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The Column

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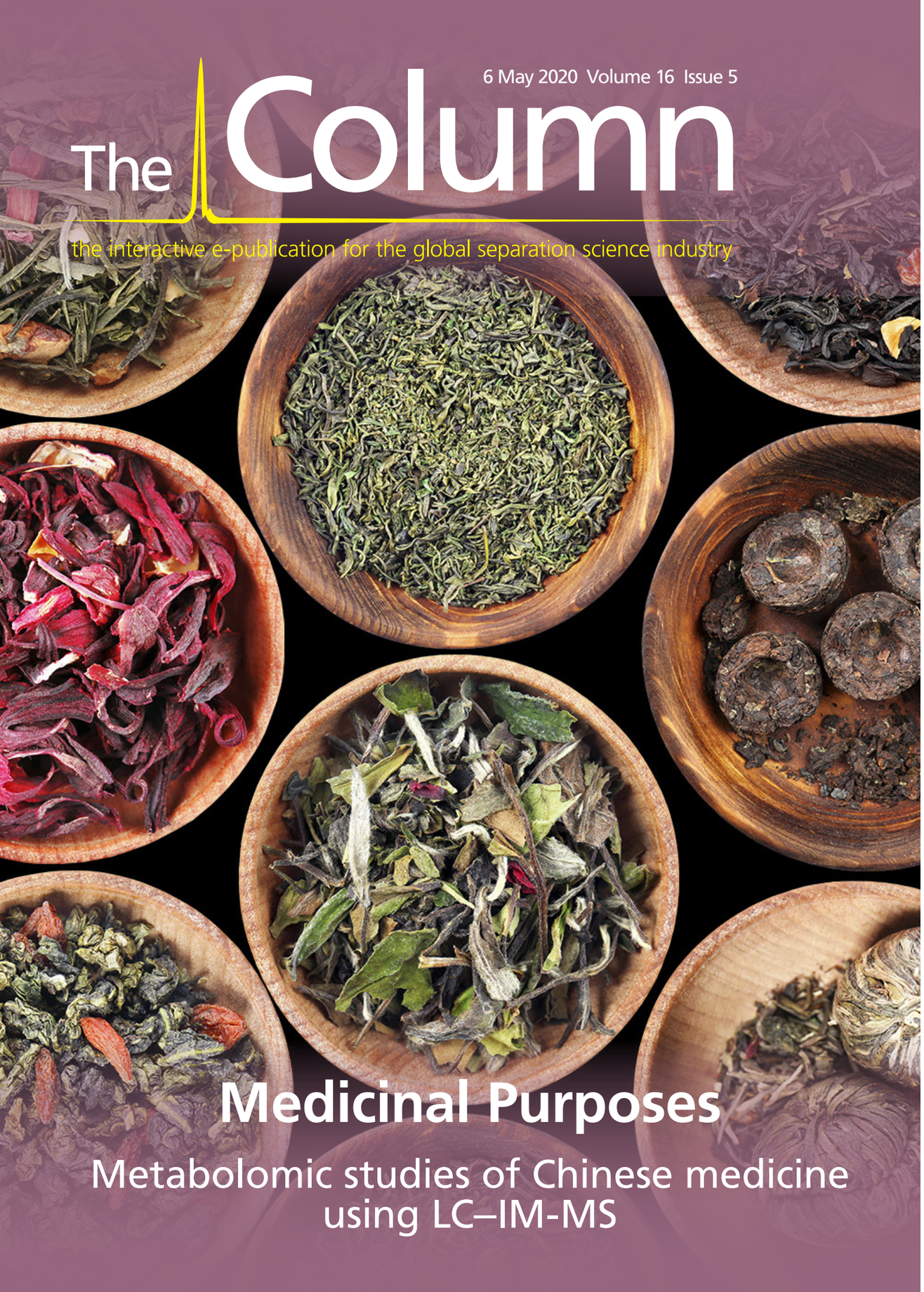


Medicinal Purposes

Metabolomic studies of Chinese medicine using LC–IM-MS

Cover Story

Features

Regulars

Metabolome Studies of Herbal Medicine Using High Performance Liquid Chromatography Ion-Mobility Mass Spectrometry

Lisa Mahdi and Oliver J. Schmitz, Applied Analytical Chemistry, University of Duisburg-Essen, Essen, Germany

In Chinese herbal medicine (CHM), traditionally, *Hedyotis diffusa* and *Scutellaria barbata* are cooked together and given to patients as tea. It is hypothesized that the interaction of metabolites from both herbs during cooking, improves the medical effect. This hypothesis was tested by preparing two tea variants which were then analyzed using high performance liquid chromatography (HPLC) coupled to ion-mobility mass spectrometry (IM-MS).

According to the World Health Organization (WHO), 70–80% of the world's population, or approximately five billion people living mainly in developing countries, are treated with herbal medicines as primary care (1). It is claimed that the combination of specific herbs results in mutual assistance, restraint, suppression, or antagonism leading to

improved medical effects or a reduction of negative side effects (2). Possible modes of actions between the different herbs have been hypothesized (3). However, the impact of these interactions on the metabolite composition and on the medical effects of certain extracts remain unclear.

The annual herb *Hedyotis diffusa* (family Rubiaceae, also known as *Oldenlandia diffusa*) contains various important phytochemicals including iridoids, triterpenes, flavonoids, anthraquinones, steroles, cyclotides, coumarins, and alkaloids (4). The perennial herb *Scutellaria barbata* (family Lamiaceae chin.: "Ban-Zhi-Lian") is one of the most important ingredients in Chinese herbal medicine (CHM). The dried aerial part of the plant is used for medical purposes. The compounds of *Scutellaria barbata* mainly comprise flavonoids, alkaloids, diterpenoids, and sugars. Important components known so far, as well as the current findings regarding their antitumour activity, are summarized and listed by Wang et al. (5). Both herbs are distributed in northeast Asia and commonly used in CHM for a variety of therapeutic activities including anti-cancer, anti-inflammation, and detoxification (6–10). In CHM, a mixture of both herbs is given to patients as a tea to treat different infections including hepatitis, sore throat, appendicitis, urethral infection, and certain cancer types (6–10).

The most commonly-used techniques for qualification and quantification of components in a complex sample are based on separation techniques such as gas chromatography (GC) or liquid chromatography (LC). However, using these methods alone can limit the separation of all components of a complex sample. To extend the separation power and provide more information on the separated compounds, multidimensional chromatographic techniques such as two-dimensional gas chromatography (GC×GC) or two-dimensional liquid chromatography (LC×LC) have been combined with different mass spectrometric (MS) techniques. A novel approach combines one-dimensional (1D-) or two-dimensional (2D-) high performance liquid chromatography (HPLC) with ion-mobility mass spectrometry (IM-MS). IM-MS allows the separation of ions according to their size-to-charge ratio as well as their mass-to-charge ratio (*m/z*). This enables the qualitative and quantitative detection of an increased number of components including isobaric species (11,12).

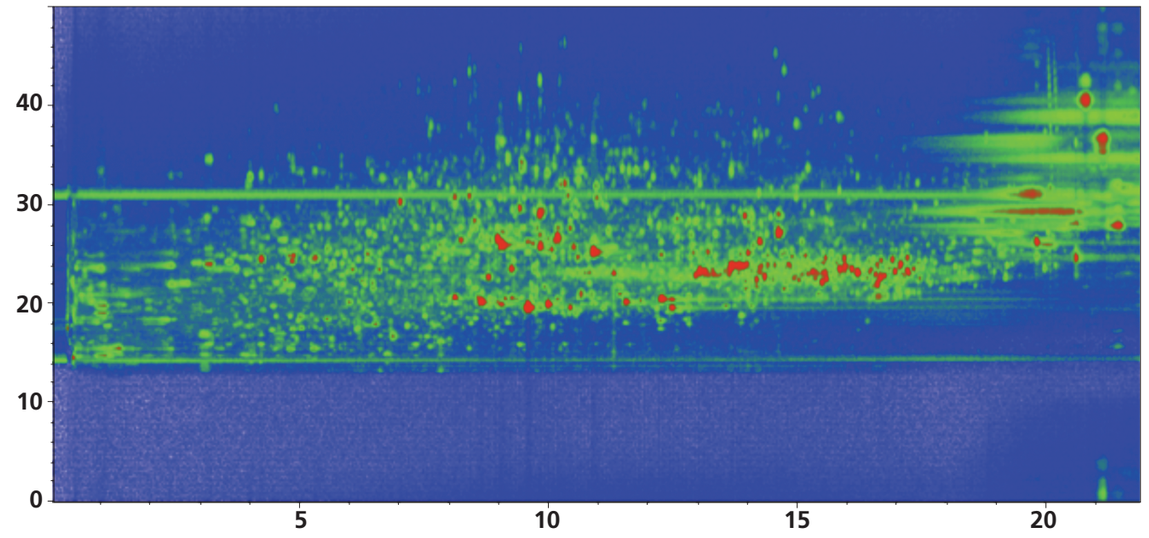
Experimental

In order to compare the metabolic compounds, two tea variants were prepared. For one tea sample, both herbs were cooked together, while for the other tea sample, the herbs were cooked separately and mixed together afterwards. Both tea samples were measured three times with a 6560 Ion Mobility QTOF Mass Spectrometer (Agilent Technologies) system coupled with a 1290 Infinity II UHPLC system (Agilent

Table 1: Column information and method parameters for LC–IM-QTOF-MS analysis

	Column	C18 column: 50 mm × 2.1 mm, 2.6-µm
	Solvent	(A) Water containing 0.1% formic acid (FA) and 5% methanol (B) methanol containing 0.1% FA
	Flow rate	0.3 mL/min
	Linear gradient	1 min: 100% (A) / 0% (B) 20 min: 5% (A) / 95% (B) 21 min: 5% (A) / 95% (B) 22 min: 0% (A) / 100% (B)
	Injection volume	10 µL
	IM-QTOF-MS	
	Ion source	Dual ATs ESI
	ESI mode	Positive
Dual ATS ESI	Gas temperature	200 °C
	Gas flow rate	3 L/min
	Nebulizer	20 psig
	Sheath gas temperature	325 °C
	Sheath gas flow rate	12 L/min
	Nozzle voltage	500 V
	V Cap	5000 V
IM Trap	Trap full time	2000 ms
	Trap release time	150 ms
	Drift gas	Nitrogen (3.95 torr)
	Max drift time	50 ms
	Mass range	50–1700 bd

Figure 1: LC–IM-QTOF-MS analysis heatmap of *Scutellaria barbata* and *Hedyotis diffusa* with the drift time (ms) on the *y*-axis versus the retention time (min) on the *x*-axis.



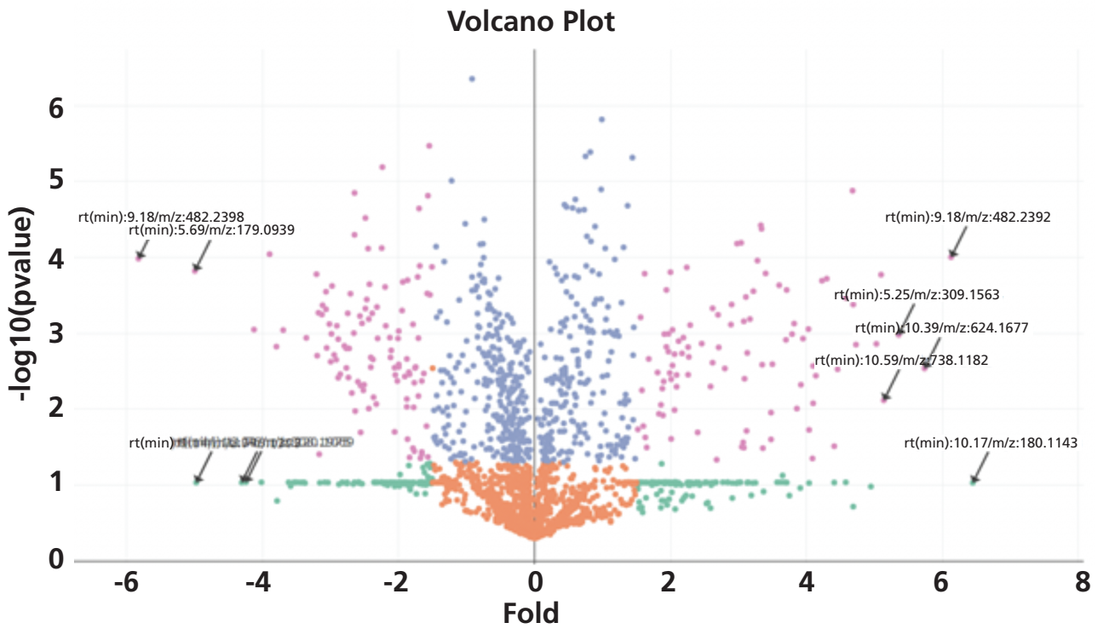
Technologies). The feature analysis was performed using IM-MS Browser B.07.01 software (Agilent Technologies). For detailed information, see Table 1.

Results

Figure 1 shows the heatmap of the HPLC-IM-quadrupole time-of-flight (QTOF)-MS analysis and demonstrates the separation power of HPLC coupled to IM-MS. Using the data obtained from the software for all six measurements (two tea sample variants with $n = 3$), a volcano plot was generated illustrating the feature volume differences between the two differently-prepared tea variants (Figure 2). This offers a quick overview on quantities of data.

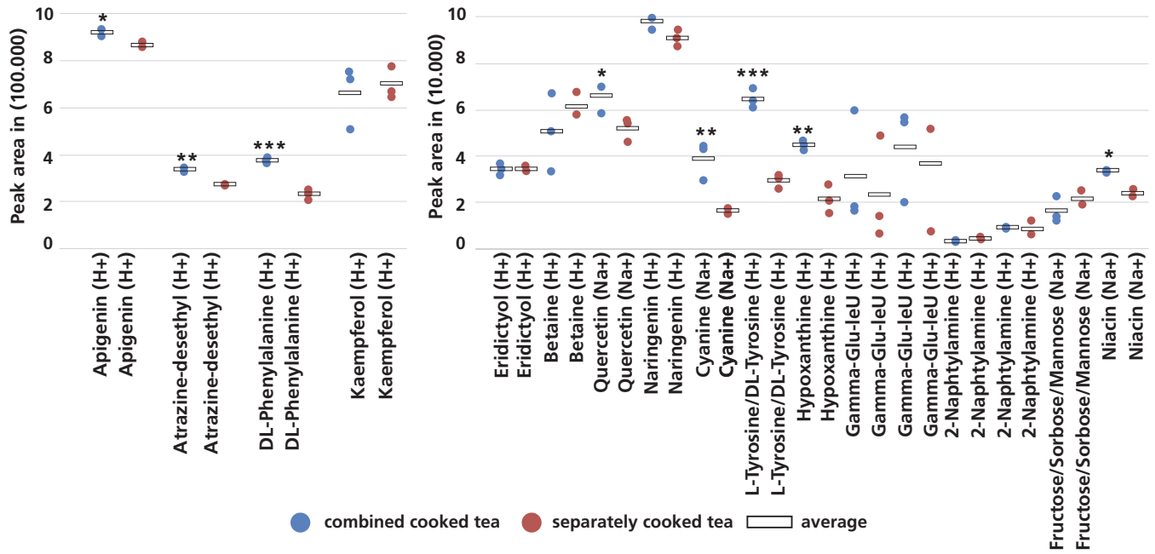
The location of each dot represents the relative volume of a detected feature. The volume describes the peak area sum of all ions created from one feature. The *x*-axis shows the abundance of each feature and the *y*-axis shows the statistical significance (*P*-value) of the abundance differences between separately cooked and combined cooked tea samples, respectively. Statistical significances were calculated using a Student's t-test. The presented data

Figure 2: Volcano plot showing the average volume of all detected features comparing the combined cooked tea with the separately cooked tea. Green dots show features that express volume differences in a fold change range; orange dots show features without significant volume differences; blue dots show features with significant volume differences; pink dots show features with significant volume differences in a fold change range. A Student's T-test was performed. Significant values show a *P*-value of < 0.05. Fold change differences are marked above a 1.5-fold threshold. Presented are features that showed peaks in at least two of three measurements.



demonstrate significant volume differences of around 200 features in a fold change range between both samples (only the determined features, which were found in at least two of the three different measurements, were used). These results strongly suggest differences in the "compound fingerprint" between the differently prepared teas and thus supports the hypothesis that the combined cooking of *Hedyotis diffusa* and *Scutellaria barbata* affects the compound composition and, in turn, the medical effects of the tea (2). Nevertheless, the correct detection of all peaks for every feature and every measurement has to be controlled manually in order to verify the results.

Figure 3: Relative amount of literature known compounds that were detected in the tea samples "combined cooked" (blue), and "separately cooked" (red), based on the respective peak areas. Each dot represents one measurement. The respective average is marked as a black bar. Significant differences were calculated comparing the two treatments for each compound by using Student's T-test and marked with asterisks (*< 0.05, **< 0.01 and ***<0.001 *P*-value).



For further evaluation, the peak areas of several known metabolites were measured manually, compared, and statistically analyzed by performing a Student's t-test. The results are presented in Figure 2. Some of the compounds detected in both samples show significant differences in the peak area (Figure 3). When both herbs were cooked together, the resulting tea contained a significantly larger amount of apigenin, atrazine-desethyl, DL-phenylalanine, quercetin, cyanine, DL-tyrosine, hypoxanthine, and niacin compared with the separately cooked tea. Flavones such as apigenin have long been described to have an anti-cancerogenic function. A lot of research has been performed in recent years on apenigin as a potential cancer prevention agent (13,14). For quercetin and kaempferol, mutualistic effects regarding their anti-cancerogenic activity have been described (15,16). Furthermore, niactin is reported to be important for genomic stability, with a great potential to reduce cancer risks (17). These results suggest that the combined cooking of *Hedyotis diffusa* and *Scutellaria barbata* is beneficial to the medical effects of the tea. Besides plant compounds, the synthetic compound cyanine and the herbicide atrazine-desethyl were detected and verified by collision cross section (CCS) values.

These results support our hypothesis that the combined cooking of *Scutellaria barbata* and *Hedyotis diffusa* causes changes in the metabolome composition of the herbal extract. The regulatory role of certain components on metabolome compositions and the underlying molecular mechanisms as well as potential changes in the medical effect remain to be elucidated in the future.

Conclusions

The results obtained by comparing the two samples clearly show significant differences regarding compounds and compound abundances. *Scutellaria barbata* and *Hedyotis diffusa* both contain large amounts of chemical compounds. Interaction of those compounds as well as the production of new compounds is therefore highly possible. The feature analysis revealed significant differences in the composition and abundance of features. The results obtained support our hypothesis, that the combined cooking of *Scutellaria barbata* and *Hedyotis diffusa* causes changes in the metabolome composition of the herbal extract. Non-target analysis has become an important tool in the field of complex samples, such as metabolomics, and food and beverage. For identification of compounds in such complex samples, often exact masses derived from high resolution mass spectrometric measurements coupled to liquid chromatography are used. The introduction of ion mobility spectrometry provides an additional separation dimension and allows the calculation of collision cross sections (CCS) of the analytes as a further physicochemical constant supporting the identification.

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Oliver J. Schmitz is a full professor at the University of Duisburg-Essen in Germany and is the chair of the Applied Analytical Chemistry group. In 2009 he cofounded a company that develops new ion sources and units to couple separation techniques with mass spectrometers. He is interested in the development of ion sources, the use and optimization of comprehensive LC, and coupling analytical techniques with ion mobility mass spectrometers. He was awarded the Gerhard-Hesse Prize for chromatography in 2013 and the Andrzej Waksmundzki Medal Award for Analytical Chemistry at the Polish Academy of Sciences and the Polish Chemical Society in 2018.

Lisa Mahdi obtained her Master's degree in biology at the University of Cologne and the Max Planck Institute for Plant Breeding Research Cologne (both in Germany) in 2018. Her interests include molecular plant-microbe interactions and cell death. The main objective of her Ph.D. is to characterize molecular mechanisms underlying root-microbe multispecies interactions and cell death regulation during the accommodation of beneficial and pathogenic microbes in the root.

E-mail: **oliver.schmitz@uni-due.de**
Website: **www.uni-due.de/aac; www.oliver-schmitz.net**

News

Agilent Sponsors the Nonprofit "My Green Lab"

Agilent Technologies, Inc. (Santa Clara, California) has become a sponsor of My Green Lab, a nonprofit organization dedicated to improving the sustainability of scientific research.

Agilent chose to support My Green Lab because of its "holistic approach to sustainability and their understanding of the interconnectivity between all the parts that make up a laboratory—from chemicals to instruments to the environment," according to the company.

"Sustainability is a key consideration in all our activities, and we look forward to ultimately passing on the benefits of our efforts and innovation to make better informed, sustainable choices", said Mike McMullen, Agilent CEO.

Agilent is currently working with My Green Lab to have the company's instruments independently audited for the organization's Accountability, Consistency, and Transparency (ACT) label. The ACT label provides information about the environmental impact of manufacturing, using, and disposing of a product and its packaging.

"We are really excited to be working with Agilent," My Green Lab CEO Allison Paradise said in a statement. "The fact that Agilent is willing to highlight sustainability through our ACT labelling program speaks to Agilent's leadership in this area."

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Investigating Contamination in Biocompatible LC Systems

Biocompatible liquid chromatography (LC) systems are generally regarded to be chemically more inert than conventional high-performance LC (HPLC) systems. However, a study into the chromatographic behaviour of some classes of compounds analysed on these iron-free HPLC systems revealed issues typically associated with metal contamination (1).

Biocompatible systems do not use stainless steel parts in the fluidic path for various reasons, including the interference of the iron solid surfaces with some biomolecules, resulting in lower sample recovery and higher peak tailing (2). Another function for these systems is to avoid rust formation when using ion exchange chromatography (IEX) and size exclusion chromatography (SEC) for protein characterization where analyses are typically run with very high concentrations of sodium chloride leading to corrosion. Instead biocompatible materials, such as polyether ether ketone (PEEK) or titanium alloys, are used, although the latter are preferred in parts exposed to high pressures.

Titanium has excellent mechanical behaviour and corrosion resistance justifying its use in HPLC systems. The titanium dioxide passivation layer which spontaneously forms when the metal is exposed to air or water prevents corrosion from high salt concentrations, and generally provides corrosion resistance. However, a number of studies have indicated that titanium may suffer from corrosion or stress-corrosion when exposed to anhydrous methanol (1), methanol-hydrogen chloride mixtures (3), and some other organic solvents (4). Importantly, this corrosion does not lead to mechanical failures. The parts where titanium is used, such as pump heads or eluent mixers, are extremely durable and rarely fail. The corrosion takes place on a microscopic scale, constantly releasing minor amounts of titanium ions into the mobile phase, potentially affecting the obtained results with the effect differing depending on the variables of the analysis.

In this particular study, unusually high peak tailing and reduced retention of veterinary drugs was observed, which indicated an unwanted metal contaminant. The aim of the study was to identify the source of this contaminant and provide a mechanism capable of explaining the loss of retention and peak tailing, as well as identifying measures to avoid the metal contamination in the future.

Corrosion of titanium alloys when using an anhydrous mixture of acetonitrile and methanol was identified as the source. The study used a polar-embedded stationary phase with no clear effects being observed in a conventional C18 reversed-phase. The mechanism by which the titanium ions were produced was thought to be based on the formation of complexes between fluoroquinolones and the titanium immobilized in the stationary phase.

Furthermore, the researchers anticipated that this effect could occur in any stationary phase capable of immobilizing titanium cations and the analyte is a metal chelating molecule.

As to potential solutions? The researchers identified literature that detailed that the effect of corrosion can be completely inhibited by including small amounts of water in the organic solvent, and highlighted the lack of corrosion in conventional C18 reversed-phases as an indicator of the importance of selecting the correct phase for the analysis.

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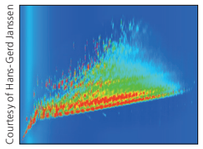
Peaks of the Month

- **The *LCGC* Blog: Silica for HPLC Stationary Phases – A Five Minute Guide**—Most HPLC columns are packed with silica onto which some form of hydrophobic ligand is bonded – these columns form the vast majority of those used for modern reversed-phase HPLC. **Read Here>>**


Courtesy of Hans-Gerd Janssen

- **Recent Application and Instrumental Trends in Comprehensive Two-Dimensional Gas Chromatography**—This critical review describes recent applications and instrumental trends in comprehensive two-dimensional gas chromatography (GC×GC), with particular (though not exclusive) attention to the period 2018–2019 and that the concept of GC×GC is inherently simple. The maturity of GC×GC and future developments are also discussed. **Read Here>>**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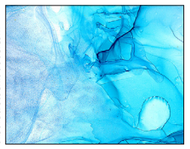
- **Pittcon Report**—Incognito observes some unexpected emerging trends at Pittcon 2020. **Read Here>>**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- **Tips & Tricks GPC/SEC Aqueous GPC/SEC for Water-Soluble Macromolecules**—Water-soluble macromolecules require water as a mobile phase, and method development therefore seems to be straightforward. At second glance, however, aqueous gel permeation chromatography/size-exclusion chromatography (GPC/SEC) is complex and the choice of the stationary phase and pH are crucial. In addition, mobile phase additives are often required to allow for interaction-free separations. **Read Here>>**

- **Modern Instruments for Improved Environmental Analysis**—Modern environmental laboratories are constantly being asked to characterize samples more quickly and with lower limits of detection. How are advancements in analytical instruments helping to alleviate these issues and provide accurate detection with high sensitivity? **Read Here>>**


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- **Nitrosamine Determination Using GC–PCI-QTOF-MS**—Researchers have developed an alternative method to reduce interferences for the analysis of *N*-nitrosamines using gas chromatography (GC) coupled to high-resolution quadrupole time-of-flight mass spectrometry (HR-QTOF-MS). **Read Here>>**

News In Brief

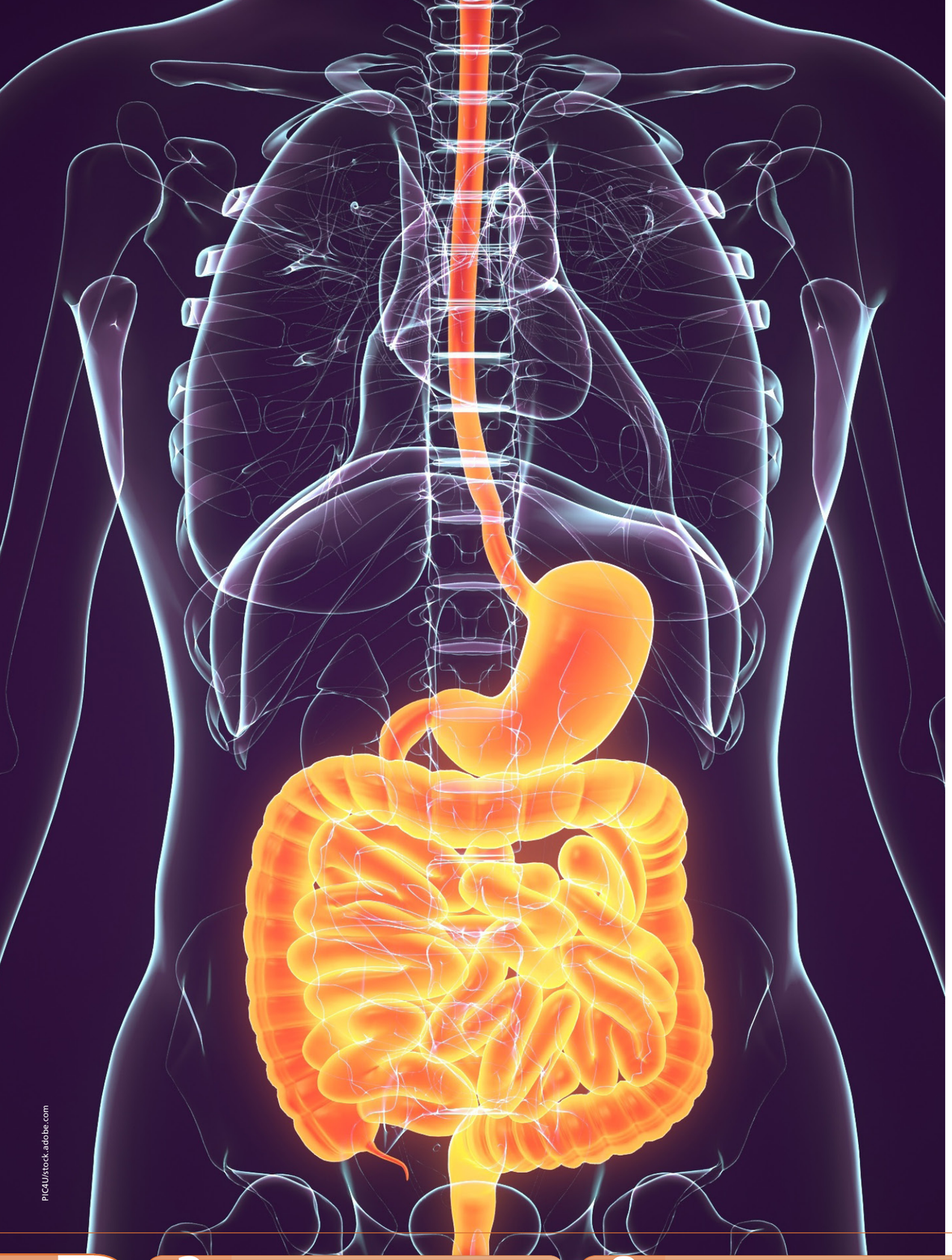
UK-based research materials supplier, Goodfellow (Huntingdon, UK) have shown their support in the fight against COVID-19 by supplying materials used to make protective equipment for frontline workers. The company donated 130 kilos of polypropylene coil to Stamford School in Lincolnshire, UK, where Design Technology teachers are voluntarily manufacturing 1000 protective face masks a day.

They have also donated to a number of other companies that are supplying protective equipment to hospitals, such as supplying PET sheet to Plastic Jesus Fabrication, an LA artist who has adapted his studio to make medical PPE to donate to local hospitals. Other companies receiving donations include Royal Mint, Brammer, TJ Morgan, and Safran Aero Boosters.

For more information, please visit: www.goodfellow.com

Trajan Scientific and Medical (Melbourne, Australia) and Queen's University (Kingston, Canada) have been awarded a Mitacs Accelerate grant to develop new multiple electrospray (MES) technologies to increase throughput and sensitivity in mass spectrometry (MS).

For more information, please visit: www.trajanscimed.com



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A New Gastro-Intestinal System to Evaluate the Effect of Food Methylglyoxal

A novel modular bioreactor for dynamic *in vitro* studies has been set-up, connecting two-dimensional (2D) scaffolds and mimicking a multi-organ model, to study the absorption/metabolization of compounds. The effect of dietary methylglyoxal, a potentially exogenous and endogenous toxic compound, on a dynamic gastro-intestinal system has been evaluated. Bioreactors represent a powerful advance in comparison with conventional *in vitro* static assays and could be a potential alternative to animal testing in the future.

—Interview by Kate Jones

Q. How did the idea of this research project arise and what are the aims of this research?

A: This project aims to develop a new dynamic and multi-compartmental millifluidic bioreactor simulating a gastro-intestinal system suitable for the evaluation of the effect (absorption/metabolization and cytotoxicity) of exogenous bioactives derived from food or drug intake.

By using this new dynamic protocol, we tested methylglyoxal (MGO), which is a potentially toxic compound, produced both in food and endogenously, responsible for the formation of the advanced glycation end-products involved in many pathological chronic implications,

such as diabetes, cardiovascular diseases, and ageing-associated disorders (1–4).

The experiments were also performed with the standardized Minekus' enzymatic digestion protocol to investigate the potential of this new dynamic system compared with static tradi-tional assays (5).

Q. What are you doing in this poster that is novel?

A: The novelty aspect of this work is the setup of a new platform that gives the opportunity to simulate continuous flow conditions, reproducing different simil-physiological flows and connecting different compartments to recreate a multi-organ model. This is the first setup of a dynamic gastro-intestinal platform and such a system represents a potential advance in the study of kinetic, metabolic, and cytotoxic profiles of different substances.

Q. What were the main analytical challenges you had to overcome?

A: First of all, it was necessary to carefully optimize the flow conditions to treat gastric (GIST-882) and intestinal (Caco-2) cells in the dynamic system when the two compartments were connected, in order to reproduce a simil-physiological digestive process.

Then, to monitor MGO metabolic fate by using a millifluidic system, it is mandatory to collect samples at different monitoring times and to analyze them by a proper analytical technique. To detect and quantify MGO in the cell medium during gastric and intestinal phases, we used a reversed-phase high performance liquid chromatography diode-array detector (HPLC-DAD) method, slightly modified from the literature and validated by us. The choice of UV detector required a sample preparation step, consisting of a derivatization procedure to obtain a UV-detectable MGO derivative (the derivatization procedure required a setup, too).

Q. What were your main findings and why are they useful?

A: The obtained results highlight that exogenous MGO is rapidly metabolized by a safe digestive process, and this is very important for human health in relation to widespread diseases, such as diabetes and ageing-associated disorders. Furthermore, by comparing the dynamic and the static processes, it emerged that the two protocols provided complementary data, and in particular the dynamic system elucidated a new role of gastric cells, which should be further investigated in the metabolization of toxic compounds.

Q. How are you planning to develop this research further?

A: We are going to submit to this dynamic digestion protocol other molecules, both toxic and ac-tive compounds, also in association. Moreover, we will probably use this platform for complex matrices, such as food samples, to test the effects of the interactions among the different food components, on the absorption/metabolization of a specific molecule.

Q. Anything else you would like to add?

A: This platform could reduce *in vivo* experiments, in particular during preliminary investigations, and find a very promising application in high-throughput cell-based compound screening for food analysis, drug discovery, and toxicity tests.

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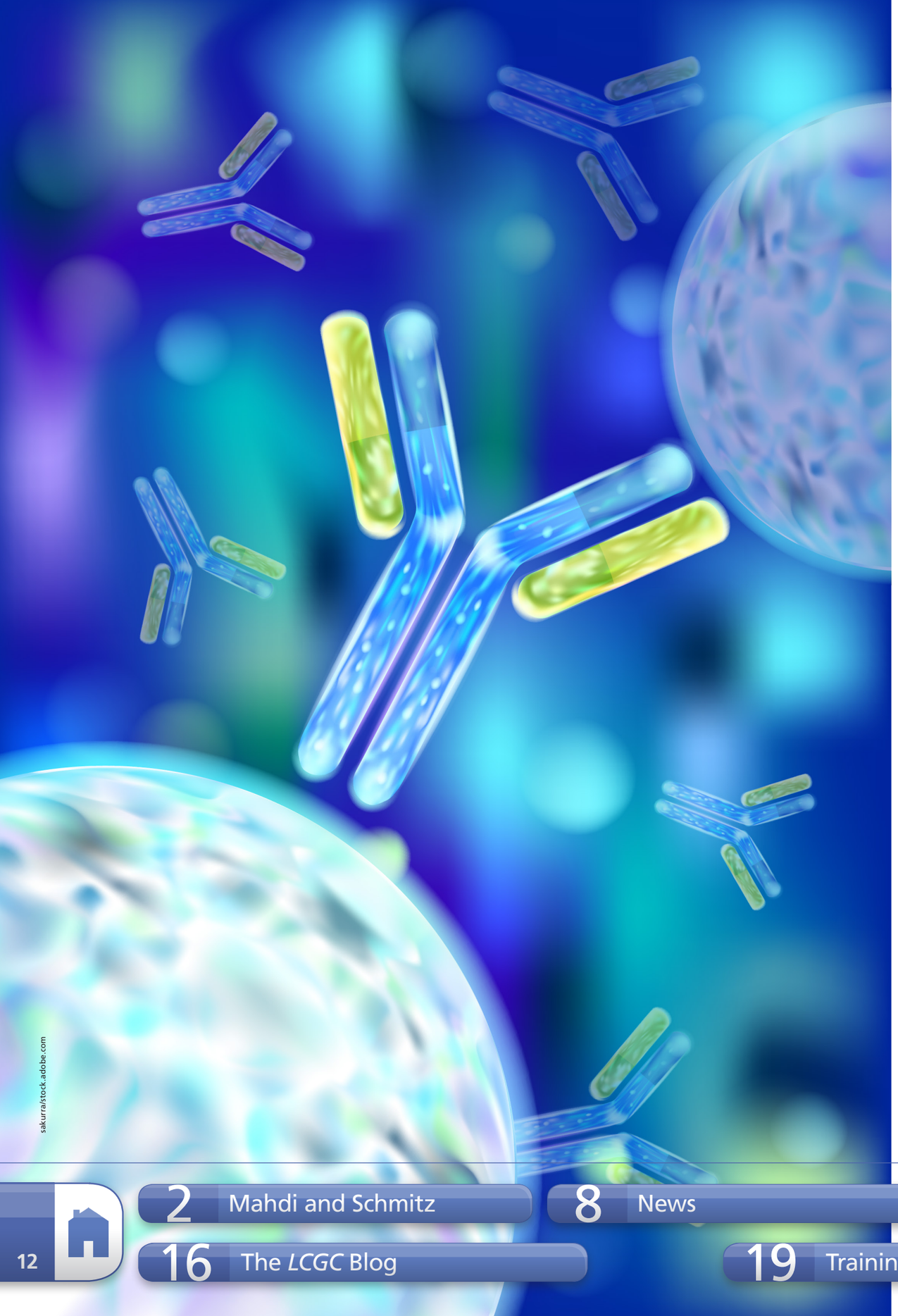
Lucia Ferron, Nutraceutical and Food Chem Toxicol Analysis Laboratory, Department of Drug Sciences, University of Pavia, Italy, FlaNat Research Italia Srl, Rho (MI), Italy.

This interview was first published in a special supplement from *LCGC Europe*: The Rising Stars of Separation Science. For more interviews with Poster Winners, go to: https://bit.ly/3aRot39

E-mail: **luci.ferron01@gmail.com**



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Glycosylation Analysis Through Released *N*-Glycan Workflows

M. Christina Malinao, Brian Rivera, and Chad Eichman, Phenomenex, Torrance, California, USA

Post-translational modifications are potential critical quality attributes (pCQAs) routinely assessed in biotherapeutic development. Glycosylation is one of the most important attributes to assess because it affects protein function as well as antigen receptor binding. *N*-glycosylation of asparagine residues is the most common pCQA assessed during monoclonal antibody (mAb) therapeutic development. There are a few protocols to assess and quantitate *N*-glycans, but the most common approach is through an enzymatic release and labelling procedure, followed by separation and detection. This article demonstrates the method development considerations for sample preparation and chromatographic analysis of *N*-glycans of therapeutic mAbs.

Glycosylation events are fundamental in biology and necessary for a plethora of biological processes. The most recent example is evident in the advent of the coronavirus (COVID-19) pandemic, where glycosylation has been predicted to contain "unique *N*- and *O*-linked glycosylation sites of spike glycoprotein that distinguish it from the SARS and underlines shielding and camouflage of COVID-19 from the host defence system" (1). In biotherapeutic development, glycosylation has many implications in safety, efficacy, and immunogenicity. In monoclonal antibody (mAb) development, potency depends on biological effector functions and glycosylation plays an important role in this regard (2). Moreover, the pharmacokinetics (PK) of mAbs is significantly altered based on the glycosylation pattern (3). Arguably, the two most

Figure 1: HILIC separation of 2-AB-labelled released *N*-glycans of spiked human IgG library

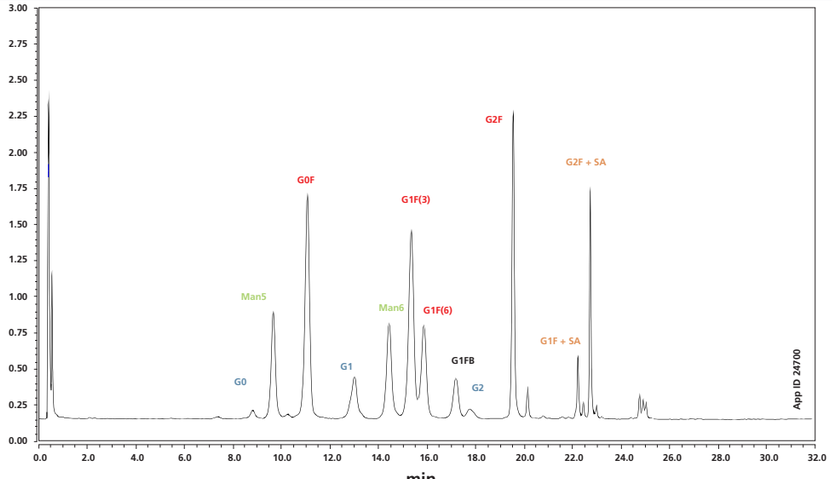
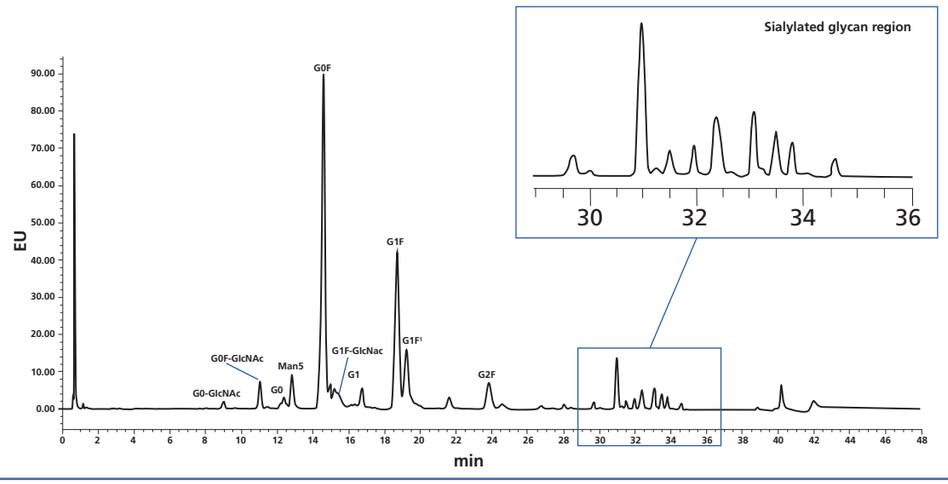


Figure 2: Procainamide-labelled released *N*-glycans of infliximab by HILIC



important *N*-glycan types are high mannose forms, such as Man5 and Man6, and sialylation because their impact in efficacy and PK profile is established. As such, researchers must consider glycosylation as one of the more important post-translational modifications (PTMs) to monitor because of these profound biological effects. Therefore, many methods have been developed for quantitation of glycans of biotherapeutics.

Structurally, in the most common type of mAb therapeutic, immunoglobulin G 1 (IgG1), glycosylation occurs covalently at the C_H2 domain at asparagine 297 (Asn-297) (4). Because this glycosylation location is inherent to IgG1 mAbs, site-specific cleavage of the Asn-297 residue can reveal the glycans attached to the mAb. The structure of these *N*-linked glycans can be assessed by intact analysis methods (5,6), however, *N*-linked glycan by release is the most accurate way to characterise the heterogeneity of glycosylation because lower level glycoforms cannot be detected by glycopeptide nor at the intact level (5). The enzyme PNGase F selectively cleaves *N*-glycans at the Asn-297 residue, which then must be labelled with an appropriate dye and subsequently separated by hydrophilic interaction chromatography (HILIC) for quantitation. This article demonstrates the released *N*-glycan analysis of proteins through different labelling techniques and focuses on high mannose and sialylation detection. Finally, an analysis of biosimilars shows the importance of *N*-glycan analysis during therapeutic development.

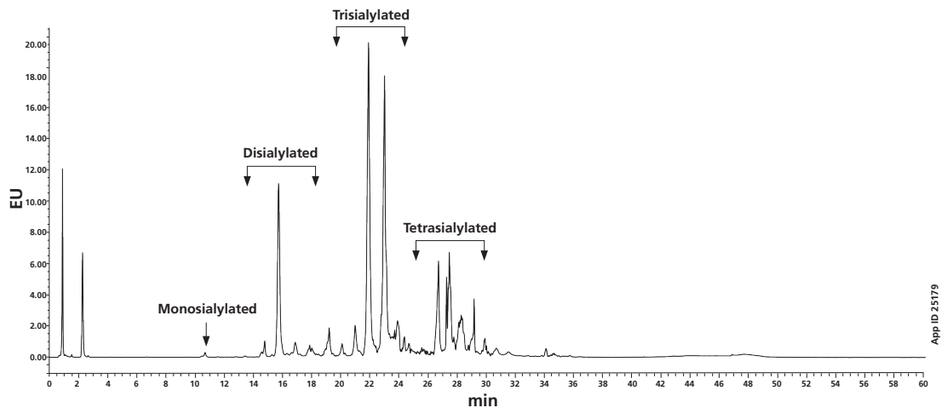
Materials and Methods

Reagents/Chemicals: Alpha-1 acid glycoprotein (AGP) sample derived from human plasma and 2-aminobenzamide (2-AB) were obtained from Sigma-Aldrich. Infliximab and biosimilars were obtained from Myoderm. 2-AB-labelled glycan libraries, PNGase F, and Gly-X *N*-Glycan Rapid Release and InstantPC kit were obtained from ProZyme (now part of Agilent). GlycoWorks RapiFluor Labelling Module was obtained from Waters. The bioZen *N*-Glycan Clean-Up plate and bioZen 2.6-µm Glycan column were from Phenomenex.

Experimental Conditions: To prepare infliximab and biosimilars for chromatographic analysis, the Gly-X and InstantPC kit was utilized. The protein was first denatured with the denaturation reagent provided and incubated at 90 °C for 3 min. Subsequent enzymatic digestion with PNGase F at 50 °C for 5 min provided the released glycans. Immediate labelling with InstantPC dye for 1 min at 50 °C followed by clean-up through a bioZen *N*-Glycan Clean-Up Plate provided the labelled *N*-glycans. 1 µL of the elution from the glycan cleanup plate was injected on a bioZen 2.6 µm Glycan column.

AGP was reconstituted to 2 mg/mL in pure water. Each sample was denatured by adding 6 µL of surfactant and heating to 90 °C for 3 min. 1.2 µL of PNGase F was then added and samples were incubated for 5 min at 50 °C. Released glycans were labelled with 12 µL of labelling reagent solution for each sample and incubated for 5 min at room temperature.

Figure 3: Sialic acid HILIC separation of *N*-linked glycans released from AGP

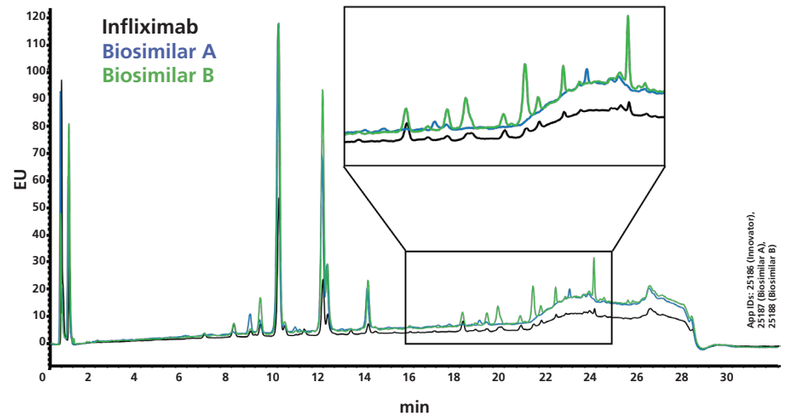


Results and Discussion

The most traditional and adopted protocol for *N*-glycan analysis deploys a 2-aminobenzamide (2-AB) label to the released glycans (7). This reductive amination process is robust and provides sufficiently fluorescent materials for analysis by a fluorescence detector (FLD). To demonstrate this technique, the prelabelled human IgG library was assessed. The 2-AB human IgG library was then subsequently spiked with 2-AB-labelled Man5 and Man6 to give a better representation of a recombinantly expressed protein which may have immature glycans. Samples were then analyzed using a HILIC LC column (Figure 1). Notably, there is baseline separation of Man5 and Man6 from other glycan species, which allows quantitative or qualitative assessment of these pCQAs pending the analytical goal.

An alternative approach to the labelling step is to use a procainamide label via a carbamate linkage, which has better sensitivity by FLD. An infliximab biosimilar was assessed using this labelling method, but still under HILIC separation (Figure 2). Notably, the Man5 species elutes much closer to G0 under this protocol indicating that the 2-AB labelling method is more efficient at this specific separation. Another aspect of this data to observe is the sialic acid region of the chromatogram. As mentioned earlier, sialylation affects the PK results, so it is important to obtain sufficient separation in this region of the chromatographic profile. To clearly demonstrate the sialic acid separation, *N*-linked glycans released from serum-derived AGP were assessed (Figure 3). This model protein is a suitable surrogate for a complex, highly sialylated glycoprotein with both neutral and sialylated

Figure 4: Comparison of infliximab and biosimilars demonstrating sialylation differences



glycans with multiple antennae. In this case, a quinolinyl label was employed which is beneficial for sensitive mass spectrometry (MS) analysis (8). The chromatogram in Figure 3 was obtained by increasing the ammonium formate in the mobile phase to 250 mM, which was necessary to partition the sialylated species.

Finally, infliximab and biosimilars were analyzed to determine glycosylation differences in these commercially-available mAbs (Figure 4). The results from this assessment indicate relatively similar levels of glycosylation of the neutral species, but significant variation in sialylation (zoomed region). Clearly, because the biosimilars will be generated from different cell lines and fermentation processes, glycosylation PTMs are expected. As these are all commercially-available therapeutics, these specific glycosylation differences are likely to have minimal therapeutic impact.

Conclusion

Monoclonal antibody therapeutics are complex mixtures of protein variants with glycosylation being a PTM of critical therapeutic importance. The most common approach to assess these glycans is through enzymatic release of *N*-glycans using PNGase F followed by labelling with an appropriate dye. This article demonstrates the use of three different fluorescent labels followed by HILIC separation, all of which are efficient at *N*-glycan quantitation. Ensuring a robust, transferrable, and reproducible method is essential for researchers that characterize and monitor CQAs. Optimization of the protocol may be necessary pending the CQA identified through orthogonal research as outlined by the sialic acid assessment of AGP. Finally, the applications presented here provide various labelling protocols for comparable released *N*-glycan analysis by HILIC separation.

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Christina Malinao received her B.S. in neuroscience and biology from the University of California, Riverside (USA). She spent some time at Schering Plough performing biomarker characterization, and several years at Agensys performing antibody and antibody–drug conjugate characterization. She joined Phenomenex as a scientist in the Phenologix application laboratory performing method development. Christina currently works at Tae Life Sciences.

Brian Rivera is the Senior Product Manager – Biologics at Phenomenex. He has worked at Phenomenex for nine years, holding other positions including technical support and sales. Prior to joining Phenomenex, Brian worked within the biotechnology industry, including positions focused on protein purification, analytical method development, and in-process analytical support. Brian has a bachelor's degree from the University of California, Davis, USA.

Chad Eichman is the Global Business Unit Manager – Biopharmaceuticals at Phenomenex, where he develops and leads the strategic plans for Phenomenex's biopharmaceutical business. He received his B.S. in chemistry from the University of Wisconsin–Madison (USA) and his Ph.D. in organic chemistry from The Ohio State University (USA). After a postdoctoral appointment at Northwestern University (USA) and an assistant professorship at Loyola University Chicago (USA), Chad joined Phenomenex in 2017.

E-mail: **ChadE@phenomenex.com**
Website: **www. phenomenex.com/bioZen**

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The *LCGC* Blog: Climbing the Academic Career Ladder: Choices at the Top

Kevin A. Schug, Department of Chemistry and Biochemistry, The University of Texas (UT) at Arlington, Arlington, Texas USA

When you've reached the top, where do you go next? In this instalment of the *LCGC* Blog, Kevin Schug explores prospects open to those of us at the pinnacle of our academic careers. Reflecting on his unique journey and offering his perspective – from the stability of academic freedom to interesting research and outside opportunities – Kevin's experience highlights several options.

For the past seven years, I have written a monthly blog article for LCGC and chromatographyonline.com. When I started writing, at the beginning of 2013, I was an Associate Professor. I had received tenure relatively recently, and I was beginning to explore a bit more of my academic freedom in terms of research and outside opportunities.

Looking back, the start of my penning of blog articles matches quite well with the start of efforts to investigate the potential environmental impacts of unconventional oil and gas extraction, and the formation of the Collaborative Laboratories for Environmental Analysis and Remediation (CLEAR, http://clear.uta.edu) at the University of Texas Arlington, USA. It also well coincided with my effort to begin some outside consulting activities, predominantly the review of forensics evidence for blood alcohol determination. Both of these activities have blossomed considerably in the past seven years, and it has become interesting

to contemplate the most rewarding way to spend my time going forward.

In 2015, I was promoted to Full Professor, and before that I had already held the Shimadzu Distinguished Professorship in Analytical Chemistry for almost two-and-a-half years. While there might be more lucrative or prestigious Professorships out there, I have reached the pinnacle of promotion for academic faculty. So, what next?

In 2017, I was offered the opportunity to be Interim Associate Dean for Research and Development for the College of Science at UT-Arlington. To me, this looked like the perfect internship position to see if a move to the administrative side of academia was palatable. My appointment was only for ten months, and for that, thank goodness! Within a month, I knew that I did not want to be a University administrator of any kind. The politics, the uncertainty of budgets and desires of upper administration, and the "one-size-fits-all" mentality from the top were among the many frustrations of that position.

There were many other frustrations, mainly spurred on by the lack of communication within and by the upper administration. It seemed that weekly a spreadsheet of some sort was thrust in front of me to complete. Invariably, I lacked any of the knowledge to complete the forms. To do that, I would need to gain the contact and coax time from people in six departments within the college. I decided very quickly that I did not go through all of the efforts to obtain a Ph.D. in Chemistry, to then rise through the ranks of academia with all of its landmines and nuances, to become a gopher to fill out spreadsheets.

In the end, I have decided that perhaps my poor experience as an Associate Dean was due to just how things run at UT-Arlington. Yet, I have had discussions since that time which lead me to believe that it can be similar in other places. I am glad that I took the opportunity to try out the administrative route, but I also feel bad for the future. At some point, I may be asked to be Chair of the department; previously, I might have accepted to give it a try. Now, no way! But everyone is different, and this was only my experience.

My short stint as Associate Dean did not have a deleterious impact on my group's research efforts. The past few years have been the most productive ever, mainly because we have not had to battle too hard to gain research funds, and the sources of those funds have not placed a lot of constraint on what sort of research questions we could try to address. We have all of the newest instrumentation and technology as a result of beneficial relationships with many leading companies in the industry. That is a perfect situation – to be able to follow your ideas and have essentially all of the resources needed to do so. I have to give a lot of credit to my friend and colleague Dr. Zac Hildenbrand, co-founder of CLEAR, for drumming up a lot of interesting research opportunities and support for our work.

As we enter into a new decade, there appear to me opportunities up ahead. These continue to build on past efforts. Last year, I helped co-organize two major conferences. One was the 2019 International Symposium on Capillary Chromatography and GCxGC conference in Fort Worth, Texas, USA, in May. I am also heading up the organization of the 2021 edition of this conference, which will again be in Fort Worth in May (though in a nicer, newer hotel).

Another was the 2nd Responsible Shale Energy Extraction (RSEE) symposium, a new conference I co-founded with Zac that we organized at UT-Arlington. In 2020, that conference will be part of half-Earth Day in October in Dallas, Texas, USA; RSEE will be rolled into a larger one-and-a-half day EnergyX conference, which will not only focus on unconventional oil and gas extraction, but also other renewable and non-renewable energy modalities. This is a whole other playing field beyond just science and technology.

Considerations also into economics, politics, and logistics of renewable and non-renewable energy sources, in addition to technical aspects, make this a very fulfilling concept in which to engage. We are always meeting new people, and what might be most interesting for LCGC readership to realize – we get a lot of respect and credit for our scientific backgrounds. We also get a close look at the technological advances and challenges, which are shaping the state-of-the-art in these different energy sectors, and it is gratifying to have the ability to understand many of the technical aspects, as well as to offer some potential solutions.

I do not ever see myself fully leaving academia. I feel like I am on the cusp of being able to do those things that people told me an academic could do in order to make a very comfortable living. I thoroughly enjoy my research group and both the fundamental and applied aspects of our analytical chemistry research. I love that this spans work in energy, the environment, pharmaceutical science, clinical chemistry, food science, instrument design, and beyond.

I also now have multiple companies. One is to handle the consulting opportunities, which come and go, and have myriad different flavours. Beyond blood alcohol, I have also had the chance to work on cases involving horse doping, patent interference, and product formulation trade secrets. The hourly rates I can charge are quite lucrative relative to my hourly rate as a Professor.

Most recently, Zac and I, together with my wife, have founded another company called Medusa Analytical, LLC. The concept of this company is to facilitate research and consulting opportunities. It is under this guise that we are organizing the EarthX EnergyX conference in April. The more and more that we have worked with industry (especially outside of the analytical chemistry industry), the more we have seen that there are vast opportunities for facilitating solutions to problems. In this case, Medusa Analytical would be best suited to facilitating solutions to problems, which my laboratory at UTA cannot specifically provide. It is also in this way that conflicts of interest and commitment with my role at UTA can be mitigated and avoided (this is a whole other topic for another time). It is important that I acknowledge UT-Arlington is my primary employer and I owe my greatest professional commitment there. But Universities give faculty some leeway to engage in outside opportunities; and if they are faithfully disclosed and present no conflicts of time, interest, or commitment, then there is nothing wrong with trying to create something new.

I initially chose the academic life with the knowledge that it would be able to provide a comfortable lifestyle for my family. My father was a chemistry professor, and I would not characterize my upbringing as wanting for anything. Now, I could go on, simply playing out my role as Professor until the end of my days, educating and training students, and developing new knowledge. I love the relationships that have been forged in this role to this point. I do not see our laboratory slowing down any time soon. That said, I certainly do have my eyes open for other interesting and lucrative opportunities. And I think they need to have quite a bit of both of those aspects (interest and monetary support) for me to bite, but that is the luxury of now being near the top.

I talk to students, especially graduate students, all the time who are scared away from the academic side of life, because it seems so stressful. Any job can be stressful. In academia, if you can climb the ladder, you can gain job security and stability. You might get a chance to patent something and strike it rich that way, but more reasonably, I think it is the potential doors that are opened as a result of being a clear expert in your field. Academic research also has no shortage of means for Professors to be lauded for their accomplishments; these bring prestige and bolster's one's credit as a quality scientist. Importantly, when the doors open, you are not compelled to run through them; that is a strong negotiating stance. The stability of the job actually takes a lot of stress out of it, in my opinion. Now, to be able to pursue some other interests, while maintaining that stability, provides a whole other level of potential learning and earning. I am excited to see where this next decade leads.

Kevin A. Schug is a Full Professor and Shimadzu Distinguished Professor of Analytical Chemistry in the Department of Chemistry & Biochemistry at The University of Texas (UT) at Arlington, USA. He joined the faculty at UT Arlington in 2005 after completing a Ph.D. in Chemistry at Virginia Tech under the direction of Prof. Harold M. McNair and a post-doctoral fellowship at the University of Vienna under Prof. Wolfgang Lindner. Research in the Schug group spans fundamental and applied areas of separation science and mass spectrometry. Schug was named the LCGC Emerging Leader in Chromatography in 2009 and the 2012 American Chemical Society Division of Analytical Chemistry Young Investigator in Separation Science. He is a fellow of both the U.T. Arlington and U.T. System-Wide Academies of Distinguished Teachers.

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AN MJH life sciences™ BRAND

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Group Vice President
Michael J. Tessalone
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Associate Publisher
Oliver Waters
owaters@mjhlifesciences.com

Sales Executive
Liz Mclean
lmclean@mjhlifesciences.com

Sales Operations Executive
Sarah Darcy
sdarcy@mjhlifesciences.com

Editor-in-Chief
Alasdair Matheson
amatheson@mjhlifesciences.com

Managing Editor
Kate Jones
kjones@mjhlifesciences.com

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Lewis Botcherby
lbotcherby@mjhlifesciences.com

Multimedia UK LLC,
Sycamore House, Suite 2 Ground Floor,
Lloyd Drive, Cheshire Oaks Business Park,
Ellesmere Port, CH65 9HQ, UK
Tel: +44 (0)151 705 7601

North America

Group Vice President
Michael J. Tessalone
mtessalone@mjhlifesciences.com

Publisher
Edward Fantuzzi
efantuzzi@mjhlifesciences.com

Sales Manager
Stephanie Shaffer
sshaffer@mjhlifesciences.com

Sales Manager
Brianne Molnar
bmolnar@mjhlifesciences.com

Editorial Director, Analytical Sciences
Laura Bush
lbush@mjhlifesciences.com

Senior Technical Editor
Jerome Workman
jworkman@mjhlifesciences.com

Managing Editor
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jchasse@mjhlifesciences.com

Associate Editor
Cindy Delonas
cdelonas@mjhlifesciences.com

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Courtney Soden
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Administation and Sales Offices
Woodbridge Corporate Plaza,
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