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Impurity Testing of Biologic Drug Products

Experts share insights on the various methods used for purity and impurity analysis of therapeutic proteins.

Adeline Siew

Impurities can have a negative impact on the stability, safety, and efficacy of protein therapeutics. "Aggregates are of particular concern, either in soluble dimer/oligomer form or subvisible particle form," notes Jay Kang, director of analytical and formulation development at Patheon, part of Thermo Fisher Scientific. "It is well documented that even a small amount of aggregates can cause a significant and sometimes life-threatening immunogenic reaction."

"Impurities may interact with the therapeutic protein in a way that blocks and/or compromises the activity and potency of the therapeutic protein *in vivo,* hence, reducing its efficacy," explain Michael Sadick, principal scientist, Catalent Biologics Analytical Services, and Michael Merges director of strategic growth, Catalent Biologics. "On the other hand, the impurity may exaggerate or enhance the therapeutic protein's bioactivity in an uncontrolled way, leading to adverse events. Some impurities (especially host cell proteins) may add an immune-stimulating or adjuvant behavior to the therapeutic, causing the patient to generate antibodies or cell-mediated immunity against the protein."

"Host cell proteins co-extracted with the therapeutic protein can contain enzymes such as oxidases and lipases that will break down the protein over time, affecting the stability of the product," adds Niall Dinwoodie, Edinburgh biologics site lead at Charles River Labs (CRL). "Other host cell proteins and binding agents carried over from purification columns may mimic the action of the therapeutic protein in assays, leading to mis-formulation of the product outside the therapeutic window."

According to Dinwoodie, some impurities have less insidious effects, but can still render the therapeutic unacceptable. "For example, trace levels of rapidly oxidized materials cause significant color change," he says. "Historically, contamination with trace amounts of metals was a problem leading to aggregation of therapeutic proteins, which can cause significant changes in efficacy, but understanding

of the phenomena has led to improved, metal-free production processes."

In general, impurities come from two major sources, observes Bérangère Tissot, general manager, SGS Life Sciences, West Chester, Pennsylvania: product-related impurities and process-related impurities. "Product-related impurities can be categorized as product variants, and basically correspond to any undesired modification of the protein amino-acid sequence or post-translational modifications," she highlights. "Variants also include forms of the therapeutic proteins in solution that are different from the intended drug product (i.e., different conformation or aggregation state). They can also be identified as a sub-form of the therapeutic protein, possessing a biological activity either higher or lower than the one of the drug product."

The second type of impurities are mostly related to the production processes, says Tissot. Dinwoodie adds that the materials used in the production process to support cell growth, extract, and purify the therapeutic protein must be removed from the final dosage form. "Residual amounts of these materials can be carried through the production process to become impurities in the final form," he points out. "Examples include growth selection agents, surfactants, purification column binding agents, and viral inactivation agents."

"The use of cells and growth media in the production process also presents risks of adventitious agents, such as viruses, entering the production system," says Dinwoodie. "Whilst removal or inactivation of these agents is considered under the production process validation as a safety issue rather than tested in the final product as a quality concern, these agents can also be considered impurities in the product."

"Process- and product-related impurities should be carefully monitored and controlled in the production of therapeutic proteins."

Process- and product-related impurities should be carefully monitored and controlled in the production of therapeutic proteins. In this roundtable discussion, industry experts share insights on the various methods used for purity and impurity analysis of therapeutic proteins.

Method development and validation

BioPharm: What is the right approach to method development and validation for therapeutic proteins?

Kang (Patheon): Two concepts are key to approaching method development and validation for therapeutic proteins: "fit-for-purpose" and "phase appropriate." A "fit-for-purpose" strategy means a method should be suitable for its intended use and phase of development. The requirement to establish an analytical method depends on whether it is for an identity test, content test, or purity/

impurity test; whether it is for release, in-process testing, or characterization. For example, the only requirement for an identity test is specificity, while specificity, linearity, range, precision, robustness, and sensitivity are mandatory for the purity test. Determining whether the method is for early-phase development or for biological license application (BLA) filing is also crucial because it will dictate the size and thoroughness of the validation data package.

Sadick and Merges (Catalent): The underpinning to this response is the knowledge that each protein therapeutic is quite different from any other protein therapeutic, whether in terms of its final tertiary or quaternary folding, biological activity, or purity profile. This is true even when considering different monoclonal antibody therapeutics. Consequently, while similar strategies may be used for different protein therapeutics, true "toolbox" approaches/platforms may not be completely successful. In the strategies for assay or method development and optimization, we consider a combination of "one factor at a time" (OFAT) to define individual factors, at least initially, and "design of experimentation" (DOE) to look at multiple and interacting factors. The use of fractional factorial DOE as soon as is practical allows for a more rapid and robust method development.

Validation would be accomplished in a phase-appropriate manner. The guideline for all phase appropriate levels would be the International Council for Harmonization (ICH) Q2 (R1) (1), although different technical platforms (e.g., enzyme-linked immunosorbent assay [ELISA] or bioassay potency tests) may have specific levels of adherence to the ICH guidelines, in addition to other guidelines, for example *United States Pharmacopeia* General Chapters <1033> and <1034> (2, 3). Validation for an investigational new drug or at Phase I level would have the more basic requirements, with fewer tests to be executed, a smaller number of repetitions, and wider acceptance criteria. Late-phase (Phase III/BLA-enabling) validation will include all appropriate test categories, as well as robustness, with an increased number of sample repetitions along with more stringent acceptance criteria. The establishment of appropriate validation acceptance criteria should be based upon data-driven decision. Those data are best generated via a prevalidation exercise conducted prior to the drafting of each phase-appropriate validation protocol.

Tissot (SGS): This is not straightforward, as validation approaches will depend on the nature of the method, its intended use, the development stage of the product, and the type of therapeutic proteins. In all cases, the method should be evaluated, prior to its validation, through a risk management process that will dictate which parameters to validate, which acceptance criteria to aim at, and all other necessary components of a validation study. These considerations include nature and number of replicates for each of the parameters, robustness conditions, and intermediate precision details among others.

Dinwoodie (CRL): The extent of development needed for a new analytical method will depend on the purpose of the method and the body of knowledge available on the product to which it will be applied. Physicochemical methods can be largely based on compendial procedures and require little development, and parameters for platform techniques such as size-exclusion high-performance liquid chromatography (SE–HPLC) can be established from an understanding of the protein's molecular weight.

Binding or potency assays, however, require the selection of suitable antibodies or modification of detector cell lines. Non-therapeutic host cell proteins can also present a considerable challenge to method development in ensuring that the polyclonal sera used provides full coverage of the range of proteins that may be extracted from the production cell line.

Method development also must consider the robustness of the approach and ensure that reagents and consumable items, such as columns, are readily available and consistent in the results they generate. Validation of the method will then serve to confirm the robustness of these elements and assess the variability introduced by different analysts and equipment.

Both the number of replicates run for the determination of repeatability and intermediate precision, and the number of batches of the product tested in each run are affected by the product's stage of development. They also must be defendable in covering all possible options for how the method will be used in the future. Other aspects of method validation are more easily derived from the guidance given in ICH Q2 (R1).

"Method development also must consider the robustness of the approach and ensure that reagents and consumable items."

Analytical methods

BioPharm: What are the commonly used analytical methods for characterizing therapeutic proteins?

Tissot (SGS): The main document used by anyone characterizing a therapeutic protein remains the ICH Q6B guidelines (4). Now these guidelines are a little outdated, mainly with regards to biophysical methods but they still remain a good basis for the design of a characterization method panel. There are many ways to address some of the key elements that need to be evaluated during a characterization study, but some of the most commonly used are listed in the following:

Physicochemical characterization:

- Liquid chromatography–tandem mass spectrometry (LC–MS/MS) following digestion for primary amino-acid sequencing, which could be completed by N-terminal sequencing using Edman degradation. The same type of methodology can be applied to the

evaluation of the most common post-translational modifications
- Liquid chromatography–mass spectrometry (LC–MS) or electrospray ionization–mass spectrometry (ESI–MS) for intact molecular weight when the therapeutic protein does not present any major challenge for ionization (such as heavily glycosylated proteins)
- Amino-acid analysis and extinction coefficient estimation
- A combination of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS), LC–MS, and other liquid chromatography with ultraviolet detection (LC–UV) or high-pressure anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) methods for the quantitative and qualitative analyses of N- and O-glycosylation
- Liquid chromatography or electrophoresis methodologies to evaluate product heterogeneity (charge variants, size variants, hydrophobicity variants etc.)
- Circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), intrinsic/extrinsic fluorescence for the analyses of secondary and tertiary structures
- Sedimentation velocity analytical ultracentrifugation (SV–AUC), size exclusion chromatography coupled to multi angle light scattering (SEC–MALS) or dynamic light scattering (DLS) for the analysis of quaternary structures.

Activity characterization:
- ELISA-based bioassays
- Cell-based bioassays
- Surface plasmon resonance (SPR) or bilayer interferometry (BLI) for binding activity.

Then we have to consider what are now called the emerging techniques, at least for their application to complex biologics in an industry context, which in a couple of years will become as common as the techniques previously listed. These techniques include:
- Hydrogen-deuterium exchange–mass spectrometry (HDX–MS), ion mobility-mass spectrometry, and nuclear magnetic resonance (NMR)
- Native mass spectrometry.

Sadick and Merges (Catalent): As protein therapeutics commonly have complex structures and are generally produced and/or modified by the host cell in several functional variations, analyses of these molecules require an orthogonal approach with multiple analytic modalities.

Process-related variants can be identified, quantified, and differentiated from process-related impurities of cellular origin via techniques such as SEC–HPLC, hydrophobic interaction chromatography (HIC), ion-exchange HPLC, and isoelectric focusing (IEF) capillary electrophoresis. Process-related impurities and residuals such as Protein A can be detected and quantified with ELISA assays, whereas host cell residual DNA can be quantified via quantitative polymerase chain reaction (qPCR) assays. Functional activity of the

protein therapeutic can be assessed and quantified with cell-based bioassays, or, in some cases, ELISA potency assays. Process-related variants and impurities may then be more fully identified and defined using mass spectrometry-dependent analyses. Host cell derived residual protein may be assessed and identified with a combination of ELISA assays (commercial assays at early phases, and then custom assays at later phase), one-dimensional and two-dimensional Western blot analyses, and more recently, MS-based analyses.

Kang (Patheon): To characterize a protein, we need to understand its content, primary and higher order structure, potency, heterogeneity, purity, and impurity. The commonly used analytical methods for characterizing these proteins include UV spectroscopy for concentration; SEC, analytical ultracentrifugation (AUC), and field flow fractionation (FFF) for aggregate measurement; capillary gel electrophoresis (CGE) for fragment measurements; capillary isoelectric focusing (cIEF) for charge heterogeneity, biochemical/cell-based assay for potency measurement; mass spectroscopy for primary structure; FTIR, CD, or HDX for higher order structure.

Testing for impurities

BioPharm: How do you ensure that the final drug product is free from impurities that affect safety and efficacy?

Dinwoodie (CRL): Designing the production process to minimize the materials introduced during manufacturing, as well as installing appropriate purification steps, are simple sounding methods for ensuring a final drug product is impurity free. In practice, additives are required for cell growth, non-target proteins will be extracted, and downstream processing will occur, so purification steps are paramount. Control of these steps must be demonstrated by validation and/or quality control checks on the bulk drug substance. Control of impurities that could arise from the fill/finish process are then assessed for the final product.

“Designing the production process to minimize the materials introduced during manufacturing, as well as installing appropriate purification steps, are simple sounding methods for ensuring a final drug product is impurity free.”

Kang (Patheon): It is very challenging to completely remove all the impurities, but the industry can make sure that the level of impurities in the final drug product are at a safe and consistent level. A key factor to ensuring this is to develop a sensitive and robust analytical method, so all the impurities can be accurately measured and the impurity-removing capability of the downstream process can be demonstrated. For

example, ELISA is the gold standard and work horse for host cell protein measurement, but it only measures the total HCP and can't give detailed information on the level of each individual host cell protein. Mass spectroscopy can fill the gap, and is, therefore, an excellent supplemental method for host cell protein analysis.

Sadick and Merges (Catalent): A "pure" protein is one that is free from any quantifiable amounts of impurities, so implementing several orthogonal methods together is necessary to assure this is the case. The complex structural properties of the protein, the nature of the potential contaminants (host cells, viruses, genetic variants, purification process), and the accuracy and appropriateness of any one given method all influence the selection of the methods used to perform the purity/impurity analysis. A subset of these analyses is executed during the purification process to assure that each purification step is performing as expected/required. The full panel is performed upon both the drug substance and the final drug product. In this way, effectiveness of and purity at each stage of processing is evaluated and assured.

Tissot (SGS): Having a product entirely free of impurities is a very arduous task, if achievable at all.

For process-related impurities, control procedures to follow the clearance of some of the process-related impurities are designed during the very early stage of the finalization of the manufacturing processes, and are refined as the processes are locked down. The use of ultra-sensitive mass spectrometry has been increasing in that very particular field, offering a greater ability to monitor such small molecule impurities at a parts per million to parts per billion level. Such methods are also commonly validated as either process-validation-related methods or even product-release methods.

For product-related impurities, the pre-IND or equivalent panel of assays, at the very early stage of the product development, includes some of the methods that will be further refined to monitor these impurities. Complementary chromatographic and electrophoretic methods using UV detection have been used to monitor therapeutic protein variants for decades, but these methods are on the verge of being replaced by multi-attribute methods (MAMs) using primarily mass spectrometry as a detection tool. The ability to not only monitor but characterize several of these variants or impurities using a single LC-MS or LC-MS/MS method will not only bring to this field more discrimination power but it is also expected to decrease the level of detection for these undesired components.

BioPharm: What are the analytical methods used for purity and impurity analysis

of therapeutic proteins?

Dinwoodie (CRL): The analytical methods used to determine the levels of impurities within a therapeutic protein are those that have both the discriminatory power to separate the impurities and the sensitivity to detect and quantify low levels of the analytes. For impurities that are not closely related in structure to the therapeutic agent, such as surfactants, for example, the method can use this difference to maximize the sensitivity. Analysis of these impurities will include steps to remove all proteinaceous material to maximize the signal for charged aerosol detection or alternative measures. Sequence and glycosylation variants are closely related, or even part of the therapeutic protein; therefore, they require highly discriminatory techniques for their quantification. Capillary electrophoresis and HPLC or ultra-high-performance liquid chromatography (UHPLC) are commonly applied to resolving these variants from the more common form of the protein. Aggregates are readily separated by SE–HPLC when the aggregation is robust. Less stressful techniques such as analytical ultracentrifugation may be required where the aggregation is more fragile. For non-therapeutic host cell proteins, cell-line specific ELISA are often used though mass spectrometry techniques can provide the discriminatory and quantification power required for these complex mixtures of impurities.

"Having a product entirely free of impurities is a very arduous task, if achievable at all."

References

1. ICH, Q2 (R1) *Validation of Analytical Procedures: Text and Methodology,* Step 4 version (1996).
2. USP, Chapter <1033>, "Biological Assay Validation," *USP 35–NF 30* (US Pharmacopeial Convention, Rockville, MD, 2011).
3. USP, Chapter <1034>, "Analysis of Biological Assays," *USP 35–NF 30* (US Pharmacopeial Convention, Rockville, MD, 2011).
4. ICH, Q6B *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products,* Step 5 (1999).

Adeline Siew, PhD, was formerly the science editor of Pharmaceutical Technology *and* BioPharm International.

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Expectations for Residual Impurity Analysis Continue to Rise

More complex biologic samples must be evaluated to ever higher levels of specificity and sensitivity.

Cynthia A. Challener

Process- and product-related impurities must be evaluated according to various regulatory guidelines during production and to enable final product release. Impurities can arise from the biological samples themselves or from the process of developing biologics, including handling of materials.

Sample-related impurities include residual host cell-derived proteins (HCPs) and nucleic acids, complexes or aggregates of the biologic (high-molecular-weight [HMW] proteins), and clipped species and half molecules (low-molecular-weight [LMW] proteins). Impurities from cell-culture media can include inducers, antibiotics, and media components.

Impurities that come from downstream processing can include microscopic particulates, metals, and any materials that have carried over from the purification process, including resin particles, surfactants, emulsifiers, and viral-inactivation agents. Biological contaminants derived from handling include mycoplasma, bacteria, and virus particles.

Some of these impurities have known structures, while others may be only partially characterized or completely unknown. Post-translational modifications such as glycosylation and phosphorylation, degradation via oxidation or deamidation, and disulfide bridge scrambling (misfolding) can occur during upstream or downstream processing or storage under inappropriate conditions, resulting in large numbers of possible impurities. Disposable equipment and plastic tubing, stoppers, and containers may be sources of leachables. For antibody-drug conjugates, free drug cytotoxins can be problematic.

The decision on whether to monitor these impurities, and to what levels, is generally risk-based, using knowledge from both analytical and biological assays, and any preclinical experience to assess the impacts of each impurity on the safety, efficacy, or stability of the biotherapeutic, according to Scott Berger, senior manager for biopharmaceutical markets at Waters Corporation.

Create many analytical challenges

Monitoring biologic production processes and analyzing products for release testing can be challenging for many reasons. For Jean-Francois Boe, scientific director of SGS Life Sciences, the greatest challenge is the vast number of potential impurities that can be formed when all of the possible chemical modifications that can occur are considered. "Tens of millions of combinations of impurities can be formed, many of which have significantly different physical and chemical properties. One unique analytical technique cannot be used. A number of appropriate analytical methods must be used to create as full a picture as possible of the impurities that are present," he explains.

"Purification of biologics is often a multi-step process, and there is no one-size-fits-all analytical methodology," adds Tiffani Manolis, director of global pharma segment marketing with Agilent Technologies. "As a result, analysis of residual impurities is often a time-consuming activity."

Another major challenge when developing methods to evaluate bioprocess residuals is matrix interference, according to Jon S. Kauffman, president of Eurofins Advantar Laboratories, a member of Eurofins BioPharma Product Testing.

"Developing a robust method for certain impurities is always a challenge. For most of the methods that support in-process or release testing of drug substances, both matrix effects and the presence of a high concentration of product are the main factors which can impact the performance of methods," agrees Jun Lu, director of analytical development at Catalent Biologics.

Matrix interference can be caused by components in the formulation buffer that interfere with the detection of the residual by suppressing the ionization in the mass spectrometer or from the residual binding to the protein, according to Kauffman. "Further," he says, "we are typically required to monitor these residuals in various steps throughout the bioprocess. The sample matrices from each step can be quite different and each pose a challenge with respect to interferences and sample preparation."

"Purification of biologics is often a multi-step process, and there is no one-size-fits-all analytical methodology."

Complicating the situation is the fact that many product-related impurities need to be monitored down to low-percentage, or fractional-percentage levels, straining traditional optical, ultraviolet (UV)-based peptide mapping assays, according to Berger. "Increasingly, this necessitates the use of liquid chromatography-mass spectrometry (LC-MS) analysis to obtain the additional the selectivity and dynamic range for detection and monitoring of critical impurities. In addition, some impurities such as clips and unfolded variants may require multiple techniques for efficient detection

and quantification, because peptide level analysis is often uninformative for these structures," he observes.

Some impurities, such as surfactants, often exhibit a broad rather than a sharp peak and can interfere with each other, making specificity difficult to obtain. "For example," notes Kauffman, "it is virtually impossible to detect poloxamer 188 in a drug substance/product that is formulated with polysorbate. In these instances, we are forced to go backward in the manufacturing process to the step prior to addition of polysorbate."

Other challenges include the need to derivatize LMW compounds before analysis, as well as the ability of some residuals to adhere to the surfaces used during sample preparation, and the instability of others. Understanding these possible issues when developing methods is extremely important, according to Kauffman.

Numerous monitoring, separation, and detection technologies

As biopharmaceutical production processes evolve, and with the complexity of new process matrices, the detection and tracking of residual impurities is becoming increasingly difficult and may require various orthogonal techniques, says Vincy Abraham, director of biologics, Catalent Biologics.

Liquid chromatography and electrophoresis remain the two main separation techniques, and immunochemical assays remain unavoidable in specific cases for the evaluation of low levels of residual impurities, according to Boe. He notes that while little has changed with these separation technologies, there are many more advanced detection methods available today. UV or visible light and infrared (IR), fluorescence, mass spec, light scattering, and more have improved capabilities.

Other separation methods include gel-permeation, size-exclusion chromatography, ion exchange chromatography, and gas chromatography. For Kauffman, LC-MS/MS performed using a triple-quad mass spectrometer connected to an ultra-high-pressure LC (UHPLC) system is the technique of choice given its sensitivity, specificity, and ability to provide quantitative results. "This instrumentation is required in most cases to be able to quantitate at the ng/mL or even pg/mL range at which residuals must be evaluated," he says. HPLC and UHPLC are, however, still used with UV, charged aerosol, or evaporative light scattering detectors for compounds of interest in the µg/mL range or higher that do not ionize.

Detection by mass spectrometry is particularly useful for evaluating residual impurities formed due to chemical modification of the biologic drug substance, according to Manolis. Depending on the specific species of interest, MS can be coupled with LC, gas chromatography, matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI).

The most common method for screening biopharmaceutical products and testing for HCPs is enzyme-linked immunosorbent assay (ELISA), a sensitive assay with a low detection limit, high level of re-

producibility, and compatibility with high-throughput screening, according to Laura Moriarty, marketing manager for Bio-Rad's Drug Discovery and Development Group. She notes, though, that because the ELISA technique does not permit identification of antigens when using mixtures of antibodies, but only provides titers, the accuracy and utility of ELISA relies on a thorough prior assessment of the antibodies used. "Accurate evaluation and validation of antibodies reactive against HCP is crucial for detecting and monitoring HCP both during the product development cycle and during manufacture of biologics," she says.

The predominant method for assessing anti-host HCP antibodies involves 1-D or 2-D electrophoresis followed by western blotting, according to Moriarty. For polypeptides with similar molecular masses in complex mixtures of proteins, 2-D electrophoresis gives much better resolution because it separates proteins in the first dimension by isoelectric point (pI), followed by molecular mass in the second dimension. Once a good purification system has been established, the final product can be routinely screened with an ELISA to make sure that impurities are continually removed from the samples.

For nucleic acid screening, quantitative polymerase chain reaction (qPCR) and droplet digital PCR are used to detect and signal the presence of nucleic acids in a sample. Mycoplasma can also be detected using PCR, as well as colometric enzyme assays. Bacteria can be detected using endotoxin testing via the limulus amebocyte lysate assay, the United States Pharmacopeia (USP) chromogenic method, and the gel-clot method. The types of viral strains to be tested are specific to the method used to manufacture a therapeutic or biological.

Biologic aggregates are typically detected using sedimentation velocity analytical ultracentrifugation (SV-AUC), size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS), or dynamic light scattering (DLS) for the analysis of quaternary structures. DLS, as well as resonant mass measurement (RMM), can also be used to detect microscopic particulates, according to Moriarty.

Multifunctional methods are important

Because there are so many different types of manufacturing processes and residual impurities from low to high molecular weight with varying chemical and physical properties, identifying multifunctional methods that can separate and detect more than one type of impurity is essential for developing optimized methods. "Mass spectrometry is becoming attractive in part for this reason; a mass spectrometer can be used for the detection of numerous different impurities well chromatographically separated or co-eluted in a single chromatographic run," Boe states.

Mass spectrometry has become the primary analytical technology applied to multiplexed analyte detection within complex samples, agrees Berger. "The additional selectivity of the mass dimen-

sion enables detection and higher dynamic range quantification of analytes, even in the presence of co-eluting species. This methodology is now starting to be applied within biopharmaceutical development against a list of targeted product or process impurities," he observes.

Recently there has been a lot of work done using LC–MS for multi-attribute monitoring method (MAM), which is designed, according to Manolis, to provide simultaneous detection, specificity, identification, quantitation, and monitoring of attributes that are relevant to safety, efficacy, and the overall quality of drug. "MAM provides residue-specific identification, quantitation, and better understanding of any post-translation modification when compared to traditional methods of analysis, thus improving overall operational efficiency, resource consumption, and time required," she comments.

As long as the transitions monitored are distinct for each compound with little to no cross talk, Kauffman agrees that newer LC–MS/MS systems and software suites allow the detection of multiple impurities at once. "The challenge in these situations is the sample prep. Often times when you optimize a method for multiple analytes, it works really well for some analytes but not for others. Finding the right sample prep that extracts all the analytes of interest can be quite challenging. Methods for sample cleanup often work for one sample matrix but not another. As a result, the rule of one analyte per method is still the preferred approach so that the method can be optimized for the analysis of that particular analyte," he says.

"With ever-increasing regulatory and compendial stringency to identify, quantify, and monitor impurities, a greater emphasis is being placed on their characterization and analysis at trace levels."

Recent advances are having an impact

"With ever-increasing regulatory and compendial stringency to identify, quantify, and monitor impurities, a greater emphasis is being placed on their characterization and analysis at trace levels," asserts Abraham. "Fortunately," she continues, "there have been parallel advancement in technologies that allow rapid characterization of impurities at levels of approximately 0.1%."

To alleviate some of the limitations with ELISA, for instance, Abraham notes that several technologies for quantitation exemplified by Gyrolab, AlphaLISA, and Octet have emerged in the past decade as viable alternatives for HCP. Each represents a different strategy for HCP quantitation.

Bio-Rad recently introduced droplet digital PCR (ddPCR) as a sensitive (picogram range sensitivity in milligrams

of recombinant vaccines) and quantitative method for quantification of residual host-cell DNA, according to Madhuri Ganta, senior global product manager in Bio-Rad's Digital Biology Group. With ddPCR, a sample is partitioned into 20,000 nanoliter-sized droplets, which makes the PCR reaction less susceptible to inhibitory substances. Unlike with qPCR, extraction of total DNA from the protein drug sample is not required; intermediates can be processed directly, and absolute quantification is possible without the need to establish a standard curve, according to Ganta.

While optical-based LC assays are still highly desirable due to the lower system cost and broader organizational accessibility of this technology, Berger observes that the increasing complexity of modern biopharmaceuticals has pushed laboratories to adopt more resolving and sensitive UPLC- and UHPLC-based separations platforms for these newer products. He adds that the additional adoption of mass detection to increase selectivity and dynamic range of these assays has been growing within regulated development and is now starting to appear in quality control for targeted monitoring of product and process attributes and impurities.

The use of mass spectrometry for the characterization and quantification of HCPs is an active area, according to Yunsong (Frank) Li, director of biologics process development at Catalent Biologics. "MS can detect the HCPs not covered by anti-HCP reagents and provide additional information such as molecular weight, theoretical isoelectric point (pI), and immunogenicity potential," he explains. ProteinSEQ technology (Thermo Fisher Scientific) has also recently been demonstrated to quantify HCPs in a much wider dynamic range than ELISA, according to Li. The combination of ion exchange (IEX)-HPLC and high-throughput western blot is also under development for quantification of low immunoreactive HCPs.

For detection of aggregates, Li adds that nanoparticle tracking analysis (NTA) can track nano-sized particles via particle-scattered light from a focused laser beam. "The system can track many individual particles and therefore count the number of particles. From the rate of the particles' Brownian movement, the size can also be calculated," he says. Flow cytometry, traditionally used for cell counting, has also been developed to count the protein aggregation particle size as low as 0.2 µm.

In other areas, traditional sodium sodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) is being replaced by capillary electrophoresis (CE-SDS) because it provides superior detection, reproducibility, and robustness, according to Manolis.

Another development, according to Abraham, involves a shift from the conventional protocol of isolation and spectral analysis to online analysis using sophisticated modern hyphenated tools, such as GC-MS, LC-MS, CE-MS, supercritical fluid chromatography-MS (SFC-MS), LC-nuclear magnetic resonance (LC-NMR), CE-NMR, and LC-Fourier-transform infrared spectrometry (LC-FTIR).

Separately, Berger points out that the use of automation for sample preparation is greatly increasing within development and quality control organizations. "In development, this automation often supports higher-throughput clone selection and quality-by-design (QbD) studies, but increasingly the reason for adopting automated sample preparation is the improved consistency of sample generation versus manual workflows. The need for a mid-tier scale of automation has become apparent," he says.

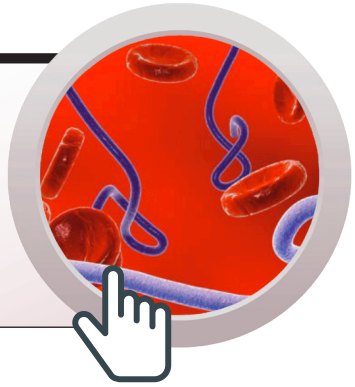
Some limitations remain

Indeed, improving the efficiency and reducing the costs associated with residual impurity analysis, which is essential to improving the overall efficiency drug development and manufacturing, requires that workflows be amenable to automation and high-throughput protocols, agrees Moriarty.

Eurofins is typically required to resolve three primary problems that are interconnected: quantitation limits, interfering compounds, and extraction of analytes of interest. "Interfering compounds and poor extraction of the compounds of interest directly affect the quantitation limits of the methods. Mass spectrometry for the most part eliminates co-eluting peaks because we can focus in on a mass transition for the compound of interest, but there are still times when compounds share the same transitions or have cross talk with transitions from other compounds. Extraction techniques have evolved over time especially with the addition of molecular weight cut-off filters and solid-phase extraction cartridges, but the more you manipulate the samples, the more chance you have to introduce error and contamination," explains Kauffman.

One challenge is the high variability in the process and sample matrix, which can contribute to out-of-specification/out-of-events, which are often time-consuming and costly, according to Manolis. Standardization of specifications for critical reagents and simplified and reproducible processes for sample digestion are also needed. For multi-impurity detection methods such as MAM, Manolis notes that improvements in systems for data processing, handling, and interpretation are needed.

Boe points to the current gap in the ability to accurately characterize and mostly quantify particles (aggregates) that are between several hundred nanometers up to 1 micrometer in size. For HCPs, he notes that the need to switch from commercial kits for HCP analysis to custom-developed methods once a candidate reaches Phase III trials is time consuming.



Currently, the greatest limitation for process-related impurity is analytical technology for HCP analysis, with the major challenge in coverage from existing anti-HCP polyclonal antisera standards, according to Li. The current approach is to develop product- and process-specific assays, which often require long lead times of at least 18 months, or combine multiple existing anti-HCP polyclonal antisera standards.

A general key challenge has been increasing the usability of more informative and complex modern analytical technologies to enable non-specialists to continue to perform these analyses, according to Berger. "While those charged with product characterization are always welcoming greater performance envelopes of their instruments, those charged with product monitoring and release now tend to be focused on minimizing user interactions with their systems and maximizing quality and reproducibility of the results," he says.

A few more thoughts

In addition to establishing methods that meet requirements for sensitivity and specificity, there are other factors that are important to consider. "It is essential to first determine the appropriate acceptance criteria and then ensure that methods can be readily transferred from R&D to commercial production. They should be robust, accurate, and precise, as well as easy to implement on equipment that will be available at the manufacturing plant," Boe asserts.

"A validation process that makes sense is also important, as is the need to consider the biological activity of product-related impurities."

A validation process that makes sense is also important, as is the need to consider the biological activity of product-related impurities. "Some impurities that are closely related to the product may have the same biologic activity as the drug substance, and therefore may not impact the safety and efficacy of the product. It may be reasonable to classify these compounds as related substances, rather than residual impurities," Boe explains.

Cynthia A. Challener, PhD, is a contributing editor to BioPharm International and Pharmaceutical Technology.

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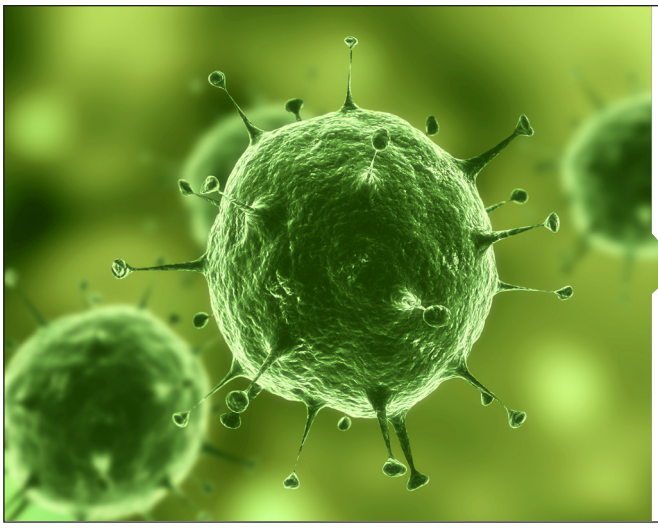


Ensuring Viral Safety of Viral Vaccines and Vectors

Viral vaccines and viral vectors used in biotherapeutic applications carry the risk of microbial contamination, which must be addressed.

Anissa Boumlic, Martin Wisher, Damon Asher, and Priyabrata Pattnaik

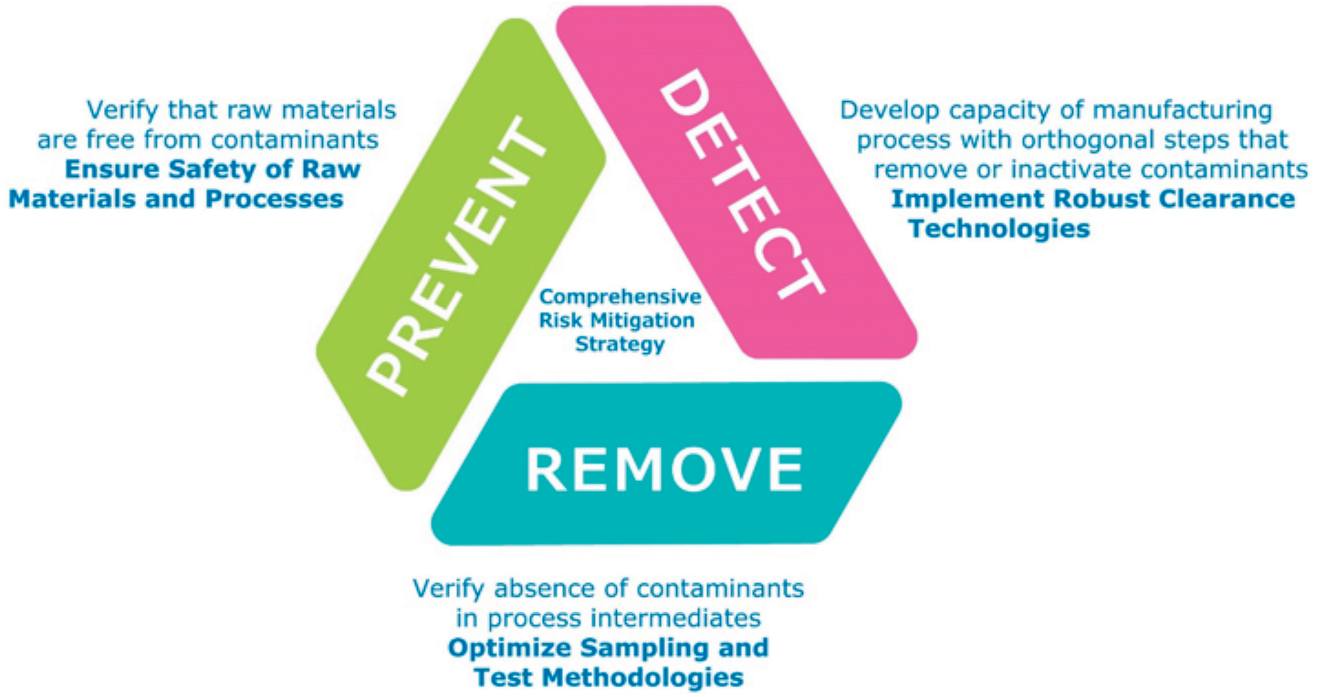
Vaccines, including viral vaccines, are a crucial invention in human history and continue to improve lives through the prevention, control, and eradication of infectious disease. Viral vaccines rely on antigenic properties of a virus or virus-like particle (VLP) to trigger an immune response against an incipient viral infection. Because of the risks associated with live and inactivated viruses, namely potential attenuation reversal or failure of inactivation, recombinant viruses have emerged in the role of either vaccines or vectors in gene and immunotherapies. However, because biological materials—cell substrates and often animal-derived products—are used in their manufacture, viral vaccines and vectors are at risk of contamination from micro-organisms, such as adventitious viruses.

In the past, a few medicines produced from biological materials, such as blood products or vaccines, were found to be contaminated with viruses. Unscreened human or animal-derived products, such as bovine serum, are now known as potential sources of bovine virus contamination (1).

Since then, better safety measures and the use of established and characterized cell lines have improved safety in biologicals. To date, no infectious virus has been transmitted to a patient by a cell-line-derived biopharmaceutical. Extraneous vesiviruses, however, have recently appeared in bioreactors (2–5), and porcine circovirus type 1 (PCV-1) contamination of oral rotavirus vaccines was first reported by a metagenomics analysis (6). PCVs entered vaccine processes via porcine trypsin, a common cell-culture reagent. In 2014, using next-generation sequencing (NGS), FDA's Center for Biologics Evaluation and Research (CBER) retrovirus laboratory identified a novel rhabdovirus in *Spodoptera frugiperda* type 9 (Sf9) cells (7). Sf9 cells are a common substrate for biological products such as VLPs.

These contamination events highlight the limitations of current technologies; more vigilance is needed. Consequences of vaccine or vectorviral contamination

Figure 1: Tripod strategy for assessment and mitigation of the risk of adventitious agent contamination in viral vaccines and vectors.



are significant, and manufacturers may be forced to recall lots or shut down facilities for decontamination, which can hurt a company's reputation. Moreover, such events could affect the broader perception of vaccine and viral vector safety.

This article outlines the risks and challenges associated with managing viral safety in virus vaccines and vectors for gene therapy, and highlights a holistic risk-mitigation approach of testing and clearance methodologies to help prevent contamination events.

Viral safety strategy and regulatory

Virus safety of viral vaccines and vectors ensures that: the vaccine product is free of unintended viruses; any residual pathogenicity of a live agent is within acceptable limits for safe use; and inactivated agents are indeed completely inactivated (8). Regulatory guidance documents (9–11) suggest that the risk of adventitious agent contamination should be assessed and mitigated through a tripod strategy (**Figure 1**):

- Preventing entry of contamination into production processes by ensuring the quality of raw materials
- Detecting contaminants by characterization of cell banks/virus seed stocks and by testing process intermediates
- Eliminating contaminants by incorporating virus inactivation/removal steps into

the vaccine purification process (challenging for whole live virus vaccines but practical for inactivated, subunit, and recombinant vaccines).

Prevent virus introduction by testing raw materials

Ensuring the quality of raw materials used in vaccine and vector production is the first step in preventing viral contamination. Animal-derived components such as bovine serum and porcine trypsin should be screened for bovine and porcine viruses. Regulatory guidelines exist for the selection, qualification, and testing of these raw materials and indicate that not only known, but also emerging viruses should be sought (12–16). There are also specific guidelines for the usage of bovine serum or trypsin in the manufacture of biologics (17, 18).

New technologies for cell culture or raw-material treatment create barriers to viruses and mitigate bioreactor contamination risk. These methods primarily target the cell-culture medium before its transfer to the bioreactor as well as raw materials of animal or human origin. Options include high temperature/short time (HTST), C spectrum ultraviolet light (UV-C), gamma irradiation, and nano- or virus filtration.

HTST is commonly used in the food and beverage industry for high-volume processing. A liquid is heated and held at 102 °C for at least 10 seconds, then cooled to 37 °C before being sent to the bioreactors. Virus inactivation efficiency has been demonstrated on several viruses; for example, a 10,000-fold reduction in foot-and-mouth disease virus (FMDV) was observed in HTST-treated milk (19). However, this method might be less effective where high levels of contamination are present (20). This technique has been applied to treat raw materials such as cell-culture media (21) and glucose (22). The impact of HTST on the properties of treated raw material and the performance of cell-culture processes needs to be assessed.

"Ensuring the quality of raw materials used in vaccine and vector production is the first step in preventing viral contamination."

UV-C irradiation has been used in the food, plasma, and biotech industries (for packaging and surface sterilization) and is effective against various biological contaminants, including bacteria and viruses (23–25). Limitation of this method is the flow rate of the fluid, which needs to be modulated for optimal results (21).

Gamma irradiation—ionizing radiation from a radioactive Cobalt 60 source—breaks bonds in nucleic acids and proteins. This method is widely used in the biopharmaceutical industry to treat bovine serum or single-use components. Certain viruses, however, have shown resistance to gamma rays (26).

Nanofiltration or viral filtration is a separation method based on size exclusion and

Figure 2: Current barrier technologies for cell-culture medium treatment, with considerations.

Technology	Robust Clearance	Media Compatibility	Point of Use	Scalability	Cost Effective
HTST (~102°C ~10 sec) 	Yes	Component dependent	Yes	Challenging for small to mid-scale	Yes at Large Scale
UV-C (254 nm) 	Organism dependent	Component dependent	Yes	Challenging at large scale	Yes at Small Scale
γ Radiation 	Organism dependent	Component dependent	No	Small batches	Yes
Downstream Virus Filters 	Yes by size exclusion Consistent LRV	Yes but designed for downstream fluids	Yes	Yes	Not for batch processes
Upstream Virus Barrier Filters 	Yes by size exclusion. Consistent LRV	Yes, specifically designed for upstream media	Yes	Yes	Yes

the usage of membranes or fiber-based filters. The biotechnology industry utilizes this technology to ensure viral clearance in downstream processing. Currently, optimized filters are considered for the filtration of thermo-sensitive raw materials and cell-culture media (27).

Because of potential impact on cell-culture performance, downstream processing, and product quality, the treatment method for the prevention of virus contamination should be chosen with care. **Figure 2** summarizes current technologies for cell culture medium treatment—and their implications.

Detect viruses in process intermediates

The second step in ensuring viral safety for vaccines and vectors is to test for viruses that could be present in the initial process, beginning with the cell bank. Testing for virus contamination is part of cell-bank characterization. The master cell bank (MCB), the starting material for the entire production process, requires a one-time, full characterization of microbial and viral contaminants. The working cell bank (WCB) requires less testing on early passages, but more on subsequent ones. And end-of-production cells (EOPC) or cells at the limit (CAL) of *in-vitro* cell age used for production—which represent the worst case for amplification of contaminants—require full, one-time characterization at production scale. This testing is summarized in **Figure 3**.

MCBs should be tested for identity (phenotypic and genotypic, if recombinant) and purity. While FDA, the European Medicines Agency, and the World Health Organization guidelines differ, testing must demonstrate

Figure 3: Test strategy for ensuring cell banks and master virus seed stocks begin and remain virus-free.

Virus Tests	Master Cell Bank (MCB)	Working Cell Bank (WCB)	Cells at Limit of Production (CAL)	Master Virus Seed Stock
Broadly specific *in vitro* assays	✓	✓	✓	✓
Broadly specific *in vivo* assays	✓		✓	✓
Species specific assays (e.g. human, simian, rodent, canine, bovine, porcine)	✓		✓	✓
Retroviruses (e.g. PCR, TEM, PERT, infectivity)	✓		✓	✓
	• Critical starting material • Full characterization, one time testing	• Small number of passages beyond MCB • Reduced package of testing	• 'Worst case' for amplification of contaminants • Full characterization, one time testing	• Neutralization pre-studies for *in vitro* and *in vivo* infectivity studies

the absence of bacterial, fungal, and viral contamination. A broad array of in-vitroand in-vivoassays may detect extraneous viruses. In a US National Institutes of Health study, lead investigator Rebecca Sheets, PhD, systematically characterized the breadth and sensitivity of routine *in-vitro* and *in-vivo* assays for inapparent viruses (28). These data should aid regulators and manufacturers in decision-making and serve as a baseline for comparison of new methods. Cell lines must also be tested for species-specific viruses, as appropriate, using antibody tests or polymerase chain reaction (PCR) panels.

Beyond these tests, other techniques for the detection of retroviruses may be implemented. Retrovirus particles can be detected using transmission electron microscopy (TEM). Reverse transcriptase enzyme activity within the retrovirus protein core can be detected using PCR-based reverse transcriptase (PBRT) assays, also called product enhanced reverse transcriptase (PERT) assays. Species-specific retrovirus screening also exists.

In addition to screening the cell lines, master virus seed stock (MVSS) must also be screened fully to detect adventitious bacteria, fungi, mycoplasma, and extraneous viruses, while taking into consideration the origin and isolation of the virus stock. Neutralizing antiserum is required for infectivity assays to specifically inactivate the master virus. Again, testing must include species-specific assays as well as testing for retroviruses (Figure 3).

Recently, NGS has been applied for viral detection in biologicals. This method allows simultaneous sequencing of millions of DNA or RNA fragments and requires no prior knowledge of potential virus contaminants (29).

Remove or inactivate viral contaminants downstream

Regulatory bodies require that the purification process for a biological pharmaceutical removes any nonproduct virus. These "viral clearance" or "viral removal" steps usually entail inactivation, chromatography, and/or virus filtration.

The fact that viral vaccines and vectors are actual viruses limits the application of removal and inactivation methods. To solve this challenge, each process must be examined to ensure that viral clearance steps do not compromise the final product.

"Regulatory bodies require that the purification process for a biological pharmaceutical removes any nonproduct virus."

Inactivation typically utilizes extreme physical (pH, temperature) and/or chemical (detergents, solvents) conditions (30). Chemical processes are typical, as with poliomyelitis and influenza viruses, and often utilize β-propylactone (BPL) or formalin (formaldehyde). Insect cell-based processes can produce 1010–12 baculovirus particles that must be removed during downstream purification. As a safety measure, some manufacturers inactivate the baculovirus prior to removal through various combinations of BPL and high-temperature or solvent/detergent treatments. In killed-virus processes, the inactivation step aimed at the antigen can also inactivate other contaminant viruses.

In adeno-associated virus (AAV) processes, any replication-competent helper adenovirus must be inactivated and removed during downstream purification. AAV particles can resist heating at 52 °C for 10 minutes, while human adenoviruses are more sensitive (31). Such treatment is effective but remaining denatured helper virus proteins may induce a cellular immune response in the patient and require removal by other methods.

Filtration is a robust method for virus removal in biologics production. However, filter size must be carefully selected in accordance with the size of the virus of interest and known and potential non-product viruses. Increasing evidence shows that removal of non-product viruses from viral-vectored vaccines or baculoviruses from VLPs expressed in insect cells can be achieved using 50-nm or 35-nm virus-retaining filters (32). This filtration, however, might not be applicable if the viruses share a similar diameter. Moreover, conditions should be adjusted to avoid aggregation or complex formation

of therapeutic viruses and, thus, product loss.

Chromatographic methods are potentially effective tools for adventitious agent clearance from vaccine viruses. Chromatography has been shown to remove PCV (33), and ion exchange and affinity chromatography have been shown to purify vectors such as AAVs. However, because achieving robust clearance is challenging using chromatography, regulatory documents urge caution but remain encouraging. European guidance states that if the virus reduction is reproducible and the influencing manufacturing parameters are well defined, chromatography could fit the criteria of an effective step (34). Chromatography processes can be optimized to separate the product from helper viruses (35). Chromatographic separation of empty versus full capsids with a similar charge remain a challenge. Density separation using ultracentrifugation is possible but is challenging at GMP manufacturing scale.

Conclusion

Viral contamination of vaccines, albeit rare, can lead to serious human health and economic consequences. Regulatory and industry expectations vary in their specifics but do entail viral safety risk assessment and mitigation in the production of these therapeutics. A three-pronged approach—verifying the safety of raw materials, monitoring process intermediates for unintended viruses, and removing viral threats from products—can help prevent the administration of adventitious virus-contaminated viral vaccines and vectors to patients.

References

1. P.P. Pastoret, *Biologicals* 38 (3) 332–334 (2010).
2. R.L. Garnick, *Dev Biol Stand* 88, 49–56 (1996).
3. Genzyme, "Genzyme Reports Progress Related to Allston Plant," Press Release, June 25, 2009.
4. A. Oehmig et al., *J Gen Virol,* 84 (Pt 10) 2837–2845 (2003).
5. Y. Qiu et al., *Biotechnol Bioeng,* 110 (5) 1342–1353 (2013).
6. J. Victoria et al., *J Virol* 84 (12) 6033–6040 (2010).
7. H. Ma et al., *J Virol* 88 (12) 6576–6585 (2014).
8. D. Mackay and N. Kriz, *Biologicals*, 38 (3) 335-337 (2010).
9. FDA, *Guidance for Industry Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications* (Rockville, MD, 2010).
10. WHO, *Recommendations for the Evaluation of Animal Cell Cultures as Substrates for the Manufacture of Biological Medicinal Products and for the Characterization of Cell Banks, Technical Report Series 978 Annex 3* (Geneva, Switzerland, 2013).
11. EDQM, *EurPh*, General Text 9.3; 5.2., "Cell substrates for the production of vaccines for human use," (EDQM, Strasbourg, France, 2018).
12. CFR, 9 *Code of Federal Regulation,* Part 113 (Government Printing Office, Washington, DC), pp. 47, 53.
13. CBER, *Guidance for Industry—Cell Substrates and Biomaterial* (Rockville, MD, Feb 2010).
14. EMA, *Guideline on Influenza Vaccines* (London, July 2016).

15. EMA, *Guideline on Quality, Non-Clinical & Clinical Aspects of Live Recombinant Viral Vectored Vaccines* (London, June 2010).
16. EMA, *Guideline on Quality, Non-Clinical and Clinical Aspects Microbial Contamination of Gene Therapy Medicinal Products* (London, May 2015).
17. EMA, *Guideline on the Use of Bovine Serum in the Manufacture of Human Biological Medicinal Products* (London, May 2013).
18. EMA, *Guideline on the Use of Porcine Trypsin* (London, February 2014).
19. P.M. Tomasula et al., *J Dairy Sci.* 90 (7) 3202–3211 (2007).
20. M. Murphy et al., *Biologicals* 39 (6) 438–43 (2011).
21. M. Schleh et al., *Am. Pharm.*13, 72–76 (2010).
22. K. Remington and C. Hernandez, *GEN* 34 (5) (2014).
23. W.A. Hijnen et al., *Water Res.* 40 (1) 3–22 (2006).
24. Q.S. Meng and C.P. Gerba, *Water Res.* 30, 2665–2668 (1996).
25. G. Chevrefils et al., *IUVA News* 8 (1) 38–45 (2006).
26. H. Stettmund Von Brodorotti and H. Mahnel, *Zentralbl Bakteriol B* 170 (1–2) 71–81 (1980).
27. C. Carbrello et al., "Cell Culture Engineering XV," presentation at Engineering Conferences International (Palm Springs CA, 2016).
28. J. Gombold, *Vaccine* 32 (24) 2916–2926 (2014).
29. C. Braxton, *Amer Pharm Rev* (2017).
30. B. Sanders et al., "Inactivated Viral Vaccine," in *Vaccine Analysis: Strategies, Principles and Control*, B. Nunnally, V. Turula, and R. Sitrin, Eds. (Springer, Berlin/Heidelberg, Germany, 2015), pp. 45–80.
31. B.A. Thorne et al., *Biologicals* 36 (1) 7–18 (2008).
32. C. Bogedain, G. Maass, and M. Hoerer, "Filtration Method For Separating Viruses," US patent 6479273. (Nov. 2002).
33. B. Yang et al., *Biotechnol Prog.* 29 (6) 1464–71 (2013).
34. EMEA, Note for Guidance on Virus Validation Studies: The Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses. CPMP/BWP/268/95, (London, revised 1996).
35. G.J. Ye et al., *Hum Gene Ther Clin Dev* 25 (4) 212–217 (2014).

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Laboratory Water Preparation—Optimized

Why process analytical matters to process development R&D.

Nadia Brandes

Introduction

Water is used for almost all applications in the laboratory. Impurities are found in all tap water and can have a negative effect on scientific analyses. This article explores the sources of water impurities and how to optimize water purity to the standard needed for specific applications. It will demonstrate how to save resources by providing a theoretical and practical understanding of how to work most efficiently with a lab water system. Topics include impurities, standards, purification, applications, and accessories and services.

Water impurities

There are five classes of water impurities:

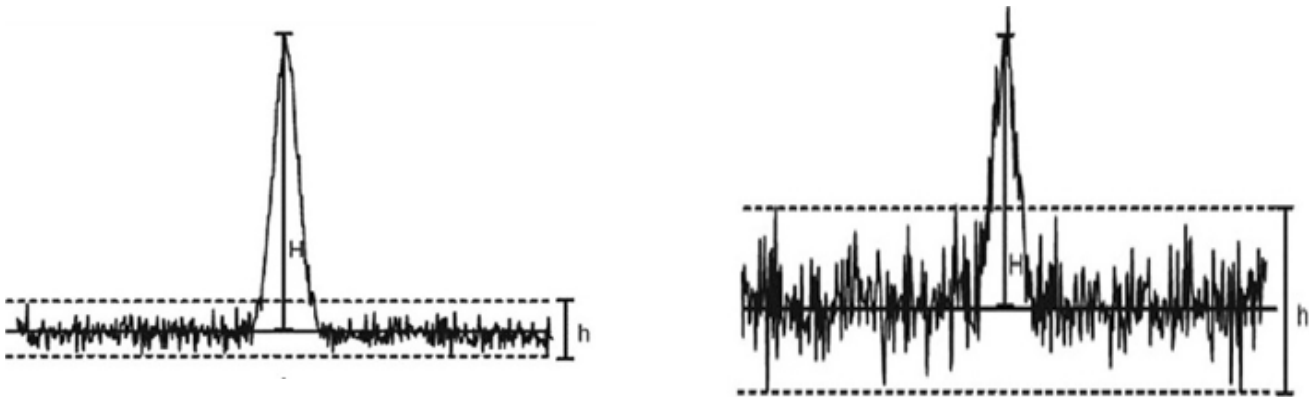
- Suspended particles, which include sand, pipe work debris, and colloids.
- Dissolved inorganic compounds including calcium, iron, and salt.
- Dissolved organic compounds such as pesticide residues and oil.
- Microorganisms, which include bacteria such as pseudomonas.
- Dissolved gases like oxygen and carbon dioxide.

These impurities can affect a laboratory's work.

Water that is adequate for rinsing vessels may not be adequate for analytical work. For example, if a laboratory is running a high performance liquid chromatographic (HPLC) analysis and the water has high organic content, it can cause background noise as shown in **Figure 1.**

Water that is adequate for running an autoclave may not be adequate for life science and molecular applications. Inorganic impurities can lead to nonspecific staining in histology slides, as well as influence enzyme functions. Some of most sensitive or critical types of applications are cell cultures. Endotoxins in the media can lead to cell death. Therefore, analysts must measure the relevant contaminants to be sure it is the correct water quality for their applications, as summarized in **Figure 2.**

Figure 1: Organic can increase background noises in chromatographic assays.



Standard and regulations

The measurement and limits for these contaminants and impurities are governed by several standards including the American Society for Testing and Materials (ASTM), the International Organization for Standardization (ISO), the Clinical and Laboratory Standards Institute (CLSI) and international pharmacopeia (including USP, EP and JP).

There are both similarities and differences among these standards. However, the ASTM is the most commonly used standard for laboratories. CLSI standards are required in clinical work and the pharmacopoeia governs the pharmaceutical industry.

The ASTM classifies four categories for water purity, overlapping the types already described here, with specific ranges for conductivity, TOC and ions. The USP classifies water in two categories: purified water and water for injection. Here, bacteria count and endotoxins are considered over conductivity. Thus, it is important to know which standards apply to a laboratory's daily work.

Water purification

Type 3. There are three water purification categories. Type 3, often referred to as *RO* (reverse osmosis) water, is used for non-critical work, where the reduction of salts, silicates, and particulates is necessary. Type 3 water requires pre-filtration and reverse osmosis. Pre-filtration uses activated carbon to remove chlorine and some organic matter. This step is immediately followed by depth-type filtration (5 µm) to remove particulates, including any activated carbon residuals. Reverse osmosis uses pressure to force-filtrate the pure water molecules through a semi-permeable membrane. While this is quite a mature technique, it remains the best filtration technology for pre-treatment because it can typically remove 98% of the calcium carbonate ($CaCO_3$), 95% of salt (NaCl) and organics, and 99% of bacteria and particles.

It is important to keep in mind that reverse osmosis has about a 40% yield. This reduced flow means a small laboratory system might produce about 20–40 liters per hour.

Figure 2: Measuring impurities.

Contaminant	Measurement	Unit
Inorganics	Conductivity / Resistivity (Measure of a fluids ability to conduct electricity)	µs/cm ≙ MΩ.cm 1 µs/cm ≙ 1MΩ.cm 0.1 µs/cm ≙ 10MΩ.cm 0.056µs/cm ≙ 18MΩ.cm
Organics	Total Organic Carbons (TOC)	ppb or mg/l
Particles	Fouling Index (Silt Density Index)	Blockage rate of a 0.45µm membrane
Bacteria	Colony count	CFU/ml (Colony Forming Units / ml)
Endotoxin	LAL (Limulus Amoebocyte Lysate) Test RPT (Rabbit Pyrogen Test)	EU/ml (Endotoxin Units / ml)

Therefore, type 3 water systems typically have a tank to ensure an adequate supply is available.

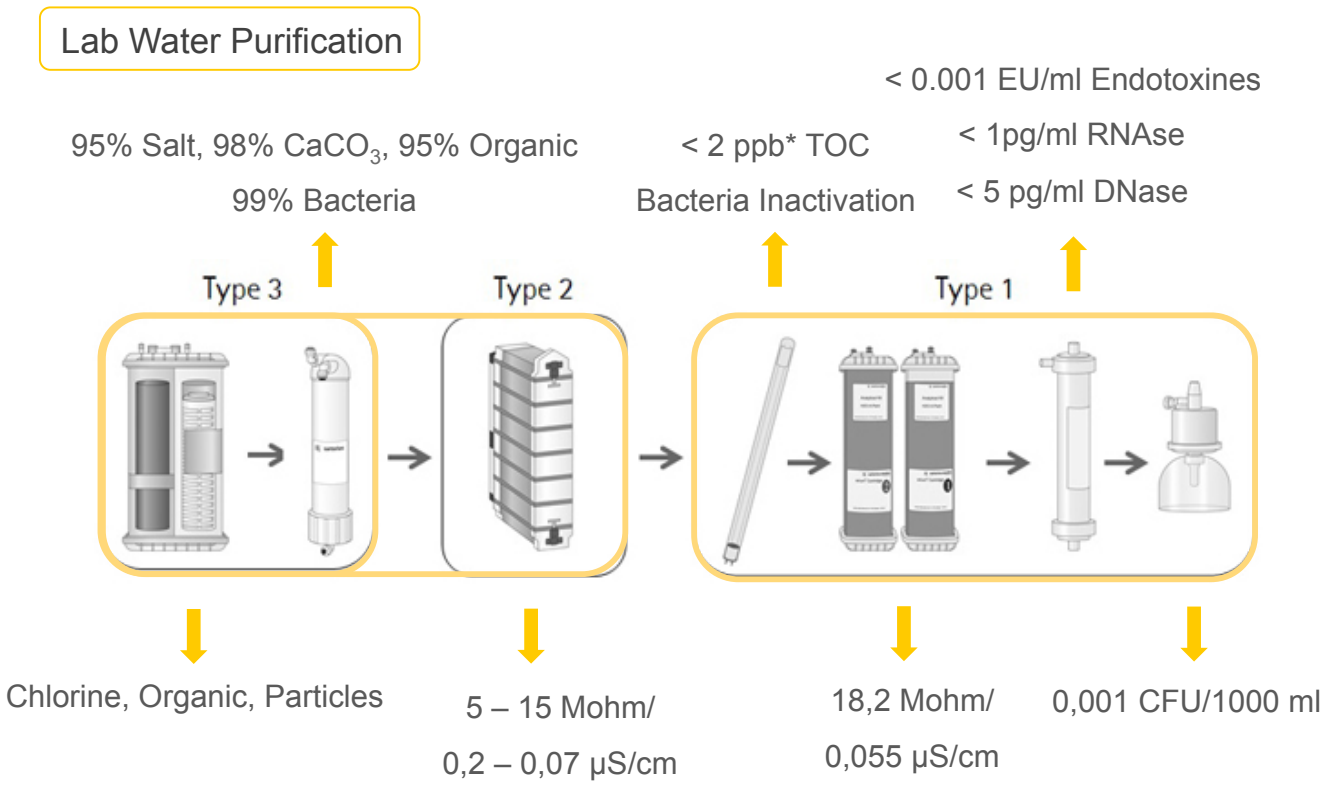
Type 2. Type 2, often referred to as *general lab water* or *DI* (deionized) water, is used for more general and less critical laboratory applications.

Next, the production of type 2 water requires the same components as type 3 water with the addition of ion exchange technology or electronic deionization (EDI) to remove anions and cations including fluoride, sodium, chlorine, ammonium, nitrate, sulfate, phosphate, and calcium. The resulting water has a conductivity of 0.2–0.07 µS/cm. Because the technology to produce type 2 water includes reverse osmosis, type 2 water systems may also have a tank to ensure adequate available flow rate.

Type 1. Type 1, often referred to as *ultrapure,* is required for critical applications. High-efficiency ion exchange is used to remove anions and cations to produce water with a conductivity of ≤0.055 µS/cm, which is a quality indicator for type 1 water.

In addition, there are optional components for type 1 water depending on the application. Long-wavelength UV (254 nm) has the greatest bactericidal action because it damages DNA and RNA polymerase, thus preventing replication. Short-wavelength UV (185 nm) is most effective for oxidizing organics, breaking up organic molecules for removal by the ion

Figure 3: Technologies used to produce the three types of water.



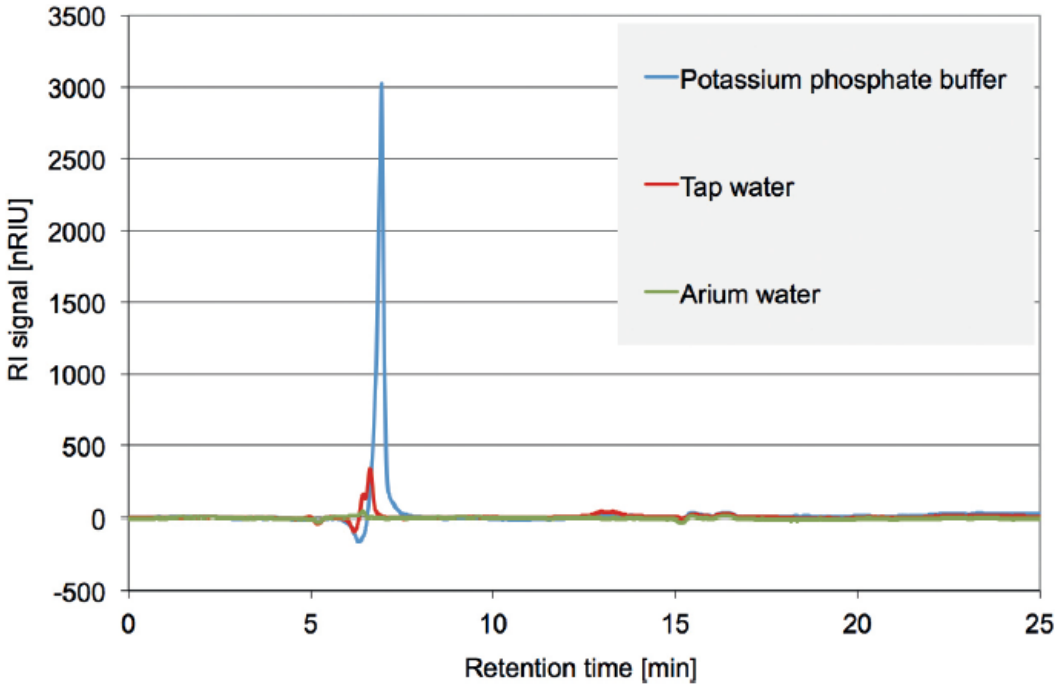
exchange beds. Thus, short-wavelength UV oxidation may be included for HPLC applications.

For bioanalytical applications, you may need ultra-filtration (UF) to reduce the concentration of microorganisms, endotoxins (pyrogens), and nucleases. UFs may be used at the "point of use" or in a cross flow. Cross-flow filters are always used in a recirculation process. As an example, for mammalian cell culture applications, endotoxins must be removed from the water to prevent apoptosis of the culture.

Finally, a sterile final filter is used to protect against secondary contamination by particles or micro-organisms. Typically, final filters have an external protection bell to minimize the risk of skin contact and avoid secondary contamination by RNase. The technologies used to produce the various types of water are summarized in **Figure 3.**

Storage. Once purified water is produced, it is important to consider storage options. Polyethylene (PE) tanks are available, which are sanitized in between use. Some suppliers like Sartorius prefer bag tanks because they can be easily replaced, therefore avoiding a long sanitization process and minimizing downtime. Also, the bag tanks are a

Figure 4: Analytical methods (HPLC) (1).



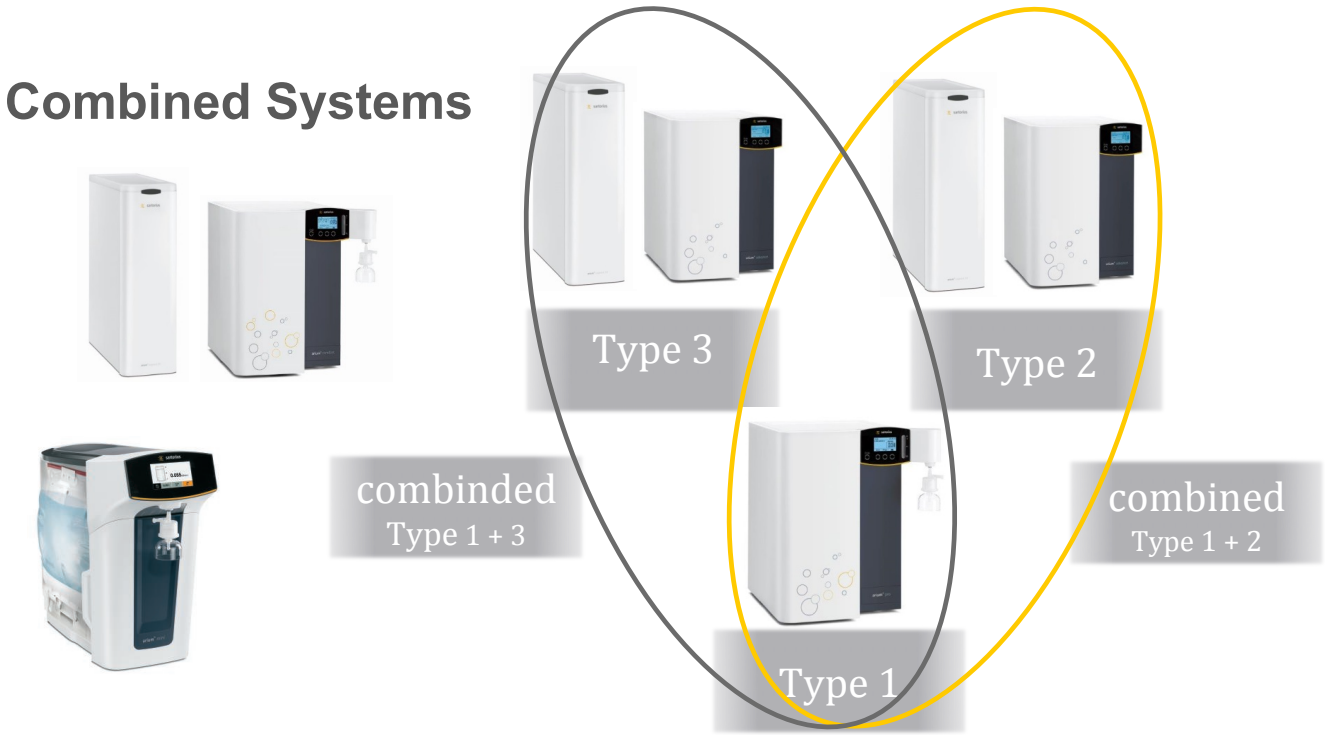
closed system, so secondary contamination is not a concern.

Water applications

It is important to understand the applications and needs to select the most efficient purified water options. Type 3 water (RO) systems are typically used for standard applications, such as washing machines, autoclaves, or for feeding certain type 1 water systems. Type 2 water (DI) systems may also be selected for standard applications. Further, type 2 water systems are required for preparing buffers and media, and for reagents and blanks, depending on the applications' requirements. Type 1 water (ultrapure) systems are mainly used for critical applications such as analytical methods, life science and molecular applications. Analytical methods include the instrumental methods, high performance liquid chromatography (HPLC), ion chromatography (IC), and inductively coupled plasma-mass spectrometry (ICP–MS). Life science methods include DNA analysis, cell culture, and histology. **Figure 4** shows a chromatographic comparison of impurities in a potassium phosphate buffer and tap water with ultrapure arium® water. These impurities can interfere with both analytical methods and life science methods, including cell culture.

Mammalian cell culture applications require ultrapure water treated by an additional ultrafilter to prepare the media because high

Figure 5: Combined water purification systems.



endotoxin values would lead to cell death. Tap water may have endotoxin concentrations about 25 EU/mL. Deionized water is better at about 0.02 EU/mL. However, arium® pro UF Ultrapure water yields endotoxin concentrations <0.001 EU/mL. Cell cultures of PER.C6 EpCAM cell lines in spinner flasks prepared with Type 1 water and grown over eight days result in higher cell density and viability than those prepared with other waters because the ultrapure water has nearly zero endotoxin concentration. Monoclonal antibody production in spinner flasks yields higher values when cultivated using media reconstituted with type 1 water than when cultivated using ready-to-use media.

The ultrapure water, free of microorganisms, with low endotoxin values, low inorganic ions, and low particles is the demonstrable choice for media preparation for sensitive cell culture applications.

When choosing a water purification system, both the

application and the consumption should be considered. Are multiple systems needed? Is there high-volume storage and production? Some firms like Sartorius make combined systems, for instance including pre-treatment (type 3) and ultra-purification (type 1) in a single compact system, thereby saving space in the laboratory (see **Figure 5**).

Accessories and services

Sartorius is dedicated to providing customers with complete solutions for their water purification needs. Recorders are available to continuously monitor and document the water quality for documentation. Dispense guns provide high flexibility and a foot switch, allowing hands-free use, like for batch preparation. Customized service solutions are also available, from installation to maintenance to qualification, including IQ/OQ (installation qualification/operation qualification).

End-user service and regular maintenance should also be mentioned and may be summarized by the following golden rules for high-quality clean water collection:

1. Dispense water before collecting and check water quality. For example, when using ultrapure water (type 1), dispense and discard at least 600 mL of water before using, to be sure all filters are flushed.
2. Use suitable extractable-free containers such as glass for ultrapure water. Avoid storing the dispensed water.
3. Avoid the formation of bubbles when dispensing the water to minimize external contamination.
4. Pay attention to regular maintenance, including the final filter.
5. Ensure that the tank size is adequate for your daily consumption.

“Some firms like Sartorius make combined systems, for instance including pre-treatment (type 3) and ultra-purification (type 1) in a single compact system.”

Conclusion

When choosing a water system, choose right size system for your laboratory’s daily consumption. From accessories and services to specific purities (with consideration for the application’s standards), look for technologies that optimize water purity and efficiency, helping to minimize downtime and cost. Removing the impurities found in all laboratory water can optimize scientific analyses.

References

1. K. Töppner, D. Hansen and E. Herbig, “Ultrapure Water for HPLC Analysis”, GIT Lab J Eur 17: April 2014.

Nadia Brandes
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Ultrapure Water for Trace Analysis

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The purity of the solvents used, primarily that of water, is a decisive criterion for interference-free and reproducible analysis by liquid chromatography and for the sensitivity of this method, especially for applications in trace analysis. In a study, different sources of ultrapure water used as eluents were compared in high-performance liquid chromatography with diode-array detectors (HPLC–DAD) and mass spectrometry (MS) systems in various experiments.

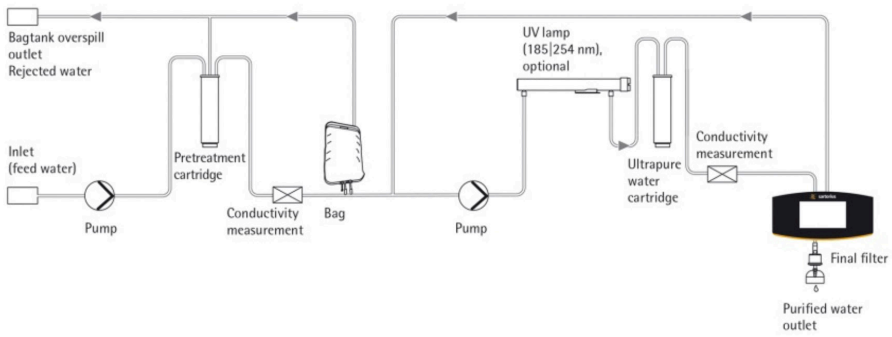
In the flavor and fragrance industry, many products are based on natural raw materials such as vanilla beans, citrus fruits, blossoms, and other materials of plant origin. HPLC systems coupled to various detectors (e.g., mass spectrometers, DAD, or refractive index [RI] detectors), are used for quality control of such raw materials in incoming goods inspection and final quality control of outgoing products, as well as in research and development of new products.

A routine analysis performed both in research and in quality control is, for instance, the quantification of vanillin in various samples (e.g., vanilla beans, vanilla extract, vanillin sugar, chocolate, beverages and flavorings) by HPLC-DAD. Besides the quantification of certain analytes, screening methods for the identification of partially unknown substances contained in raw extracts and natural products, among others, play a significant role in research. LC systems are predominantly used in such methods and are coupled to high-resolution time-of-flight (TOF)-MS instruments.

Avoiding high background noise

The purity of the solvents used, primarily that of water, is a decisive criterion for interference-free and reproducible analysis by LC analysis and for the sensitivity of this method, especially for applications in trace analysis. Contaminants in the eluent can result in relatively high baseline noise originating from the detector and thus to a poorer signal-to-noise ratio (S/N) of a peak. In DAD, such contaminants are, for example, organic compounds that absorb light

Figure 1: Arium mini plus ultrapure system and flow chart. To enhance the clarity of this diagram, the valves and process controllers have been omitted.



(photo and diagram courtesy of Sartorius)

in the UV/Vis range. In LC–MS applications, the concentration of ions (Na^+, K^+) should be kept as low as possible to prevent the formation of adducts with analytes during ionization. Water contaminants that are enriched in the stationary phase can also be eluted with a higher percentage of organic solvent and occur as potentially co-eluting peaks on the chromatogram.

For these reasons, a number of specially treated and filtered types of ultrapure water are commercially available in different grades (HPLC and LC–MS grades). An alternative to these grades of water that are usually filled in 1- or 2.5-liter bottles is to use water purified by ultrapure water systems such as the Arium mini plus ultrapure water system (**Figure 1**).

Use of ultrapure water freshly produced by such a system to prepare an eluent for HPLC-DAD and MS systems was compared in various experiments with two commercially available brands of bottled water of LC–MS grade. For this purpose, the background signal in the chromatogram—usually detectable as a baseline—was examined after a relatively long accumulation phase for each particular water sample used in the chromatographic system and subsequent gradient elution performed on two different detectors (DAD and TOF-MS). In addition, representative routine analyses, such as the analysis of vanillin by HPLC–DAD and screening of a natural product by LC–MS, were run with three different sources of ultrapure water as part of the mobile phase and compared.

Production of ultrapure water using Arium mini plus

For the production of ultrapure water, Arium mini plus (Figure 1) is directly connected to the tap water feed to purify this

Table 1: Device parameters of systems 1 and 2 for analysis of contaminants present in the different water sources

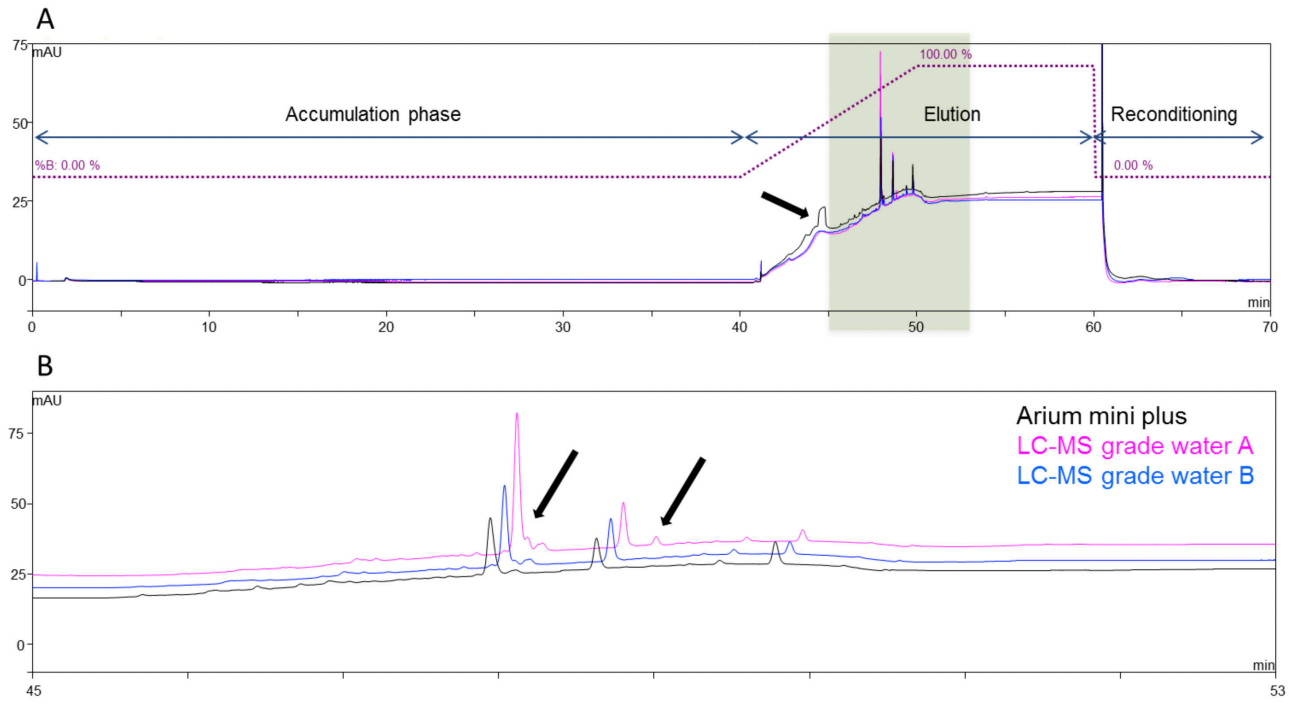
	System 1	System 2
Device	Agilent 1290 with DAD	Waters Acquity UPLC, Bruker microTOF II
Separation column	Grom™ Saphir C18, 150 x 2.1 mm, 5 µm (Grom™ analytical + HPLC, Herrenberg, Germany)	Kinetex RP-C18, 100 x 2.1 mm, 1.7 µm (Phenomenex, Aschaffenburg, Germany)
Mobile phase	A: Water B: Acetonitrile	
Gradient	0 min. 100% A / 0% B 40 min. 100% / 0% B 50 min. 0% A / 100% B 60 min. 0% A / 100% B 70 min. 100% A / 0% B	0 min. 100% A / 0% B 16 min. 100% A /0% B 19 min. 5% A / 95% B 23 min. 0% A / 100% B 26 min. 100% A / 0% B 30 min. 100% A / 0% B
Temperature	40°C	50°C
Flow rate	1 mL/min.	0.55 mL/min.
Detection	200 nm	50 – 1,600 Da (ESI- and ESI+)

water in a two-stage process. In the first stage, this compact system produces pure water, reverse osmosis (RO) water, and in the second stage, ultrapure water. As lower flow rates are reached during RO purification and this stage therefore has a limiting effect on such rates, an Arium bag is connected as a reservoir between the two stages (see flow diagram in Figure 1).

In the first stage of the Arium mini plus system, feed water is passed from the system inlet through a pretreatment cartridge, an RO module, by using a diaphragm pump. The RO module has two outlets, one for the permeate flow and the other for the concentrate flow. The latter flow path is connected to the system's outlet to drain off the water removed from the RO purification stream, "rejected water." The permeate flow is purified RO water (i.e., pure water) that fills the bag and is monitored in the process by a

"The purity of the solvents used, primarily that of water, is a decisive criterion for interference-free and reproducible analysis by liquid chromatography and for the sensitivity of this method, especially for applications in trace analysis."

Figure 2: (A) HPLC-DAD chromatograms (detection: 200 nm) after a 40-min. accumulation phase for each of the three water sources tested in the column and subsequent elution of the contaminants performed with acetonitrile. (B) Magnified view of the colored section in A; differences in the peak profile are identified by arrows.



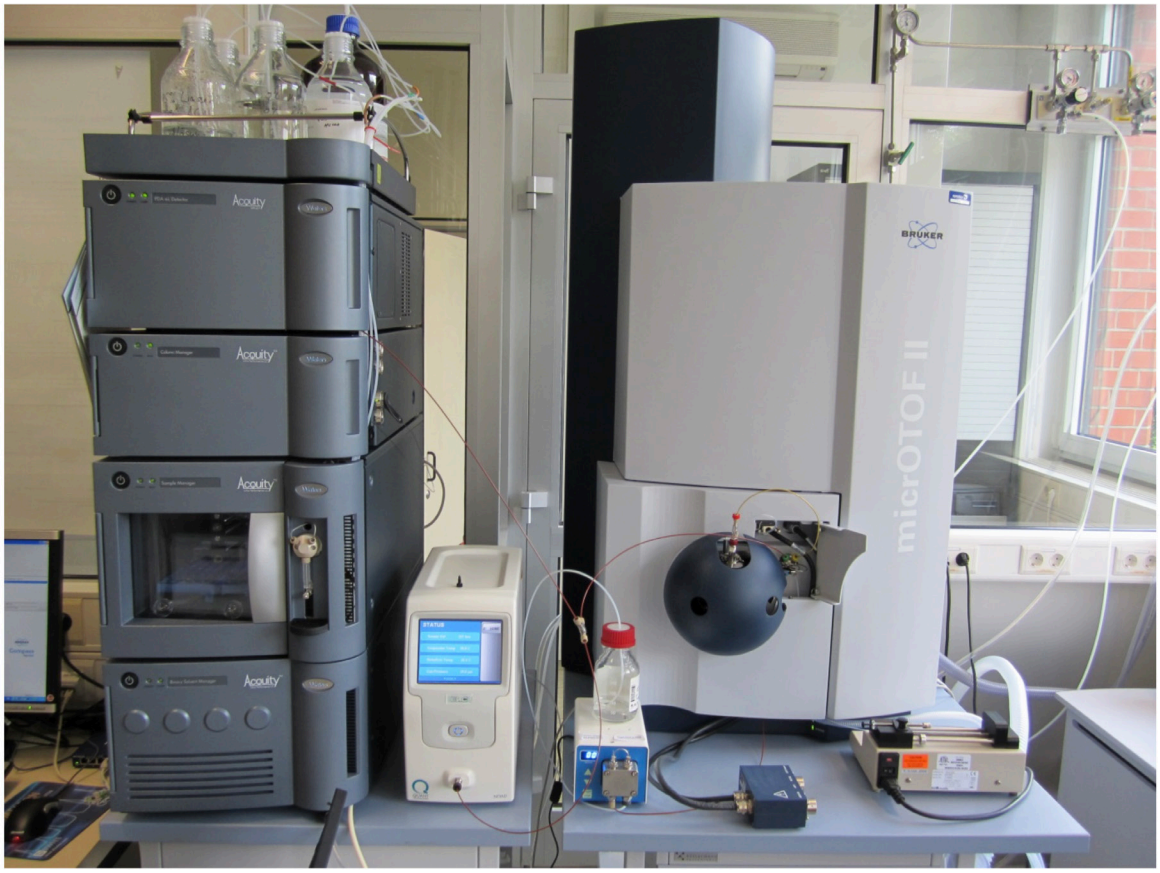
conductivity cell.

In the second downstream stage, the pure water obtained is transported by a further pump out of the bag to the actual purification cartridge for generating ultrapure water. Here, pure water is transformed into ultrapure water using an optional UV lamp (has an oxidizing and germicidal effect at wavelengths of 185 nm and 254 nm, respectively) and by passing through a cartridge filled with active carbon and ion exchange resin. During purification, the quality of ultrapure water is continuously monitored by a second conductivity cell to maintain a conductivity of 0.055 µS/cm (corresponds to a resistivity of 18.2 MΩ x cm), compensated to 25°C. Then in the last purification step, purified water is dispensed via a final sterilizing-grade filter. This process is shown as a schematic diagram in Figure 1.

Materials and methods

The water sources tested included two commercially available, certified water grades for LC–MS applications (LC–MS grade water A and B) besides ultrapure water freshly produced by the Arium mini plus system. To examine the background signal in the HPLC–DAD and LC–MS chro-

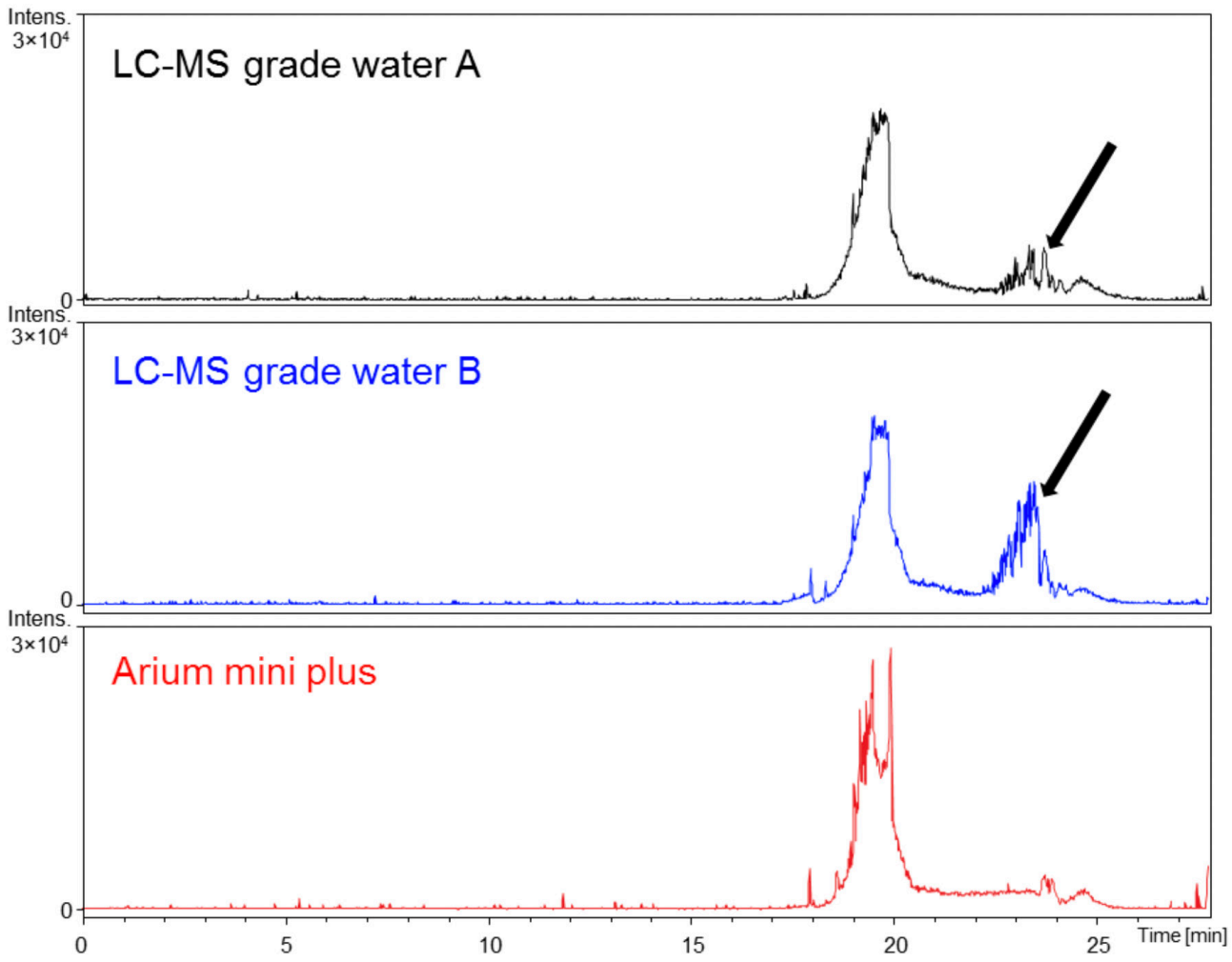
Figure 3: Waters Acquity UPLC with Bruker microTOF II (location and photo: Symrise)



matograms, the different water sources were each passed through an RP-C18 column, under standard flow conditions and without the addition of modifiers (e.g., formic acid or buffer), for a period of 40 min. for the HPLC–DAD method and 16 min. for the LC–MS method, respectively, to concentrate any contaminants present in each water source (accumulation phase). Then the potential contaminants were eluted by running a gradient of water used as the solvent to 100% acetonitrile. At the end of each run, the column was reconditioned with the respective water source. This was carried out on two different systems: HPLC-DAD (system 1 supplied by Agilent based in Waldbronn, Germany) and LC/TOF-MS (system 2 supplied by Waters, Eschborn and Bruker based in Karlsruhe, Germany). The device parameters are listed in **Table 1.**

In routine analysis, system 1 is used, for example, to quantify vanillin, whereas system 2 is mainly employed for screening of compounds, such as those in natural products. For these two applications, trial

Figure 4: LC/TOF-MS chromatograms after the accumulation phase for each of the three water sources tested in the column and subsequent elution of contaminants with acetonitrile. Ionization was performed in the ESI- mode; the mass range shown is 70–1,600 Da. Differences in the peak profile are identified.

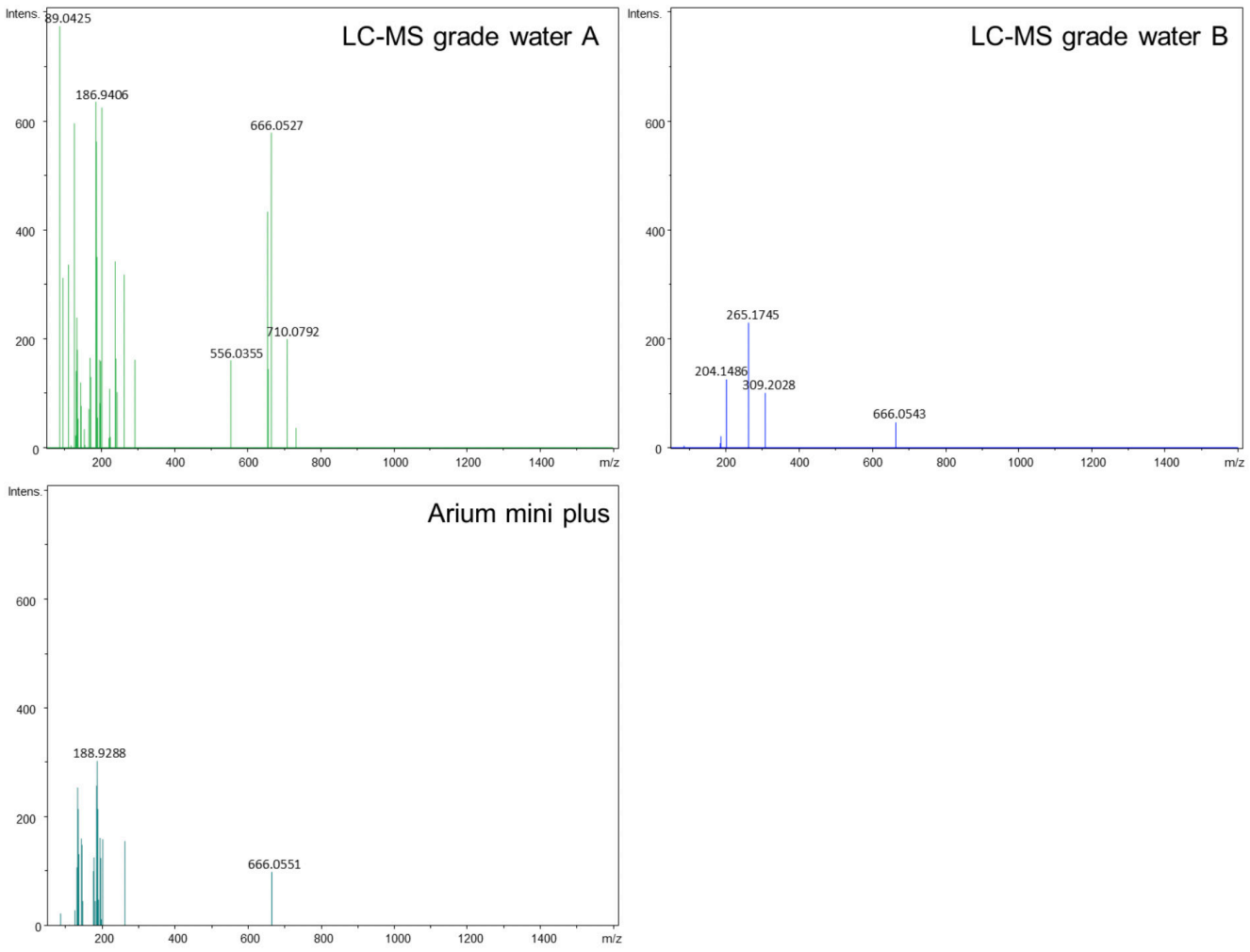


runs were performed using the different water sources.

Results

Background signal in UV and MS detection. The resulting chromatograms of the trial runs conducted on the HPLC–DAD system (system 1) are shown in **Figure 2**. In the top graph (Figure 2A), the chromatograms of the three samples of ultrapure water are overlaid, and the gradient profile is marked. On the chromatograms, both the commercially available bottled water grades and ultrapure water freshly

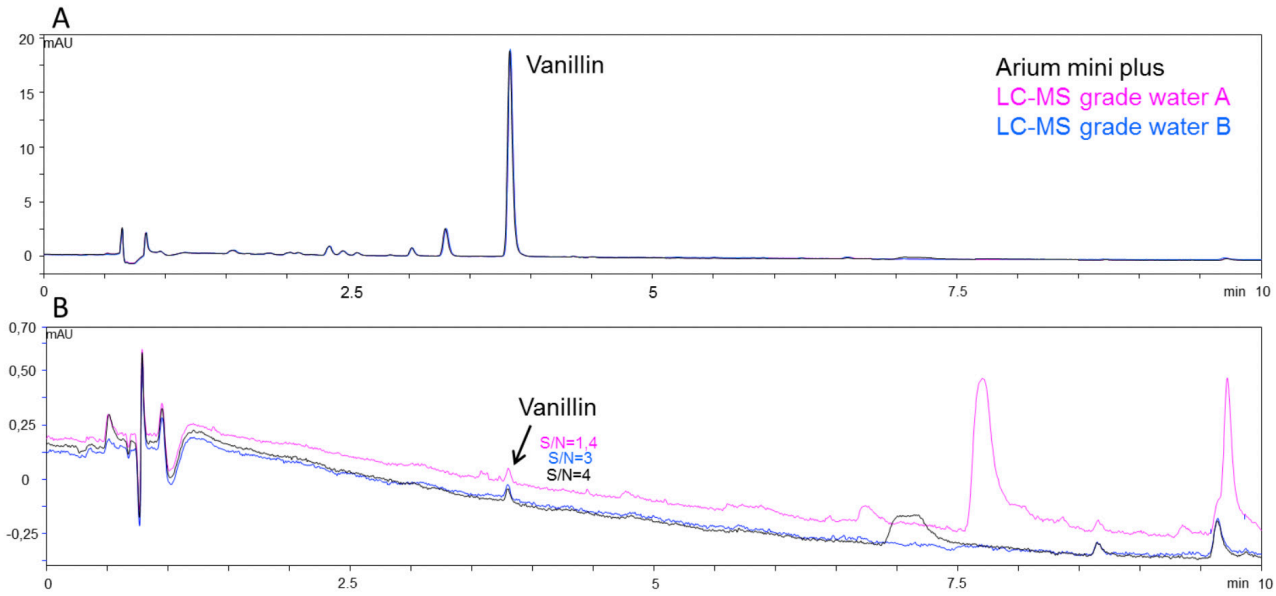
Figure 5: Mass spectra of each of the tested water sources, obtained after direct injection by syringe pump following electrospray ionization in the negative mode (ESI-).



produced by the Arium mini plus system show similar contaminants that were accumulated in the separation column. At approx. 45 minutes, a broad peak was observed, which exhibits a substantially higher peak area for accumulation using Arium mini water and, due to its asymmetry, indicates overlay of several contaminants. In the colored section (45–53 min.; see magnified view in Figure 2B), there are slight differences in the peak profile. Here, the profiles for Arium mini water and water B are comparable, whereas in water A, contaminants that cannot be observed in the other chromatograms are detected. This accumulation experiment delivered reproducible results (n = 5).

This trial conducted to examine the

Figure 6: HPLC–DAD chromatograms of a vanilla extract (A) and an aqueous vanillin solution (B, 9 ng/mL) obtained with the different water sources used in the mobile phase (detection: 280 nm; column: Poroshell 120 SB-C18, 2.7 µm, 100 x 2.1 mm; eluents: acetonitrile and water with 0.1% formic acid in the gradient mode; flow rate: 0.4 mL/min.

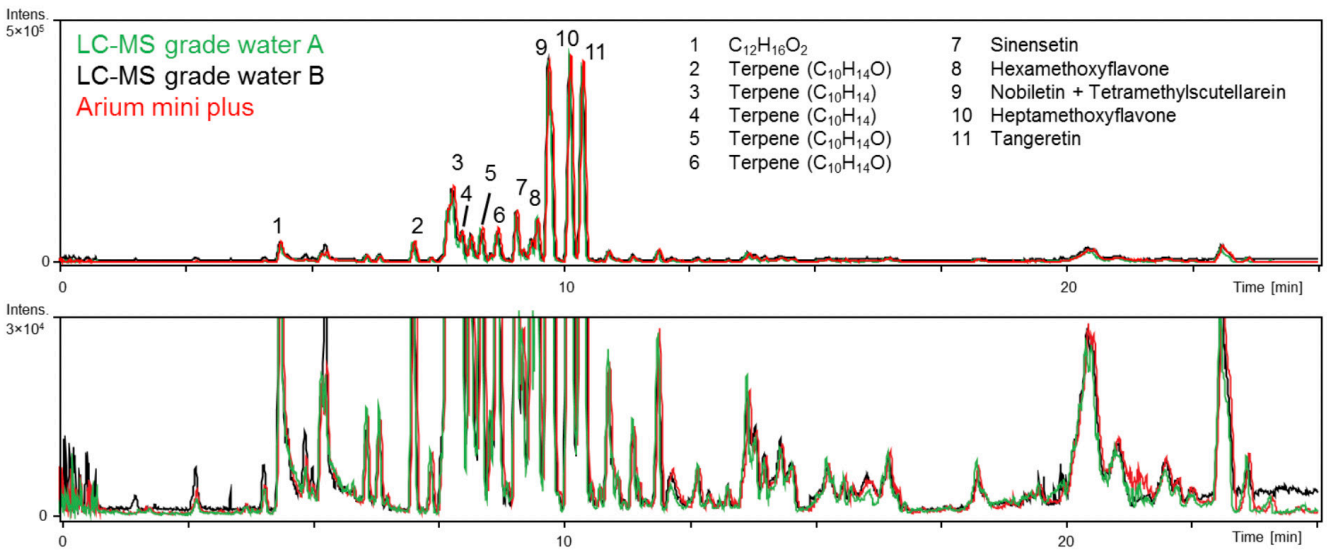


background signal after accumulation of the contaminants for the various samples of ultrapure water on an RP-C18 separation column was additionally performed with a high-resolution TOF-MS system (system 2, **Figure 3**). After electrospray ionization in positive mode (ESI+), hardly any differences can be seen between the peak profiles (data not shown). By contrast, differences can be seen in the peak profiles obtained in the ESI- mode (**Figure 4**). Thus, the chromatogram for Arium mini plus water in the range of 22–25 min. shows fewer peaks of contaminants in comparison to those obtained for the commercially available brands of bottled water.

In addition, the particular water samples were injected by a syringe pump directly into the TOF-MS system (Bruker microTOF II). As special experiments are carried out by direct injection just as is generation of reference spectra both in the scan and MS/MS modes, it is also important in these cases that the quantity of interfering ions produced from the solvents used be kept as low as possible.

The spectra recorded were averaged by software over a time span of 1 min. **Figure 5** shows examples of the spectra obtained in the ESI- mode. By comparison, LC–MS grade water B and water from the Arium mini plus system show fewer signals of potentially interfering ions, whereas LC-MS grade water A generates

Figure 7: LC/TOF-MS chromatograms (BPC, base peak chromatogram; 70–1,600 Da) of an orange oil (diluted 1:20) obtained with the respective water sources in the mobile phase (detection: 50–1,600 Da (ESI+); column: Kinetex RP-C18, 1.7 µm, 100 x 2.1 mm; eluents: acetonitrile and water, each with 0.1% formic acid in the gradient mode; flow rate: 0.55 mL/min; lower chromatogram: magnified view.



considerably more signals. This can also be observed in the ESI+ mode (data not shown) and supports the observations made in assessing the chromatograms depicted in Figure 4.

Comparison of water sources in routine analyses

To test the usability of the different ultrapure water sources in routine analyses, these sources were each employed as solvents in sample chromatographic runs.

Quantification of vanillin in vanilla extract. After diluting with methanol (approx. 1:1,000), vanilla extract was analyzed using each of the three different water sources as a component of the respective eluents run through system 1. The vanillin concentration of each injected solution corresponded in this case to approx. 4 µg/mL. The resulting chromatograms (**Figure 6A**) are nearly congruent, and the peak areas of vanillin do not differ at all. However, if the vanillin concentration is within the range of the detection limit (9 ng/mL), the background signal, as a result of the water purity, does play a role, and substantial differences can be seen (**Figure 6B**). In view of the baseline curve and signal-to-noise ratio (S/N) of the vanillin peak, Arium mini plus water and LC-MS grade water B are comparable, whereas the chromatogram for LC-MS

grade water A shows a higher baseline and more potentially interfering peaks. This confirms the observations made in the experiments described above with regard to the background signals in the HPLC–DAD system and after direct injection into the TOF-MS system.

Screening of orange oil using LC/TOF-MS. Furthermore, the three water sources were tested in a qualitative screening method by high-resolution LC/TOF-MS (system 2) to identify individual compounds in mixtures. A specific type of orange oil was used as the sample material. The chromatograms are shown in **Figure 7.** Comparable performance regarding the peak height/area, retention time and separation was observed. Likewise, in view of the baseline curve and signal-to-noise ratio, hardly any differences are seen (see Figure 7, magnified view).

Discussion

Based on the experiments conducted, it could be shown that Arium mini plus ultrapure water is excellently suited for use in chromatography and mass spectrometry (MS). In view of the potentially co-eluting peaks and the background signal in UV and MS detection, it was observed that Arium mini plus water is comparable with the tested quality grades of commercially available bottled water.

The background signal, which primarily depends on the purity of the solvent used in chromatographic analysis, must be as low as possible as this signal is highly significant for the sensitivity of the analytical method and for reliable quantification. Besides LC–MS grade water B, Arium mini plus water with a higher S/N excels especially in trace analysis requiring high sensitivity, as shown in the example of the vanillin peak obtained on the HPLC–DAD chromatogram.

Unlike commercially available, bottled ultrapure water, an ultrapure water system offers the considerable advantage of being able to freshly purify water in any quantity on demand. From an economic point of view, this feature is thus a good alternative to purchased ultrapure bottled water. Fresh purification also prevents water from standing in opened bottles for long periods because such water stored in opened bottles can be contaminated by absorption from the laboratory atmosphere (1, 2) and dissolve CO_2 from air, among other contaminants. Organic contaminants in water are detectable by an increase in the TOC level (total organic carbon). At high TOC levels, identification and quantification of trace components can be compromised (1), for instance, by shifts in the baseline (1, 2) or by the occurrence of ghost peaks (3).

In addition, if bottled water is stored for relatively long periods, Na-cations, for example, can leach from the glass bottles, which, in turn, can lead to increased formation of adducts during ionization in LC–MS systems. An accordingly lower yield of ions used for evaluation (usually $[M+H]^+$ or $[M-H]^-$) ions can have a negative impact on the sensitivity of the method [4].

The high suitability of fresh ultrapure

water, produced by Arium pro systems, in different chromatography techniques (see also, e.g., 4 and 5) and the increasing use of these technologies in the most diverse applications will very likely contribute to the growing acceptance and pervasiveness of laboratory water purification systems.

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References

1. S. Mabic, J. Krol, and C. Regnault, *LCGC Europe*, 18 (7) 410-414 (2005).
2. M. Tarun and S. Mabic, "A practical guide to the effects of water purity on HPLC, LC-MS and related techniques," 2011, Millipore Corporation.
3. W. Gottwald, "RP-HPLC für Anwender," publisher Gruber, U. and Klein, W., VHC Weinheim, 1993.
4. T. Iven, K.Feußner, C.Herrfurth and E.Herbig, LABO, 2014, 12, 44-47 et al., *Bioprocessing J*, 14 (4) 2015/2016.
5. K. Töppner, D. Hansen, and E. Herbig, LABO, 2013, 6, 22-27

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