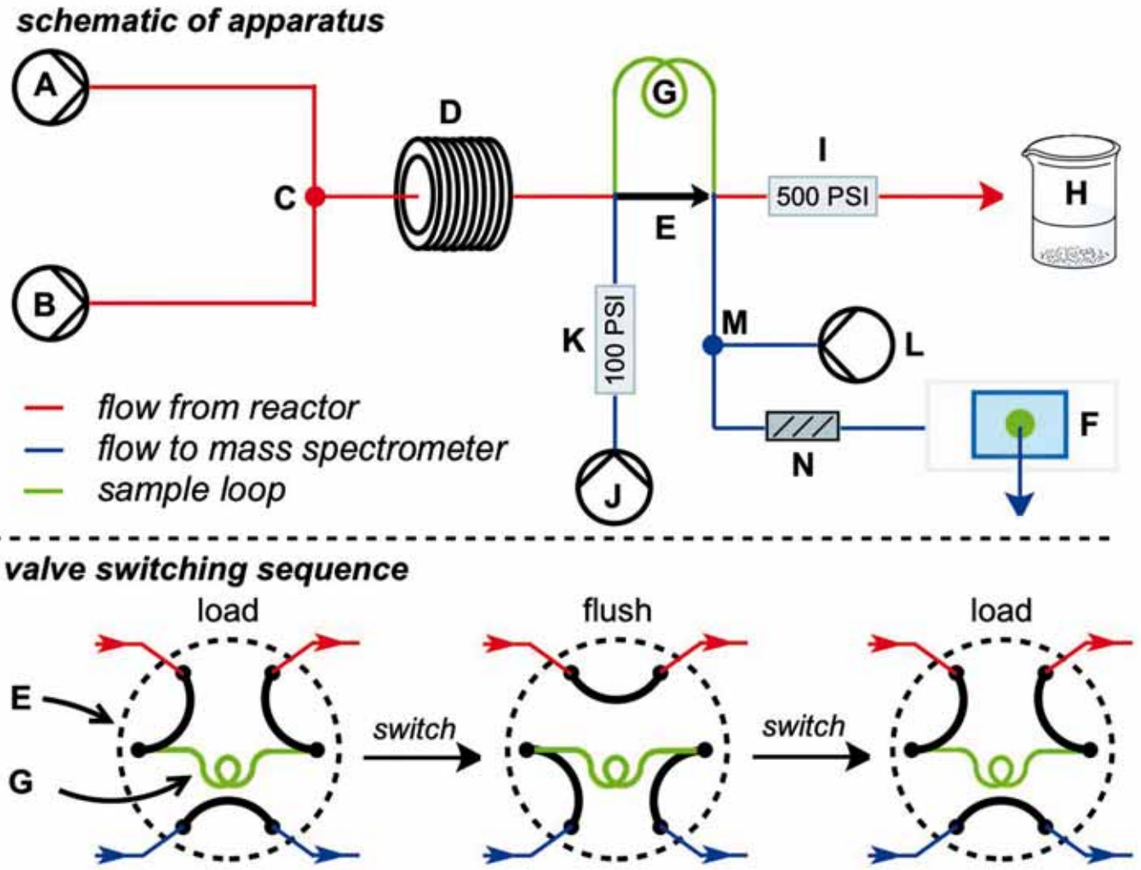


Figure 2: A schematic of the miniature mass spectrometer coupled to a flow chemistry system. The reactant solutions were pumped through high pressure pumps (A and B) to a mixing tee (C). The flow stream then flowed through the reactor coil (D) and sampling loop (G), which was fitted across two ports of the six-port valve (E). After passing through a back-pressure regulator (I), the solution was discharged into a collection vessel (H). When the six-port valve was switched to the inject position, a third high-pressure pump (J), which was stabilized by back-pressure regulator (K), pumped acetonitrile through the loop to flush the contents into mixing tee (M). A 50:50 (v/v) mixture of acetonitrile and water with 0.1% formic acid was pumped into the mixing tee by a fourth high-pressure pump (L) to further dilute the sample and modify the solution to aid the ESI process. An in-line filter (N) removed particulates before analysis of the sample by the miniature mass spectrometer (F).[9]

One of the keys to successfully incorporating the miniature ESI–MS system with the preparative flow equipment was in the mode of sampling the output flow stream. To periodically sample the flow stream leaving the reactor coil, a six-port switching valve fitted with a 5 µL loop was used. To ensure the gaseous products remained in solution, a high fluidic pressure was maintained. Approximately 10 s after activating the valve mass spectra corresponding to the sample loop contents were observed. As a demonstration of how this apparatus can be used the generation of benzyne and its subsequent reaction with furan was investigated. Benzyne was prepared *in situ*, via diazotization of anthranilic acid using tert-butyl nitrite. Figure 2 shows a schematic of the miniature mass spectrometer coupled to a flow chemistry system.

Data and Results

The experiment found that the explosive diazotized intermediate was detected by the mass spectrometer at both low coil temperatures and short residence times. The optimal reactor temperature and residence time for production of the desired Diels-Alder product are 50 °C and 3–5 min, respectively. There are competing reaction pathways leading to the formation of acridone and several other by-products.

The data produced from the experiment was used to deduce a number of other interesting by-products, allowing the exploration of other avenues and probing of the reaction mechanism. Interestingly, several of the low-level background reactions observed by the miniature mass spectrometer device have been previously exploited and reported as discrete reactions of benzyne in numerous other papers. In other words, the set-up permits the observation of useful background reactions, each of which could be delineated and optimized separately. Often, such low levels of by-products can be overlooked and potentially new and exciting reactions missed.

The data and the noted trends permitted optimization of the flow reaction conditions so as to minimize any observation of the dangerous material in the flowing output. As a result of the rapid collection of real-time data, it is also possible to observe trends for other masses at the same time.

Conclusion

The cited article[9] and the results demonstrated here highlight how a miniature mass spectrometer may be used to advance flow chemistry research. An on-line mass spectrometer enabled the flow conditions to be quickly tuned for safe operation and optimal generation of the desired product. The validity of this approach was supported by off-line liquid chromatography–mass spectrometry (LC–MS) analysis of a flow sample. On-line MS provides

an in-depth snapshot of the materials discharged by a flow chemistry reactor system.

Generally, the results are encouraging and demonstrate that compact MS technology is an important development. It is apparent that through this technique a flow chemistry system can be set up in such a way that it can generate reactive intermediates that can then be diverted by another reactant flow stream. This is a particularly exciting prospect — the potential to observe and intercept such fleeting and reactive entities paves the way to discovering untapped synthesis methods.

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Duncan Browne moved from an early career Doctoral Prize Fellowship sponsored by the EPSRC (Sheffield, UK) to assume the position of postdoctoral research associate within the group of Professor Steven V. Ley FRS CBE at the University of Cambridge (Cambridge, UK) in 2010.

It was in the Whiffen and ITC laboratories that he learnt how to incorporate continuous flow processing into organic synthesis. With a strong interest in the physical engineering and assembly of flow chemistry apparatus, he was able to learn a lot from his colleagues.

During this time he has had the opportunity to test numerous pieces of equipment from commercial suppliers and evaluate them in the context of flow chemical synthesis.

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News

Cell talk

High performance liquid chromatography coupled with mass spectrometry (LC–MS) was used to investigate cell-to-cell communication. The results, published in PLOS ONE, outline how proteins in the cell membrane of a specific cell line are modified to change their function. [1]

The study analysed the proteome of E14.Tg2a, focusing specifically on glycoproteins. The cell line is an important tool in the study of the physiology and pathology of human Lesch-Nyhan disease, a neurological disease characterized by mental retardation and self-mutilation.

Glycoproteins are added to other proteins to change function, and so play an important role in cell-to-cell communication, as well as facilitating interactions with pathogens, such as viruses. The analysis found that most of the glycoproteins were cell surface proteins, which are thought to be difficult to study because of their complex structure and poor solubility, according to lead author Bingyun Sun.

As well as providing detailed molecular information about the E14.Tg2a stem cell line, the scientists also discovered a novel relationship between the levels of glycosylation and the function of proteins within the cell. Advances in understanding how membrane proteins are adapted by glycosylation could improve drug design and development.

According to Sun, the findings will help further our understanding of glycoprotein function, which are implicated in a number of roles from signalling, sensing environmental cues, cell-to-cell communication, development and defence. Sun told *The Column*: "This finding has also been conserved in evolution, when we further examined other four evolutionary distinct species, such as fly, worm, fish and human, which indicates the importance of it to biological systems."

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Vitamin D supplements' potency varies widely

The amount of vitamin D in supplements can be anything from 9% to 146% of the stated dose, according to a research letter published in the *JAMA Internal Medicine*.[1] Investigators from Kaiser Permanente (Portland, Oregon, USA) tested 55 bottles of over-the-counter vitamin D supplement tablets from 12 different manufacturers using high performance liquid chromatography (HPLC).

Vitamin D supplements are often prescribed for vitamin D deficiencies, but are not regulated by the Food and Drug Administration (FDA). There are a range of reports suggesting that a vitamin D deficiency may have links to a number of health problems from neurological disorders to hypertension, as reported in *The Column*[2] last year. Hypovitaminosis D, a deficiency of vitamin D from either dietary or sunlight sources, is now widely recognized to be an affliction of the western world as a result of low sunlight exposure and poor diet.

Supplement tablets purchased from Portland pharmacies were analysed by HPLC and found to contain 9–146% of the labelled concentration. This variation was found not only between tablets produced by manufacturers but also between tablets contained within the same bottle.

"We were surprised by the variation in potency among these vitamin D pills," said study co-author Erin LeBlanc, lead author and investigator, Kaiser Permanente Center for Health Research (Portland, Oregon, USA). "The biggest worry is for someone who has low levels of vitamin D in their blood. If they are consistently taking a supplement with little vitamin D in it, they could face health risks."

Manufacturers can voluntarily submit supplements to accreditation by US Pharmacopeial Standards (USP). These standards require tablets to contain between 90% and 110% active ingredient. According to the report, these may be sparsely distributed.

"The USP verification mark may give consumers some reassurance that the amount of vitamin D in those pills is close to the amount listed on the label", commented LeBlanc. "There are not many manufacturers that have the USP mark, but it may be worth the extra effort to look for it."

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World's biggest allergy study

The University of Manchester (Manchester, UK) has announced the start of the world's biggest allergy study. Eurofins (Brussels, Belgium) is one of the main industry laboratory participants in the four-year study sponsored by the European Commission. Eurofins were selected following validation of liquid chromatography–tandem mass spectrometry (LC–MS–MS) for this type of research.

The project has attracted funding of 9 million euros, advancing on an earlier 14.3 million euro research study. Researchers from across Europe, Australia and the US are taking part in the European Commission-sponsored research known as the Integrated Approaches to Food Allergen and Allergy Risk Management (IFAAM) to formulate a standardized management process for companies involved in food manufacturing.

Professor Clare Mills of the Allergy and Respiratory Centre of The University of Manchester's Institute of Inflammation and Repair will head-up the study. Mills said, "This is a massive research project which will have far-reaching consequences for consumers and food producers. The evidence base and tools that result from this will support more transparent precautionary 'may contain' labelling of allergens in foods which will make life easier for allergy sufferers as they try to avoid problem foods."

For more information please visit: www.eurofins.com

AB Sciex and Leco support metabolomics

AB Sciex (Massachusetts, USA) and Leco (Michigan, USA) have formed a co-marketing partnership to provide support for researchers working in metabolomics in Europe and North America Metabolomics researchers often use a systems biology approach that requires both liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS).

"We interact with metabolomics researchers all over the world, and we understand that there are times when metabolomics researchers want to use both GC–MS and LC–MS," said Aaron Hudson, Senior Director, Academic and Omics Business, AB Sciex. Hudson added that the joint "bundles " from AB Sciex and Leco provide users with improved felxibility in chromatography while obtaining the rich information that MS–MS delivers."

Jeffrey Patrick. Director of Marketed Technologies at Leco, said his company's GC–MS technologies complement the AB Sciex offerings and the combined portfolios offer expertise, services and support for metabolomics researchers.

The companies will offer "bundled" solutions for researchers requiring both separations. The application and service support teams from each company will work together.

For more information please visit: www.leco.com or www.absciex.com

The heart of "Richard the Lionheart" analysed

The heart of Richard I — King of England from 1189–1199 and nicknamed "Richard the Lionheart" — has been analysed by gas chromatography–mass spectrometry (GC–MS), combined with an array of other bioanalytical techniques. The team of scientists, led by forensic scientist Dr Philippe Charlier, published their findings in the journal *Scientific Reports*.[1]

The heart of Richard I was first found in the 19th century, contained within a small lead box with the inscription "Here is the heart of Richard, King of England". This is somewhat inaccurate; in reality what remained within the lead box was a white-brown powder.

The team used an array of bioanalytical approaches to show that the heart had been embalmed and mummified. The breakdown of the tissue is likely to be because of an incomplete seal on the iron box.[1]

GC–MS analysis of the powder revealed the presence of triterpenoid compounds including α- and ß-boswellic acids characteristic of frankincense. In addition, GC–MS analysis detected phenolic derivative compounds indicating the presence of creosote, noted for its antiseptic and preservative properties. Other biomedical techniques suggested the use of daisy, mint, mercury and lime.

The results indicate that the aim was to preserve the tissues, with materials inspired by biblical texts, according to the authors. The findings are important as they provide insight into the post-mortem processes used at that time.

Reference

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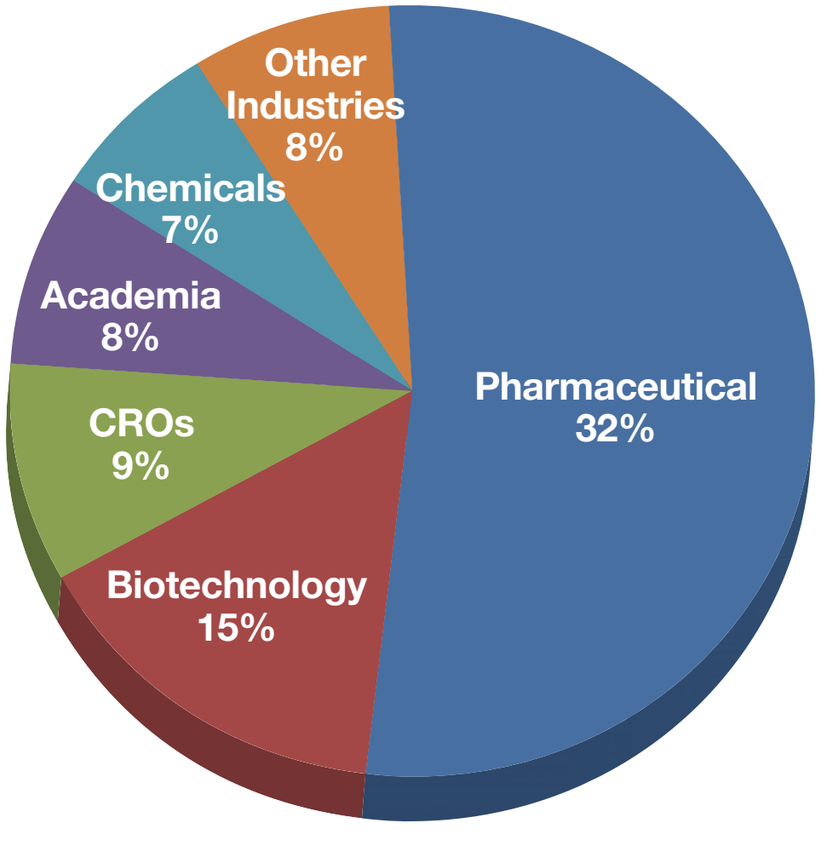
Photo Credit: tgden/Getty Images

Market Profile:
Flash Chromatography

Flash chromatography is a type of preparative liquid chromatography commonly used in the separation of organic compounds. It utilizes a plastic column filled with some form of solid support, usually silica gel, with the sample to be separated placed on top of this support. The rest of the column is filled with an isocratic or gradient solvent that, with the help of pressure, enables the sample to run through the column and become separated.



2012 Flash Chromatography Demand by Industry

Flash chromatography has become a popular method of normal-phase separation through purification. While flash chromatography is typically a low-pressure technique, scientists are using vacuums or pumps at medium pressures to speed up the separation process. The columns are packed with a silica adsorbent of defined particle size, usually between 30–60 µm, although other packings with other particle sizes are also used. Mobile phases with low viscosity require smaller particle sizes.

Demand for flash chromatography comes primarily from the pharmaceutical industry and accounts for over half of the market. Biotechnology, CRO, academia and chemical industries complete the top 5 demand for flash chromatography. The technique is commonly used for the separation of synthetic organic compounds, which are particularly useful in drug discovery. Flash chromatography systems assist laboratories focusing on combinatorial chemistry to streamline their purification steps.

The foregoing data was extracted and adapted from SDi's recently published report titled *Laboratory Sample Preparation Techniques: Breaking the Productivity Bottleneck - Prep Chromatography, Extraction & Concentration*. For more information, contact Glenn Cudiamat, VP of Research Services, Strategic Directions International, Inc., 6242 Westchester Parkway, Suite 100, Los Angeles, California 90045, USA, tel: +1 310 641 4982, fax: +1 310 641 8851, e-mail: cudiamat@strategic-directions.com

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Tips & Tricks:
GPC/SEC Method Optimization

Daniela Held, PSS Polymer Standards Service GmbH, Mainz, Germany.

During the development of a gel permeation chromatography/size exclusion chromatography (GPC/SEC) method, when selecting the optimum stationary and mobile phase an optimization of the default conditions is a good idea. Depending on the sample molar mass and polydispersity several adjustments are recommended.

Many scientists consider the development of a gel permeation chromatography/size exclusion chromatography (GPC/SEC) method easy, especially when compared to high performance liquid chromatography (HPLC) applications. This is true in the sense that normally only isocratic conditions are applied, so that a time-consuming gradient optimization is not required. However, there are many measures that can be taken to improve the quality of an existing method and the reproducibility.

Once the stationary and mobile phases of a GPC/SEC method have been established (where the sample chemistry plays the most important role), method optimization with respect to sample molar mass and polydispersity is desirable. Special care is required, if high molar mass samples above 1 million dalton are to be analysed.

Stationary Phase, Solvent Additives and Modifiers

The importance of a matching stationary phase for a robust method has been discussed in previous GPC/SEC Tips & Tricks instalments.[1,2] GPC/SEC ideally relies on interaction-free chromatography. To reach this goal for a range of sample chemistries and molar masses, a variety of stationary phase polarities is required.

Many GPC/SEC methods, especially in medium polar and polar solvents (for example, DMF and water) require the addition of a low molar mass additive or modifiers.[2] Column manufacturers provide recommendations for the concentration of these additives that will work for the majority of samples. In some cases, this concentration might be either too high or too low. Therefore, varying the additive concentration may be useful to learn more about the influence of this concentration.

If the peak shape and the elution volume of the peak maximum do not change when a higher or lower concentration is used, the lowest additive concentration with constant conditions should be used. This helps to protect the pump seals or other parts of the instrumentation. If, on the other hand, a small change in additive concentration changes the peak shape and the elution volume, this indicates that the method is not robust and needs further development.

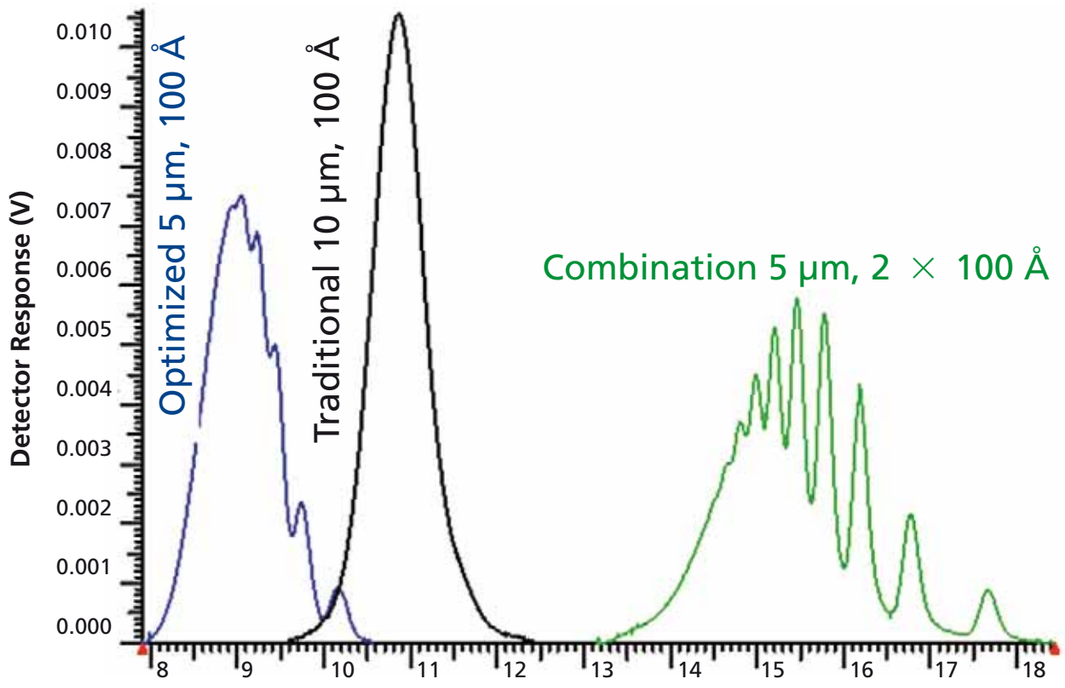
Tools to Increase the Resolution

Additional Columns: The accessible pore volume is the parameter that mainly influences the separation in GPC/SEC. Pore volume can be added by adding more separation columns to the system, with the disadvantage that the analysis time and solvent consumption will increase with the resolution.

For better resolution in the actual molar mass separation range, a column with the same porosity (for linear columns a column of the same type) has to be added.

To separate system peaks or residual monomer/solvent further away from potential oligomers, a column with a smaller porosity can be added. However, the combination of the porosities of the columns should be dislocation or

Figure 1: Oligomeric dextran separated on a traditional 10 µm aqueous phase (black line) and an optimized 5 µm phase with one (blue line) and two (green line) 100 Å columns. The reduction of the particle size and the flow rate dramatically increased the resolution.



mismatch free. Therefore, linear columns should **not** be combined with columns with single small porosities, as in the majority of cases this will result in a mismatch or a dislocation and artifical peak shapes.[3]

Smaller Particle Size: When applicable to sample molar mass and system back pressure, smaller particle sizes can be used to gain a better resolution. They are particularly useful for the analysis of oligomers, low molar mass polymers and proteins.

They should not be used if there is a danger of shear degradation, for example, when very high molar masses are analysed. However, recent data showed that the risk for shear degradation is less than previously reported, opening up new characterization paths.[4]

Flow Rate: The typical flow rate applied in GPC/SEC on analytical columns with

Table 1: Recommended sample concentrations with respect to molar mass and polydispersity index (PDI).

Sample	Guideline concentration [mg/mL], (%)
narrow PDI (100–10 000 Da)	2 (0.2%)
narrow PDI (10 000–1 000 000 Da)	2–1 (0.2–0.1%) (lower for higher molar masses)
narrow PDI (> 1 000 000 Da)	0.5 (0.05%) (lower for higher molar masses)
broad PDI (2 and higher)	4–5 (0.4–0.5%)

an inner diameter between 7.5 mm and 8 mm is 1 mL/min. This flow rate is a good compromise between time and eluent consumption and resolution. However, lower flow rates can be advantageous to increase the resolution.

A reduced flow-rate is required for high molar mass samples. As a rule of thumb, above 1 million dalton a flow-rate of 0.5 mL/min or less should be used. This reduces the risk for potential shear degradation and irregular retention at the same time.

Temperature: When using highly viscous solvents like DMAc, a temperature increase improves the separation because of the reduced solvent viscosity. Only a few polymers, for example, polyethylene glycol in aqueous systems, show better resolution at lower temperatures. To ensure trouble-free detection, the columns and detector temperature should be 15–20 °C lower than the boiling point of the solvent.

Figure 1 shows the separation of an oligomeric dextran on an aqueous GPC/SEC system. Separation on a traditional 10-µm column material (black line) was compared to separation on the latest 5-µm column material with one (blue line) or two (green line) separation columns. To obtain the highest resolution, a reduced flow rate of 0.25 mL/min has been applied.

Tools to Increase the Raw Data Quality

A high signal-to-noise ratio can be obtained by using high-quality detectors and by increasing the overall injected mass, which is the product of sample concentration and injected volume.

Table 2: Recommended injection volume with respect to number of analytical separation columns.

Number of analytical columns	Guideline injection volume (µL)
4, 5 or more	200–250
3	100
2	50
1	20

However for good chromatography, the injected mass should be as low as possible.

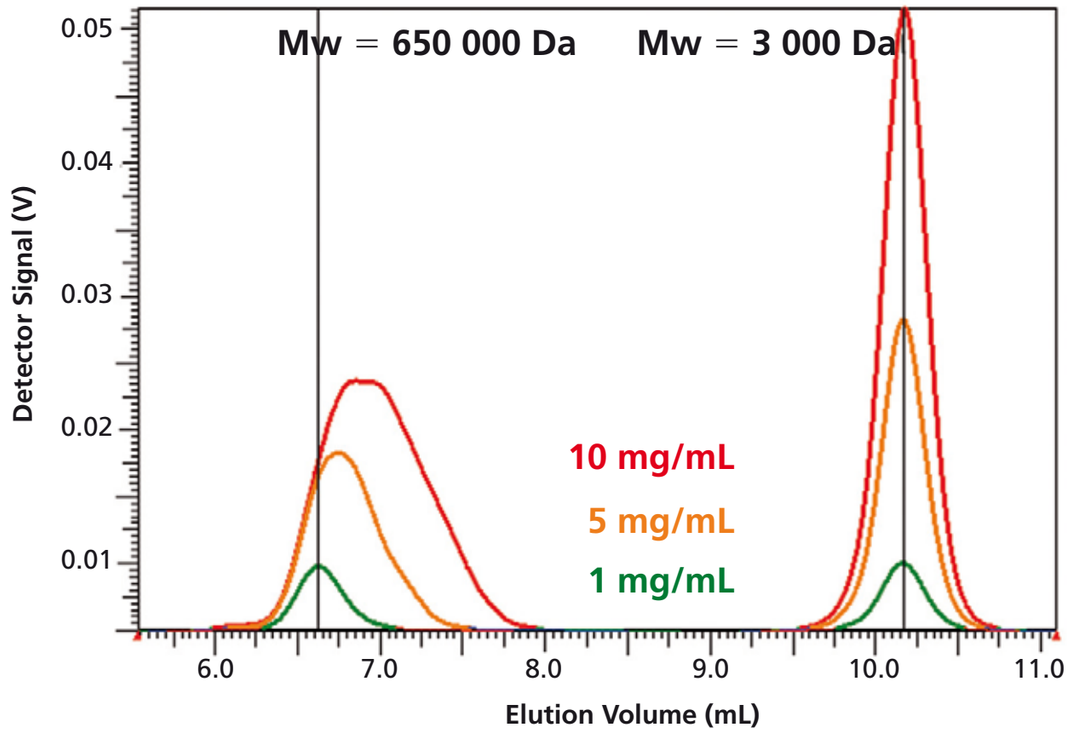
As it is always better to see something in the detector than not to be able to analyse at all, users of GPC/SEC and hyphenated techniques should be aware of the consequences when they increase concentration or injection volume. Too high an injected mass can produce artifical peak shapes and wrong elution. These effects are more pronounced for higher molar masses. Figure 2 shows the effect of an increased concentration for two samples with a low polydispersity around D = 1.1 and with two different molar masses. While the elution volume of the peak maximum and the signal shape change only slightly for the low molar mass sample, the high molar mass sample shows a shift to higher elution volume and artifical shoulders. Not only does the elution volume shift, but also the chromatography can be disturbed, resulting in erroneous distribution information, even when using such techniques as triple detection or on-line light scattering.

Tables 1 and 2 show the recommendations for sample concentration and injection volume depending on the sample molar mass and polydispersity and on the number of analytical columns used for separation.

These conditions should be applied if the raw data quality and the signal-to-noise ratio for all detectors allows the determination of results with low result uncertainty.[5] If one or more detector requires more injected molar mass they can be changed depending on the sample molar mass:

- For high molar masses the injection volume can be increased while the concentration should be kept as low as possible. A low concentration ensures that

Figure 2: Concentration effect for a 3000 Da and 650,000 Da sample. Increasing the concentration too much results in a peak shift and artificial shoulders, especially for higher molar masses.



the individual polymer chains can occupy the hydrodynamic volume corresponding to their molar mass.

• For low molar masses and oligomers the sample concentration can be increased while the injection volume can be kept as low as possible. A small injection band has advantages for a good separation.

Fortunately the risk of having too high an injected mass is less for samples with a higher polydispersity. Therefore, for many industrial samples the concentration can be increased significantly.

As usual there are some exceptions to the given general recommendations. The values in the tables are based on flexible, linear chains. As it is the viscosity of the sample that mainly influences the elution behaviour, molar mass is only one of the parameters. Branching and chain stiffness must also be taken into account.

Because of the lower viscosity of a branched sample compared to that of a linear one of the same molar mass, branched samples can be analysed at higher concentrations than their linear counterparts. On the other hand, for stiff chains the concentrations above might already be too high and these samples might require an even lower concentration.

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Authentication of Organic Milk

Robert Packer and Avinash Dalmia, PerkinElmer, Shelton, Connecticut, USA.

A growing number of people are purchasing organic milk, partly because of concerns over substances added intentionally or unintentionally to conventional milk. This, coupled with high food and fuel prices, has led to shortages in organic milk supplies. As demand outstrips supply, a gallon of organic milk can cost 25–100% more than conventional milk, which presents the opportunity to defraud consumers. This article illustrates how chromatographic and mass spectrometry techniques can be used to establish milk authenticity.

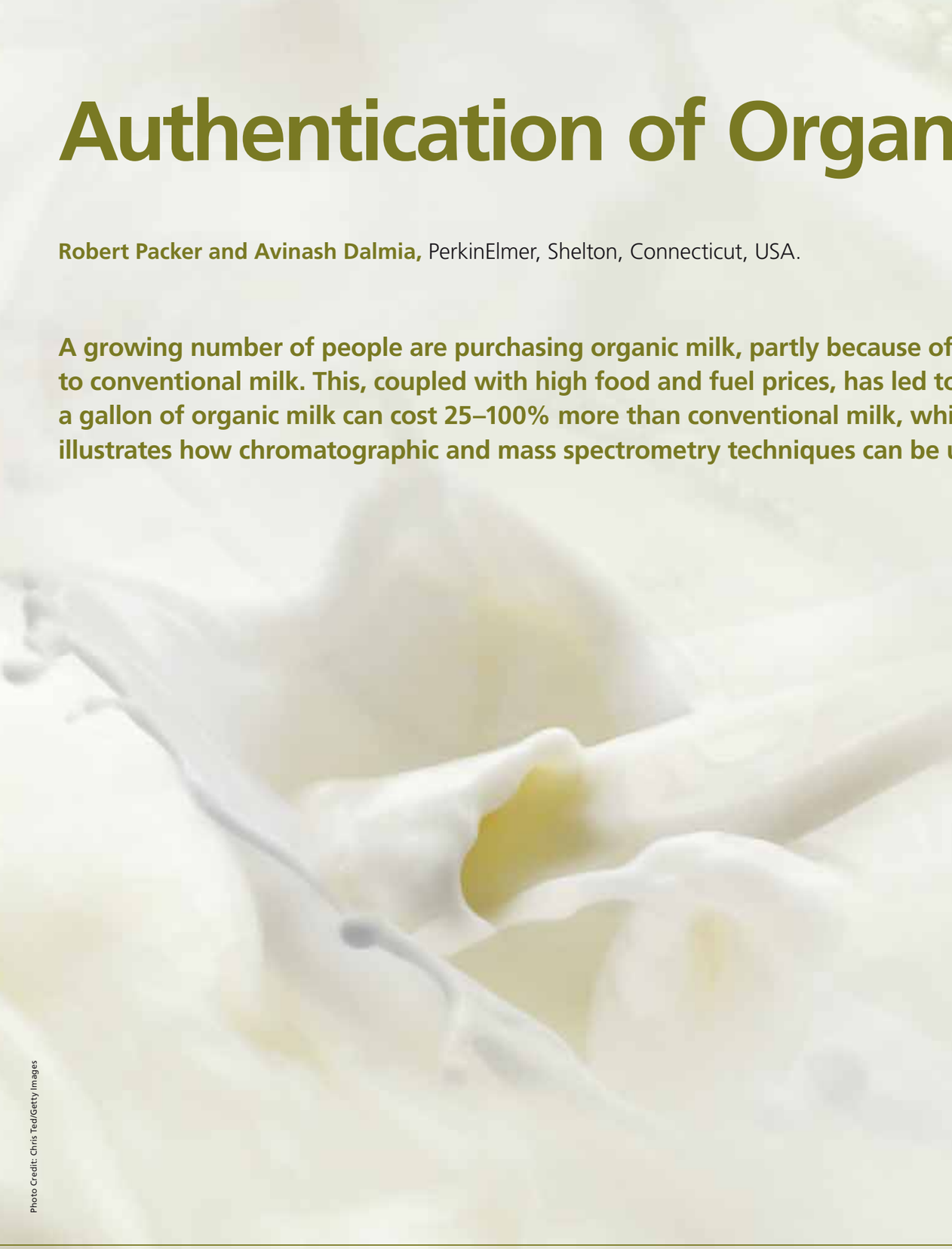


Photo Credit: Chris Ted/Getty Images

Sales of organic whole milk rose in the US by 17% between January 2011 and October 2011, with reduced-fat organic milk increasing by 15%.[1] This surge in popularity, coupled with high food and fuel prices, has caused shortages in the supply of organic milk.[2]

In the US and Europe, stricter policing of farming practices has been adopted to ensure authenticity, but this is not the case for all countries.

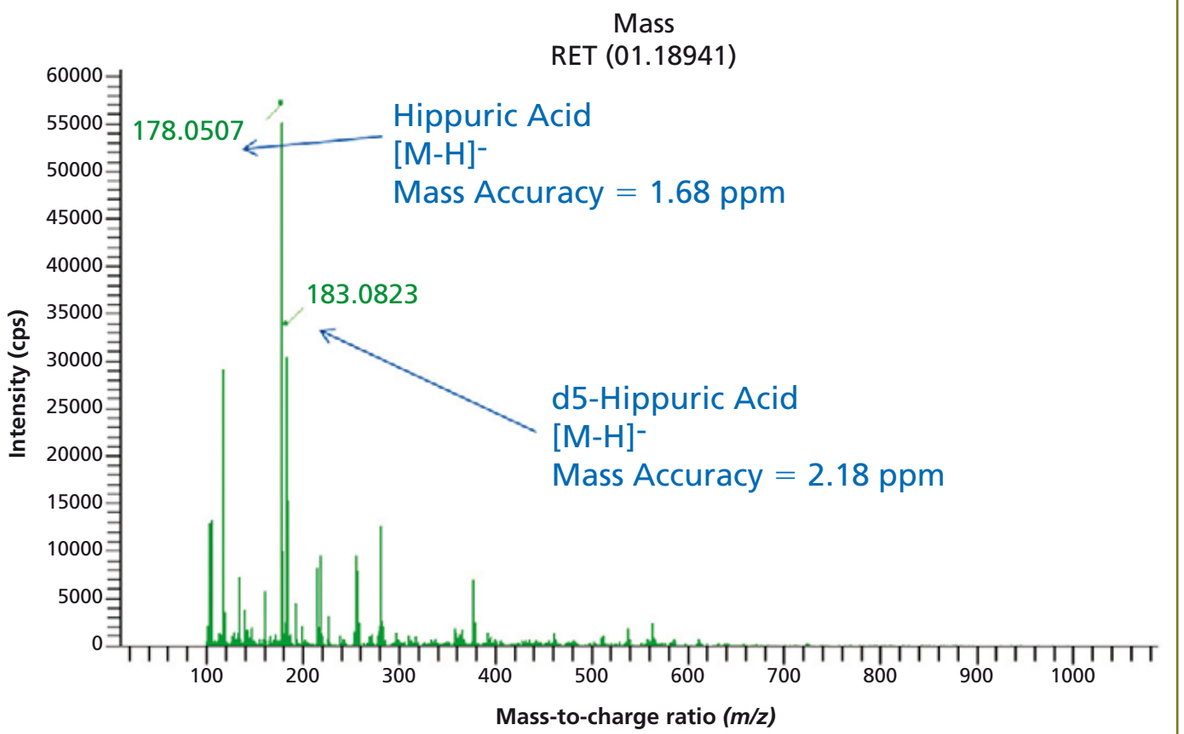
As imports of organic milk powder are growing, fraudulent powders can find their way into the West through distributors or processed foods, such as chocolates. These products also command a higher price if labelled as organic. These substitutions do not commonly cause health problems, but consumers are defrauded and hardworking organic farmers lose business.

What is the Difference Between Organic and Non-organic Milk?

When producing organic milk, farmers must adhere to specific rules, and these vary from country to country. Here are the guidelines for the USA:

- *Grazing time* — Farmers have to ensure that at least 30% of their cows' diet comes from pasture grass during the mandatory grazing season (no less than 120 days).[3]
- *Antibiotic use* — Organic dairy cows are not to be routinely treated with antibiotics. If required, the animal is not allowed back in the milk production rotation until 12 months of antibiotic-free certification have passed.
- *Bovine Growth Hormone (BGH)* — Dairy cows from organic farms are not allowed shots of BGH.
- *Pesticide use* — The use of pesticides on an organic dairy farm is forbidden.

Figure 1: Representative mass spectra of an organic milk sample spiked with 5 ppm internal hippuric acid standard.



The organic cow cannot consume pesticide-treated feed.[4]

How Can We Detect This?

Chromatographic techniques, such as liquid chromatography–mass spectrometry (LC–MS) and high performance liquid chromatography (HPLC), can be used to detect traces of pesticides, antibiotics or even growth hormones in the milk or animal feed. Determining if a cow has been fed a predominately commercial feed diet, rather than fresh grass or silage, is more difficult. One of the techniques used to characterize organic compared to non-organic milk is isotopic ratio mass spectrometry (IRMS).

IRMS can identify the type and even origin of feed, but requires large databases and has not been explored in enough depth to make definitive conclusions. Recent work has been looking at the levels of minor acids in the milk. One study used gas chromatography–mass spectrometry (GC–MS) to measure levels of phytanic acid in organic and non-organic milk. Organic cows consume more fresh green matter than non-organic cows, so consume higher levels of phytol. which is part of chlorophyll. Phytol is broken down in ruminant's stomachs to phytanic acid. The study found that, on average, organic milk had double the amount of phytanic acid levels than conventional (approximately 300 mg/100 g for organic milk compared with 150 mg/100 g for non-organic milk).[5] Another study focused on goats' milk proposed measuring hippuric acid, which is suggested to be found at higher levels if more grass and silage were consumed.[6]

Table 1: Experimental parameters for the analysis of hippuric acid in milk.

DSA Parameters	Value
Heater temperature	350 °C
Auxiliary gas pressure	80 psi
Drying gas flow rate	3 L/min
Drying gas temperature	25 °C
Corona current	-5 µA
TOF Parameters	**Value**
Mode	Pulse (Negative)
Mass range	100–700 *m/z*
Capillary exit voltage	-100 V

Figure 2: Representative extracted ion chromatogram (EIC) of hippuric acid and d5-hippuric acid (5 mg/L) for the organic milk sample.

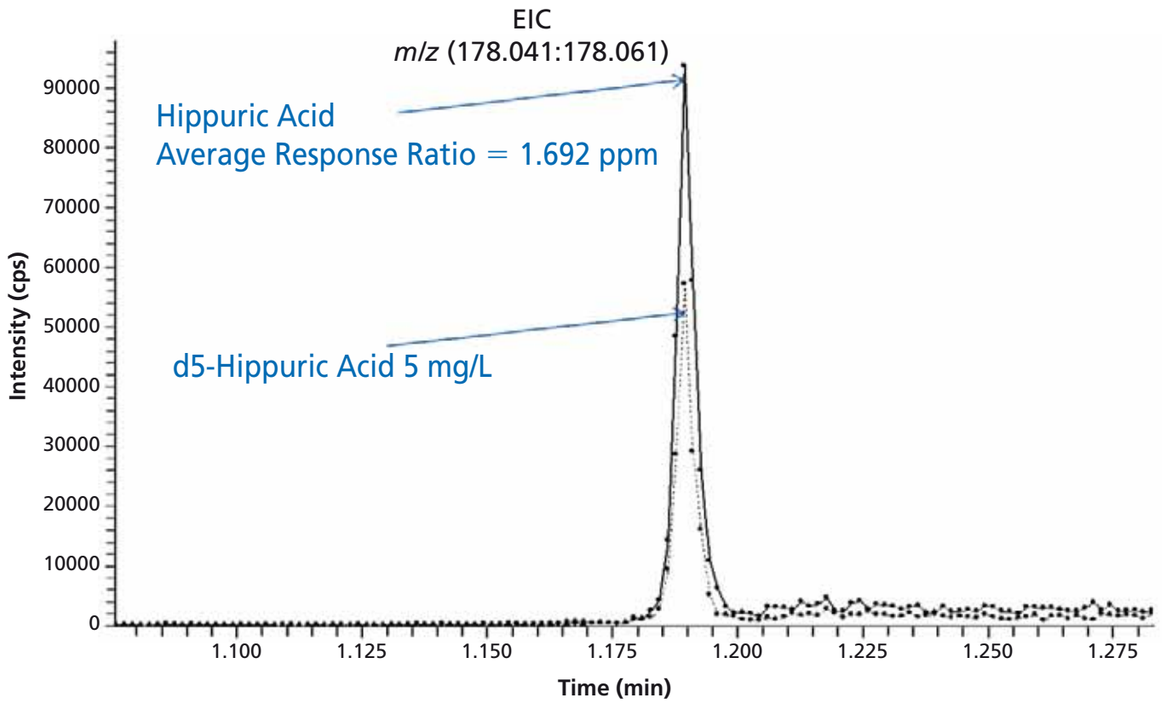
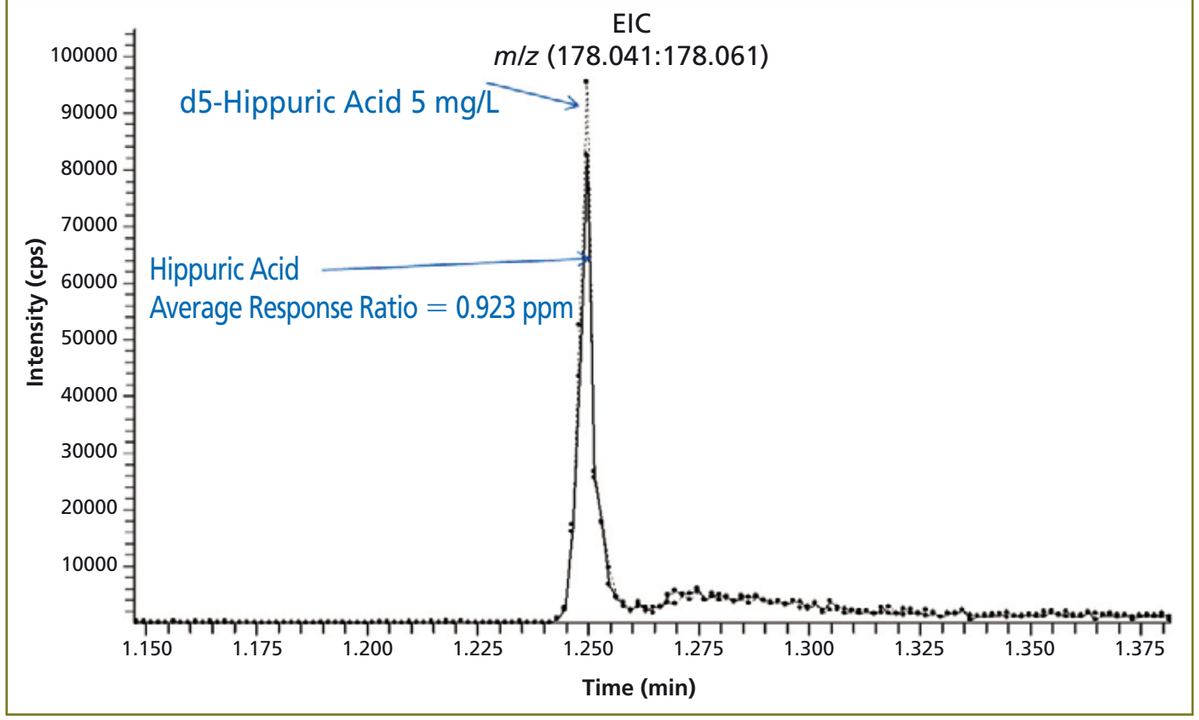


Figure 3: Representative extracted ion chromatogram of hippuric acid and d5-hippuric acid (5 mg/L) for the conventional milk sample.



Experimental

Most of these techniques involve some time waiting for separations to take place. In this investigation, ambient ionization mass spectrometry using an AxION Direct Sample Analysis (DSA) system (PerkinElmer) integrated with an AxION time of flight (TOF) mass spectrometer (PerkinElmer) was performed. Samples were directly ionized and drawn straight into the mass spectrometer to reduce the time of analysis to 30 s.

Three organic and three conventional milk samples were purchased from a local supermarket. All milk samples were subjected to the same preparation: 1 mL of milk was diluted with 2 mL of acetonitrile and 1 mL of methanol to carry out a protein precipitation. The samples were then centrifuged for 10 min at 7800 RPM. Finally, 1 mL of the resulting supernatant was diluted and then spiked with an internal standard, d5-hippuric acid, to give final concentrations of 5 mg/L of internal standard in each sample. Ten µL of each protein precipitated sample was then pipetted directly onto the stainless steel mesh of the system ready for ionization and analysis. The experimental parameters are shown in Table 1.

Results

Figure 1 shows the mass spectra of an organic milk sample. It is clear that the dominant signals are from the hippuric acid and the deuterium substituted hippuric acid standard.

If these peaks are examined in more detail and then overlaid as shown in Figure 2, it can be seen from the area of the peaks that the response from the hippuric acid is 1.692 times that of the deuterated standard. If this is repeated for conventional milk (Figure 3), we see that the ratio is closer to 1 at 0.932.

Discussion

This was repeated for all 6 samples with the results shown in Table 2. For all

Table 2: Levels of hippuric acid in organic and conventional milk samples.

Type of milk	Response ratio with respect to 5 mg/L d-5 hippuric acid	Hippuric acid in milk (mg/L)
Organic milk brand 1	1.692	33.48
Organic milk brand 2	1.837	36.73
Generic organic milk brand 3	**0.864**	**17.28**
Conventional milk brand 1	0.923	18.45
Conventional milk brand 2	0.998	19.96
Conventional milk brand 3	1.075	21.51

three samples of conventional milk, the hippuric acid concentration was on a average 1:1 (v/v) ratio with the reference standard. Factoring in dilution, this means that hippuric acid concentrations in the conventional milk were around 20 mg/L. For organic milk samples 1 and 2, it was clear that the levels of hippuric acid were higher as previously hypothesized. These samples had around 35 mg/L which is approximately 1.75 times that of conventional milk. One organic milk sample (sample 3) even had lower levels than conventional milk. This could be because this particular sample was the supermarket "home" or generic brand and therefore not as organic as suggested on the label. Ideally a wider study is needed to support this theory. For example: split a herd of cows into two groups, feeding one group an organic diet and the other a conventional feed-based diet over a year. Over the year, hippuric acid levels could be measured in the resulting milk, in a similar way to that used for the goats' milk study.[6]

Conclusion

This work has shown that it is possible to measure hippuric acid levels in milk by ambient ionization TOF–MS using a reference standard to ascertain relative concentrations (rather than using a calibration curve). There is also evidence that hippuric acid levels could be used to determin whether cows have been fed an organic diet; however, a much wider study would be needed to prove this conclusively.

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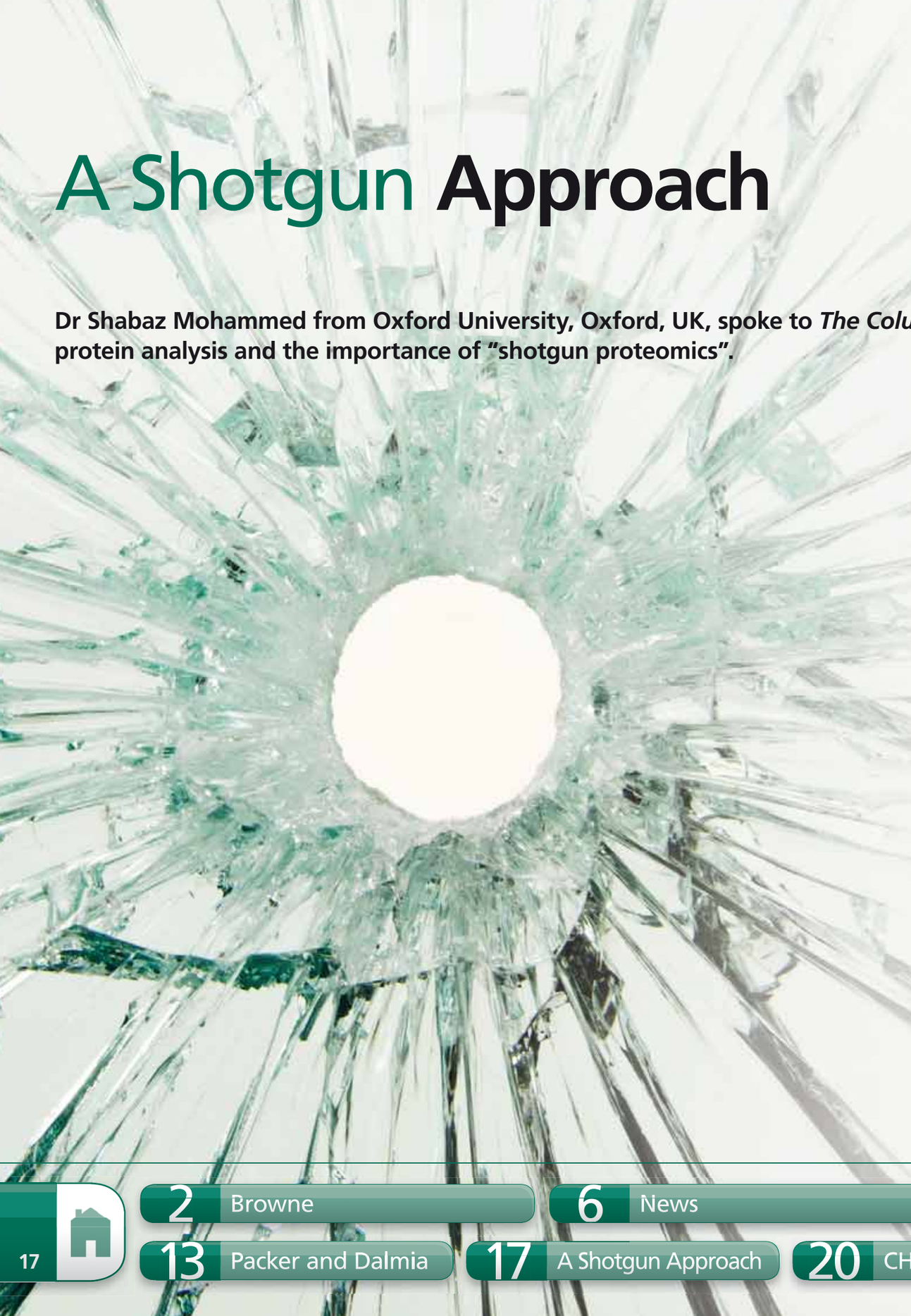
A Shotgun **Approach**

Dr Shabaz Mohammed from Oxford University, Oxford, UK, spoke to *The Column* about his group's activities in protein analysis and the importance of "shotgun proteomics".



Q: Why are you interested in researching proteins?

A: Proteins represent a major class of biomolecules that aid in the functioning of cells. Their activity is modulated by changes to their structure that are performed by the chemical modification of various amino acids. Such changes are often referred to as post-translational modifications or PTMs. Each cell contains over 10000 proteins spanning seven orders of magnitude in abundance: the proteome. Each protein can be present in multiple forms because of these PTMs. The primary mandate of our group is to develop and improve protein characterization and quantitation techniques. Such complexity, both in terms of number of unique biomolecules and variety in chemical composition, requires complexity reduction through the development of separations/enrichment and improvements in mass spectrometric characterization techniques.

Q: Shotgun proteomics is considered to be a rapidly growing field of research, rivalling that of genomics. Do you agree and, if so, why do you think this is?

A: Certainly, shotgun proteomics is the *de facto* method to characterize proteomes. Advances in mass spectrometers and the required computational tools have also improved dramatically. The current generation of mass spectrometers are capable of sequencing up to 50 peptides per second and can detect over 4 orders of magnitude. A single proteomic experiment will now generate millions of sequencing events. As our understanding of the generated data has improved so have the algorithms. We can now, without human intervention, assign peptide sequences to the mass spectrometric data for the majority of the common peptide classes. These same algorithms can also then assign these

Photo Credit: Richard Goerg/Getty Images

peptides to proteins with the help of genomic data. The current state of play is that within a week, we can now identify and quantify over 10000 proteins. Our laboratory and a few others can perform the same task in a few days primarily as a result of further improvements in chromatography. This level of data is rivalling the level of information generated by genomics yet proteins are the main protagonists in a cell. Thus, we can now study the behaviour of cells in a more biologically relevant manner in the context of their environments and their reactions to stimuli.

Q: Shotgun proteomics could potentially be used in biomarker identification methods in the diagnosis of disease. Do you think this is feasible in a clinical setting? Are there other potential applications?

A: A significant number of diseases and cancers are caused by proteins malfunctioning which has a direct knoc-on effect on other proteins. Detecting the malfunctioning of a protein and its direct consequences is useful for both diagnosis and prognosis as well as therapies. A number of these proteins can be shred from tumours into the bloodstream too. The massive improvements observed in proteomics are now allowing efforts to be made to detect such aberrant processes initially in cell lines and tumours but also, ultimately, in blood. Of course, understanding how cellular systems operate (and their impact on physiology) is the greater goal.

Q: You have recently optimized a protocol for the use of Zwitterionic chromatography-cholinate hydrophilic interaction chromatography (ZIC-cHILIC) for sample separation in proteomics analysis. Can you outline the basic principles of the approach you used and how this is different to currently used protocols?

A: A proteomic experiment usually involves a number of rounds of peptide fractionation followed by LC–MS where the optimal configuration for this final step is the use of nanolitre flow-rate reversed-phase (RP) chromatography and a high sequencing rate mass spectrometer with an electrospray source. The upstream fractionation must be orthogonal to this reversed-phase chromatography. Furthermore, sensitivity of an experiment depends heavily on minimizing surface areas and sample dilution. Developing a high-resolution separation that operates at very low flow rates is desirable. Ion exchange chromatography (IEC) has been the preferred strategy for a decade. It has excellent orthogonality to RP but has a poor separation power for peptides and is difficult to miniaturize. ZIC-cHILIC allowed us to operate with salt-free eluents thus allowing miniaturization, and it also provides an excellent separation that is mostly orthogonal to reversed-phase chromatography.

Q: Single-cell proteome analysis is the ultimate goal for the development of research protocols. How will optimization of sample preparation protocols advance research to this?

A: The ability to analyse material levels approaching single cell populations is the holy grail of most biotechniques. However, most cell behaviour depends on their immediate environment and they are also constantly communicating with their surrounding cells. I would suggest the goal has slightly changed to measuring the proteomes of cell populations at the resolution of a single cell. Nevertheless, the argument still boils down to sample handling, reducing sample losses, improving sensitivity and speeding up the process. Miniaturization and automation will improve sample handling and that requires constant improvement in separation power and robustness. Through improving separations upstream sample manipulation can be reduced. The added bonus of better separations is sharper peaks which lead to less ion suppression in mass spectrometry, improved dynamic range and better sensitivity. Better separation also means faster analysis, which is important because sample throughput is a major challenge too.

Q: Is it plausible that with the information encoded within the proteome of a single cell that disease biomarkers may be identified?

A: I don't think a single cell is particularly informative; however, catalogues of shred proteins from tumours into the blood stream are being generated in a number of proteomic laboratories. Enzyme-linked immunoassay absorbent (ELISA) has shown that such proteins can be used for prognosis/diagnosis of diseases and, I believe, the current improvements in proteomics will allow the mass spectrometer to replace

ELISA with the added benefits of superior specificity, sensitivity and reproducibility.

Q: What are the challenges you face in your field of research?

A: Although great strides are being made in the analytical tools for the characterization of proteomes they are far from comprehensive. Over 200 PTMs exist and methods are only available for a handful. Much work needs to be done before we have a complete picture. An equally significant challenge is stitching all the data together. Bioinformatics is always lagging a little behind what can be experimentally generated, which is primarily a cause of the unknown territory that is being explored.

Q: Is there anything you would like to add?

A: I would add that there is great excitement in the field because we can now identify proteins at a similar depth as genomics and there is now much we can explore about the cell that wasn't possible in the past. Furthermore, there are still many things to explore in terms of analytical tools to address the trials ahead. Like all scientists, we love a challenge and it's great because there is a feeling we are up to the task.

Dr Shabaz Mohammed received his PhD specializing in mass spectrometry from the University of Manchester, Manchester, UK, before joining the University of South Denmark as a post-doctoral researcher. He was an assistant professor at Utrecht University, Utrecht, The Netherlands, and a theme leader at the Netherlands Proteomics Centre until 2013. He is now a group leader at the University of Oxford, Oxford, UK. Mohammed's primary research focus is on the development of proteomics techniques to answer biological questions.

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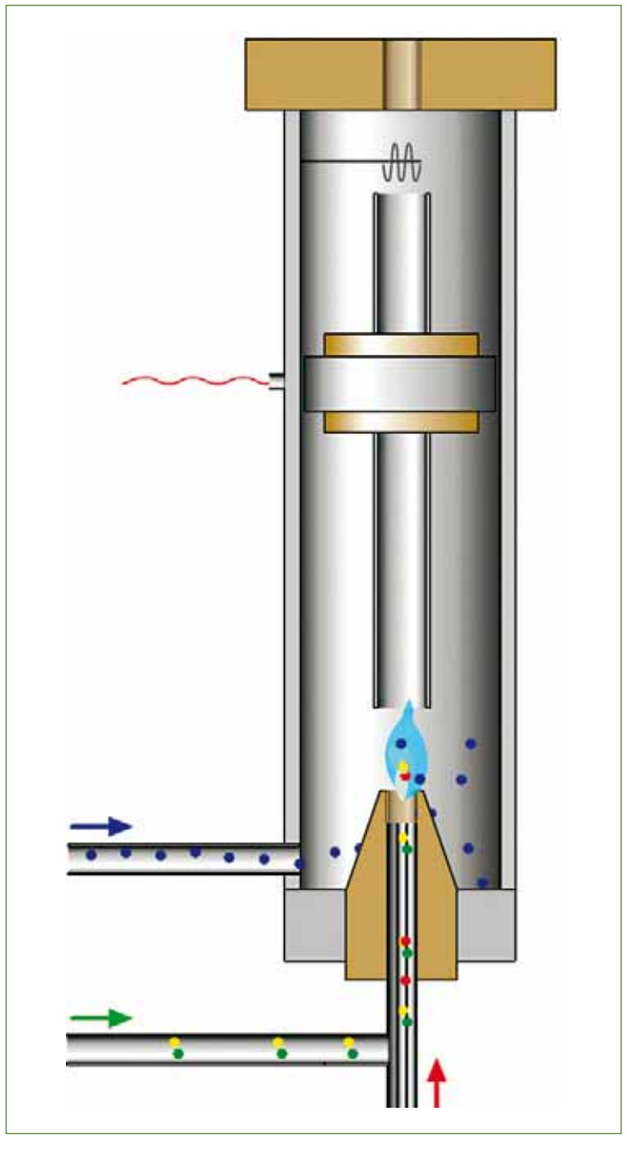

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