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Compendium of articles
Solutions for mass spectrometric detection

Leading the way for over a century

Our journey began in 1904 with the development of an aluminum oxide for adsorption chromatography. Over the decades, numerous products and techniques followed. Many set new milestones. Each was recognized as an important advancement in the field.

This spirit of innovation still continues relentlessly. Today, EMD Millipore is among the leaders in the science of liquid chromatography and is committed to the further development of sorbents, analytical and preparative columns, sample preparation, thin layer chromatography as well as LC-MS grade solvents and reagents.

Whether for research and development, quality control or purification purposes, EMD Millipore products are widely used throughout the world. How did we get where we are today? Through uncompromising quality and a strong customer focus. This obsession with quality is the reason chromatography columns, plates and mobile phases from EMD Millipore are found in mass spectrometry laboratories worldwide.

This compendium of selected articles is intended to help and support users of chromatographic separation methods in mass spectrometric laboratories worldwide, by providing an overview of different actual topics in this field of analytical science.

Contents

Introduction

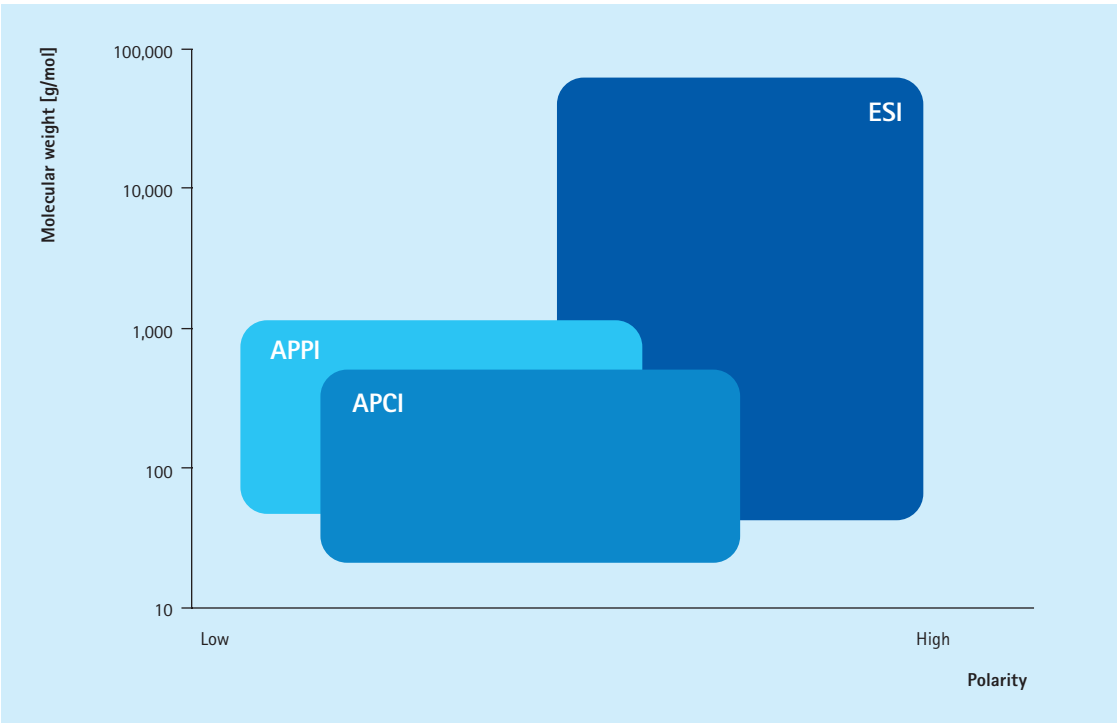
Mass spectrometry (MS) is nowadays an established routine detection method that can be coupled to various techniques such as liquid chromatography (LC), thin layer chromatography (TLC), gas chromatography (GC) or inductively coupled plasma (ICP). The utilization of MS in combination with LC is by far the most frequently utilized setup. In contrast to more simple detectors such as ultraviolet-visible (UV-Vis) or fluorescence, MS generates data about molecular masses and detailed structural parameters and is by far more sensitive in the wide majority of applications. Many sophisticated as well as affordable systems are available, covering various fields of use such as quantification and qualification experiments.

High performance liquid chromatography (HPLC) is handling dissolved analytes under ambient pressure (760 Torr) and delivers the sample to the mass spectrometer, where the detection of the gaseous, ionized samples is performed under high vacuum conditions (10^{-5}–10^{-6} Torr). The transfer of the analyte solution from the LC to the MS system is accomplished via an interface. This device stepwise converts the sample to an aerosol, ionizes it and removes solvent. Depending on the physical properties and the molecular mass of sample molecules, different types of interfaces are applied. They vary in the ionization method and the pressure applied during this process.

All frequently used methods utilize ionization under ambient pressure; less common techniques such as electron ionization (EI) and chemical ionization (CI) work under high vacuum conditions and are also suitable for GC-MS.

Ionization techniques performed under ambient pressure are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix assisted laser desorption / ionization (MALDI) and the less prominent atmospheric pressure photo ionization (APPI). All methods vary in both the underlying principle as well as the specific interface needed and can be picked according to the sample properties (Figure 1). The two former principles are the most widely used in LC-MS.

Figure 1



Ionization techniques in LC-MS analysis.

Electrospray Ionization (ESI)

In ESI mode solutions of charged or polar substances are sprayed utilizing a metal capillary ("spray needle") and a nebulizer gas (nitrogen). Resulting droplets are dried (desolvatization) and isolated analyte ions are transferred to the detector. Thermal stress is very low in this case and hence analyte molecules do not decompose. The method is almost unlimited regarding molecule size and suitable for medium to strong polar molecules (e.g., amines, carboxylic acids, heteroaromatics, sulfonic acids). It is applied when fragmentations are unwanted and molecular masses of biomolecules have to be determined. ESI MS is well suited for hyphenation with LC and, as long as flow rates do not become too high (max. 1 – 2 mL/min), attainable sensitivity is very high.

In solution the analyte is either ionized or becomes protonated or deprotonated by additives. Depending on the polar solvent and additive utilized, positive and negative ESI modes are available (positive ESI mode: e.g., 0.1 % formic acid in eluent, protonation of analyte; negative ESI mode: e.g., 20 mM ammonium acetate pH 4.70, deprotonation of analyte). Classically, positive mode is applied in combination with more basic molecules, while acid compounds are analyzed in negative mode. It is a prerequisite that all solvents and additives are volatile in order to avoid contamination of the mass spectrometer.

Atmospheric pressure chemical ionization (APCI)

This technique is comparable to ESI and also utilizable in LC-MS hyphenation, but in contrast to ESI the analyte solution becomes vaporized prior to ionization. Subsequently solvent molecules (aqueous-organic, e.g. methanol, propanol, acetonitrile, acetone etc., combined with 2 – 20 mmol of a volatile organic buffer such as formic or acetic acid, ammonium acetate or triethylamine) become ionized with a corona needle and then transfer their charge to the analyte molecules via proton transfer or abstraction. APCI is also suitable for the analysis of less polar, weakly ionizable substances with small or medium molecular weight (analytes without acid or base centers, e.g. hydrocarbons, alcohols, aldehydes, ketones, esters) and therefore complementary to ESI, as long as the sample is thermally stable and vaporizable.

Fragmentations are observed with APCI. Highest sensitivity is achieved with acetonitrile, methanol or water as solvents; the degree of analyte ionization can be optimized via eluent pH. APCI can be combined with LC flow rates up to 1 – 2 mL/min.

In contrast to ESI, where conductivity of the eluent is necessary, the utilization of acetone or acetic acid esters as solvents in APCI allows for a coupling of this method with normal phase chromatography.

Tips and tricks for mass spectrometry

The choice of a specific MS setup including the detector itself and the source type depends on the field of application and the properties of the sample, respectively. This means that MS technologies applied can of course differ significantly from lab to lab. Anyhow there do exist a number of easily avoidable handling pitfalls, which are independent of the MS technique used when combining it with LC.

General considerations

Electrospray ionization is the most popular MS technique and needs highly volatile and protic, polar solvents.

In positive ESI mode possible protic solvents include water and methanol, mixtures of acetonitrile (ACN) / water; methanol / water or isopropanol / water will also work. These solvents are combined with volatile acids such as formic, acetic or propanoic acid.

Strong acids such as hydrochloric acid or nitric acid are unsuitable for two reasons: They form ion pairs with analyte molecules (analyte signal suppression) and display strong oxidizing properties. Trifluoroacetic acid (TFA) is a special case. It is widely used as an ion pairing reagent to improve the liquid chromatographic separation of peptides or proteins. On the other hand, TFA can cause strong ion suppression in mass spectrometry (mainly in negative ESI mode) and also contaminates the LC-MS system. Unfortunately both a quantitative estimation of these effects as well as general recommendations are not possible, as their strength strongly depends on the MS system used. Triethylamine as an alternative additive behaves in a similar manner.
If the use of TFA is unavoidable, a weak acid (such as propanoic acid) or isopropanol can be added to the eluent to decrease the signal suppression effect.

In negative ESI mode acetonitrile / water, isopropanol / water or n-propanol can be used as solvents, combined with basic volatile compounds such as ammonium formate or acetate (c ≤10 mmol), ammonia or other volatile bases. Note: in at least all types of MS analyses the signal intensity in negative ESI mode is lower as compared to positive mode – but so is the noise intensity.

Classically, the physical properties of the analyte molecule(s), e.g. acidity or basicity, determine the choice of eluent composition (acid to neutral for columns with stationary phases based on silica) and ESI mode (positive or negative). Nowadays modern mass spectrometers are highly sensitive and hence can be operated in either ESI mode without adaption of eluent composition. This property also allows for simple MS operation utilizing polarity switching in single LC runs.

It is a commonplace, but purest solvent and reagent quality available has always to be used and their contamination has to be avoided by careful handling; any impurity will cause signal suppression and / or adduct formation with target molecules and therefore decrease sensitivity (signal-to-noise ratio) and / or increase complexity of the mass spectrum.

ESI source condition settings are used to optimize vaporization of the eluent and to achieve optimum signal intensity. Depending on the flow rate the LC system delivers to the MS (e.g., in the range of 1 – 1000 µL/min), nebulizer gas pressure, drying gas flow and drying gas temperature of the source can be adjusted. These settings differ from system to system and reference points can be found in the respective manuals. When working under highly aqueous conditions the low vapor pressure of water can negatively affect sensitivity or even cause a complete breakdown of the ESI spray. Keeping water content below 80 % helps to avoid these drawbacks, especially when operating at flow rates in the range of up to 1 – 2 mL/min. Of course, water is a standard solvent in ESI mode and for chromatographic reasons it sometimes is a prerequisite to perform a separation under highly aqueous conditions. In this case there are several options to keep the ESI spray working.

Figure 2
Various LiChrolut® cartridges suitable for the sample preparation of samples with high salt and matrix load. The type of stationary phase (e.g., reversed phase, cyano) can be chosen according to the specific needs of each sample.

These are:

- Decrease of the eluent's surface tension by addition of a volatile organic solvent (ACN, methanol etc.) after the LC system and before the mass spectrometer using a T-union.
- Split of flow coming from the LC system via a T-union, maximum tolerable flow rate for MS instruments is 1 – 2 mL/min. Alternatively the internal diameter of the column used for separation can be decreased to lower the eluent volume delivered to the MS source.
- Optimization of system settings: Increase dry gas temperature and / or decrease flow rate (see Table 1) to avoid a breakdown of the spray by insufficient vaporization.

Column type	Dimension (i.d.)	Recommended flow rate range
Capillary	0.05 – 0.2 mm	0.1 – 20 µL/min
Microbore	1.0 mm	50 – 100 µL/min
Narrow bore	2.0 mm	0.1 – 1.0 mL/min
Analytical bore	4.6 mm	1 – 6 mL/min

Table 1 *Column types, dimensions and recommended optimum flow rates.*

Next to the problem mentioned above there are some further limitations which have to be taken into account when using water: Less than 5 % organic solvent in your eluent will lead to microbial contamination of your eluent bottle, tubing and LC system, therefore at least 5 % of organic eluent should be added if chromatographic conditions allow this. On the other hand, it is recommended to keep 5 % of aqueous eluent in the organic mobile phase to avoid buffer precipitation in the system, e.g. in valves, and subsequent tedious cleaning procedures.

Any buffer used to adjust pH of eluents has to be volatile. The total ionic strength of the eluent should not exceed 20 mmol/L, therefore buffer concentration in the aqueous solvent has to be adjusted accordingly.
But buffers do not only adjust the pH of the eluent and lead to ionization of a target molecule M, they can also form adducts with the analyte. Adducts [M+buffer], e.g. with ammonium, alkali, halogens, formate or acetate, will lead to the detection of an additional peak in the MS spectrum; even a complete suppression of the analyte signal is possible when the vapor pressure of the resulting adduct (mainly alkali) is decreased significantly.

As a reason of this and in order to keep the ESI source clean, volatile buffers are recommended. Nonvolatile salts such as phosphates, borates, sulfates or citrates will precipitate in the MS source, block it and cause tedious cleaning procedures. For samples with high salt load (e.g., food, body fluids or tissue) a desalting (sample preparation step) using LiChrolut® cartridges (Figure 2) is recommended. This procedure helps to remove undesirable salts and to keep both sample and MS spectrum simple. A list of some recommended and unsuitable buffers is presented in Table 2.

Volatile Suitable for LC-MS	**Nonvolatile** Avoid in LC-MS
• Ammonium acetate	• Borates
• Ammonium carbonate	• Citrates
• Ammonium formate	• Phosphates
• Ammonium hydroxide	• Sulfates
• Trimethylamine	

Table 2 *Selection of volatile and nonvolatile salts for buffer preparation.*

Adjust eluent pH

In addition to the buffers listed above formic or acetic acid, ammonium hydroxide or trimethylamine will work to adjust eluent pH. The choice of acid, base and buffer type strongly depends on the application, some examples of which are listed in Table 3.

Table 3

Eluent pH	Compounds of (buffer) system
2.0	Formic acid
2.3 – 3.5	Pyridine / formic acid
3.5 – 6.0	Acetic acid / ammonium acetate
6.8 – 8.8	Trimethylamine / hydrochloric acid

Eluent pH and compounds of respective (buffer) systems.

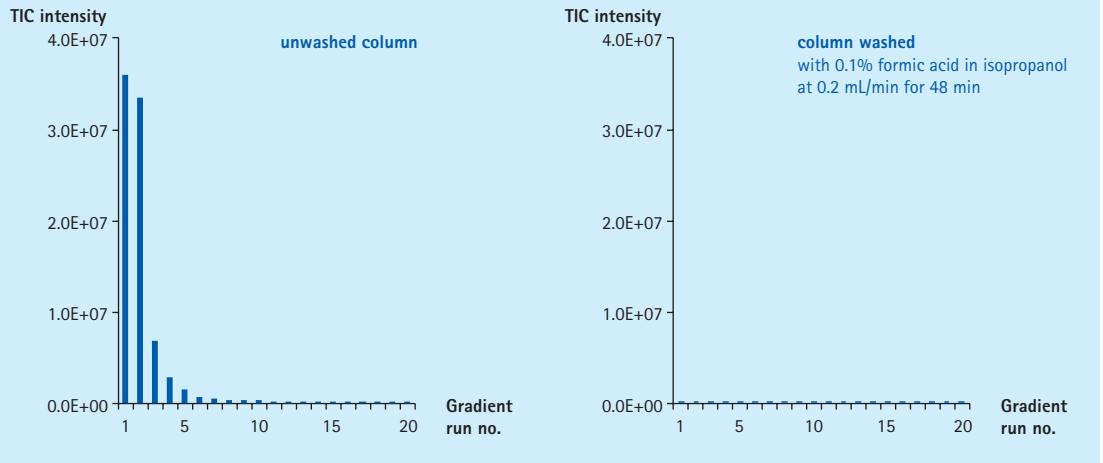
Note
Eluent pH has to be chosen in accordance with your stationary phase to avoid damage to modification or column bed (for, e.g., a silica based stationary phase a pH in the range of 2 – 7.5 is recommended).

The HPLC system has to be flushed with organic eluent (preferably isopropanol or methanol, ACN can polymerize and block valves if the system is stopped for several weeks) regularly to prevent microbial contamination and its negative effects on mass spectrometric results. The interval of flushing depends on the utilized eluents and buffers and should be between two to four weeks.

Pump debris is collected in the pump inlet filter. These compounds might not be visible using UV detection, but it is likely that they become detected via MS. Therefore the filter has to be replaced every 1 – 2 months or after changing from ACN to methanol (or vice versa) in order to obtain lower baseline noise and to generally protect the system. Various manufacturers provide filter frits which can be attached to the eluent tubing to protect the LC system from particulate matter. Here, stainless steel or PTFE filter frits should be used rather than glass frits: Cleaning of the latter is time consuming as buffer residue is hard to remove. In addition silica and alkali are dissolved from the glass filter and form adducts $[M+X]^+$ with analyte molecules.

Column washing

Figure 3



Chromatographic conditions	
System	Bruker Esquire 6000plus mass spectrometer with ion trap and electrospray ionization (ESI) source
Detection	Pos. ESI-MS, m/z range 50 – 600
Flow rate	0.4 mL/min
Mobile phase	A: Milli-Q® water from water purification system + 0.1 % formic acid 98 – 100 % for analysis EMSURE® (Cat. No. 100264) B: Acetonitrile hypergrade for LC-MS LiChrosolv® (Cat. No. 100029) + 0.1 % formic acid
Gradient	0 min 95 % A, 3 min 5 % A, 5 min 5 % A
Temperature	25°C

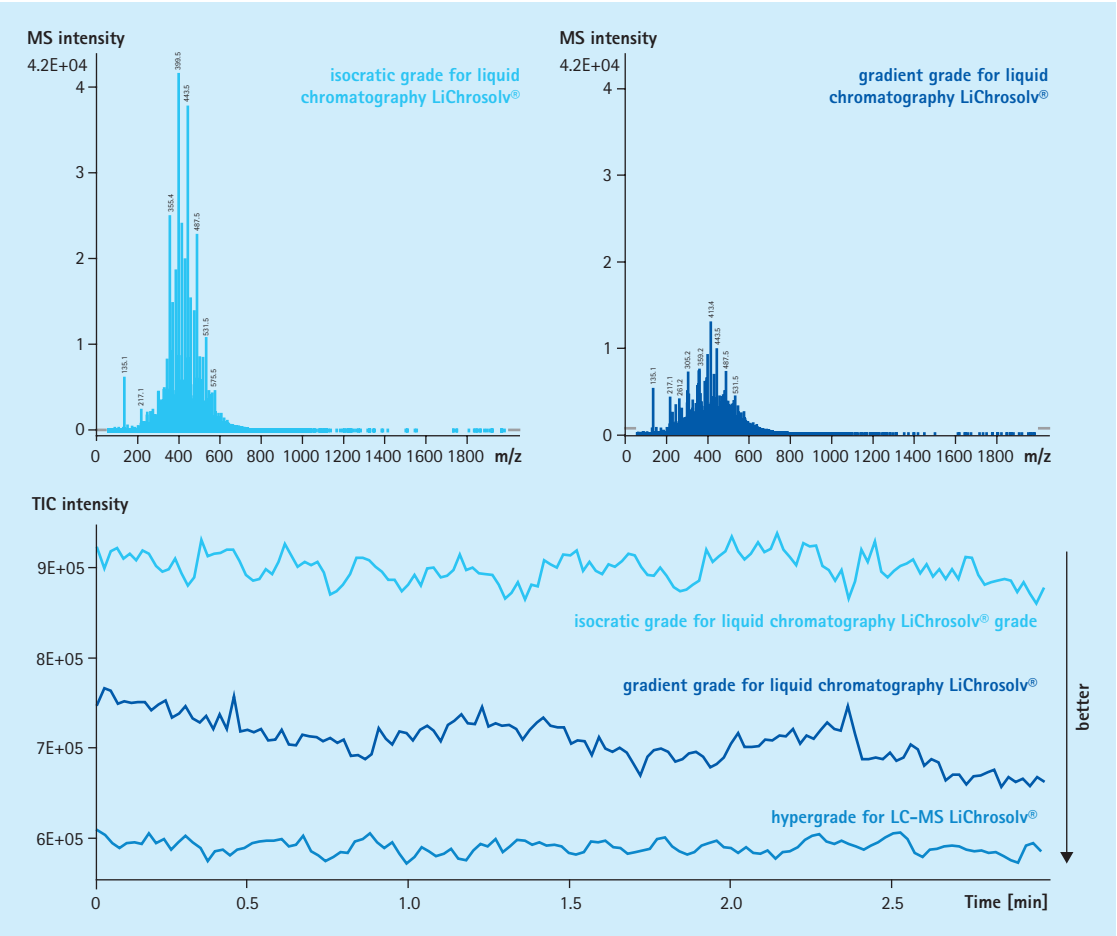
Total ion current (TIC) of a competitor column after 20 gradient runs; left: unwashed column, right: column washed with 0.1 % formic acid in isopropanol at 0.2 mL/min for 48 min.

Unless an LC run is performed utilizing a normal phase column, the stationary phase of every HPLC column contains covalently bound organic entities altering its physical properties. Depending on the quality of both phase modification and a subsequent washing step these entities (e.g. octadecyl, cyano, phenyl) can be washed off the column during a chromatographic run and cause weak to severe interfering signals. This unwanted phenomenon is referred to as "column bleeding" and leads to decreased sensitivity in MS. It can be avoided by flushing of a column prior to analysis using isopropanol and 0.1 % formic acid as a solvent at half optimum flow for one hour. This process removes unbound or weakly bound organic entities, minimizes column bleeding and hence increases sensitivity by decreasing background noise (Figure 3).

Mobile phase quality and contamination

Figures 4 and 5 display the influence of LiChrosolv® acetonitrile quality on the background noise intensity in mass spectra. EMD Millipore solvents labelled "hypergrade for LC-MS LiChrosolv®" are dedicated to the use with MS systems and deliver minimized contaminant peaks, ion suppression, adduct formation and background noise and therefore maximize sensitivity. Gradient grade solvent quality labelled "gradient grade for liquid chromatography LiChrosolv®" is suitable for LC-UV gradient runs, while isocratic grade solvents are optimized for isocratic separations.

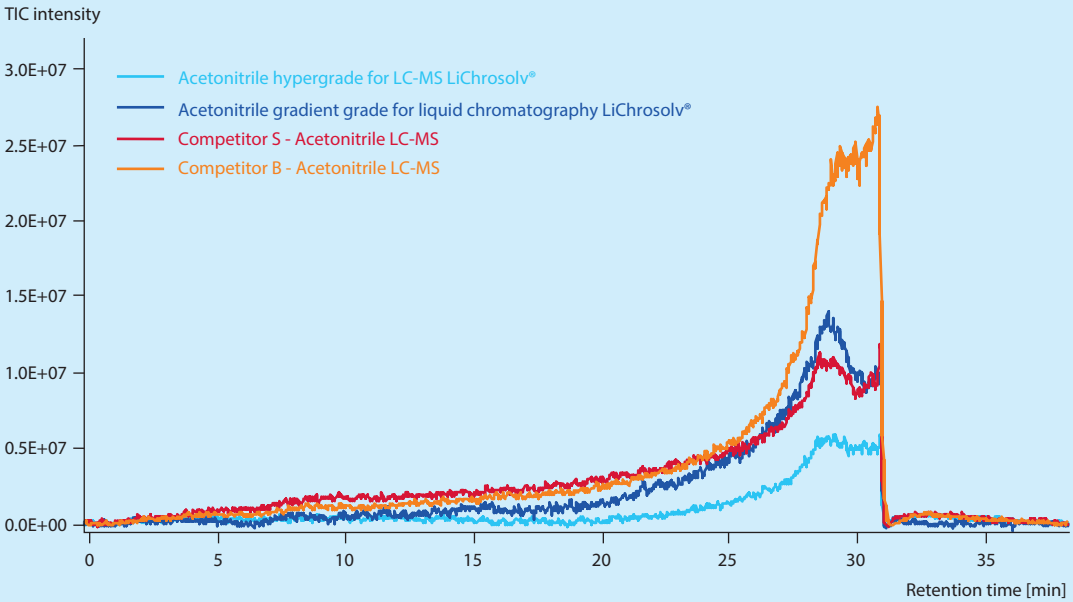
Figure 4



MS spectra of the analysis of two different ACN qualities (top) and combined TICs of the analysis of three different acetonitrile qualities (bottom). All solvents were directly injected into the MS source via a syringe pump during three minutes.

Instead of performing gradient runs with different organic solvents, a specific standard can be mixed with the organic solvent and injected into the MS via a syringe pump in order to compare solvent qualities. In many cases reserpine is utilized as a standard compound. The obtained signal of reserpine is then compared to the background noise (signal-to-noise ratio). Under negative and positive ESI conditions the highest signal-to-noise ratio (= lowest signal suppression) is obtained using hypergrade quality ACN (Figure 6, for detailed information see also section on organic solvent purity).

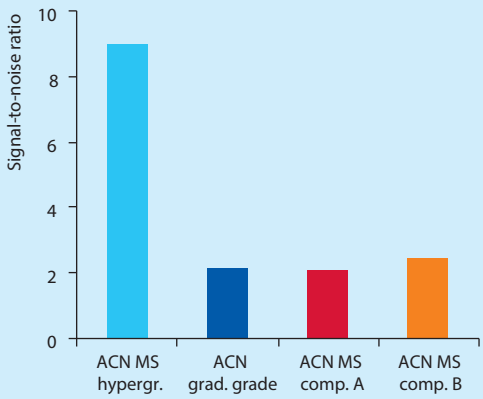
Figure 5



Chromatographic conditions	
System	Bruker Esquire 3000+ ion trap MS
Detection	Pos. ESI-MS, m/z range 50 – 2000
Flow rate	1.0 mL/min
Mobile phase	A: Water (**VWR Cat No. EM1.15333.1000**) B: Various ACN qualities as indicated in the graphics
Gradient	0 min 98 % A, 30 min 0 % A, 38 min 98 % A
Temperature	25°C

Combined TICs of the blank runs of four different acetonitrile qualities. All solvents were delivered to the MS source via an LC system.

Figure 6



Analysis of four different acetonitrile qualities via direct injection of respective reserpine solutions into the MS (ESI conditions). Hypergrade acetonitrile displays best signal-to-noise ratio (purest solvent).

Contaminations in solvents and additives such as acids, bases or buffers can accumulate on the stationary phase – depending on the chromatographic conditions applied for a specific separation and on the equilibration time prior to a run. Figure 8 shows the accumulation of plasticizers dissolved in the eluent on a reversed phase column after equilibration for 0, 15 and 60 minutes. While these compounds would become eluted as very broad peaks under isocratic conditions (and cause an increased background noise), they elute as distinct, intensive peaks under gradient conditions and can interfere with analyte signals. To avoid such ghost peaks use pure solvents and additives and avoid excessive column equilibration (column flushing with approximately 10 column volumes is sufficient, alternatively one blank gradient run including subsequent equilibration is an option).

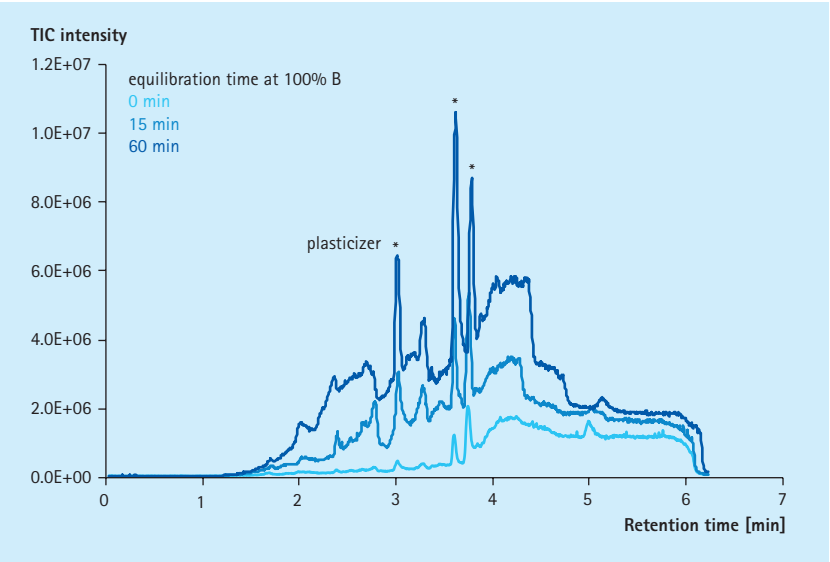


Figure 8

Accumulation of contaminants on an HPLC column for various periods of time and elution via a gradient profile.

Chromatographic conditions	
System	Bruker Esquire 6000plus
Detection	Pos. ESI-MS, TICs
Flow rate	0.4 mL/min
Mobile phase	A: Water (**VWR Cat No. EM1.15333.1000**)/acetonitrile (**VWR Cat No. EM1.00029.1000**) 95/5 (v/v) + 0.1 % formic acid (**VWR Cat No. EM1.00264.1000**)
	B: Acetonitrile + 0.1 % formic acid
Gradient	0 min 100 % A, 3 min 5 % A, 5 min 5 % A
Temperature	25°C
Sample	Plasticizers (*) were added by the immersion of plastic tubing in aqueous solvent A

Figure 9

Surface treated amber glass bottles utilized for the delivery of all EMD Millipore LC-MS grade solvents.

Solvent storage

Low solvent contamination can best be achieved by storing all eluents (water and organic) in either surface treated amber glass bottles (Figure 9; this is the original packaging all EMD Millipore LC-MS grade solvents are delivered in) or in borosilicate glass (if solvents are to be decanted). In standard glass bottles silica and alkali dissolve and form adducts $[M+X]^+$ with analytes. Utilization of professional EMD Millipore HPLC bottle caps / adapters with tube connections and membrane filter helps to protect both solvents and environment. They should ideally be mounted directly on the original amber glass bottle. If possible any decanting step has to be avoided, as it is a possible source of contamination. Home-made solutions to fix solvent tubing utilizing unsuitable materials will cause leakages and / or release contaminants to the eluents.

The ionic content in ultrapure water varies depending on the laboratory environment and the material of the container used for storage. The effect of the sodium content of a sample on signal suppression and a simultaneous increase in adduct formation can be seen in Figure 10. A solution of 500 pmol Glu-Fibrionopeptide in 50/50 ACN/water (v/v) was spiked with different amounts of sodium chloride and the mixture was analyzed via direct injection into the mass spectrometer.

Utilization of plastic devices such as bottles or funnels during handling and storing of solvents, buffers etc. leads to leaching of ubiquitous additives such as anti-static agents or stabilizers and causes ghost peaks and an increased background noise. An example of proper and unsuitable water storage is shown in Figure 11.

Figure 10
Signal suppression and increase in adduct formation in a solution of 500 pmol Glu-Fibrionopeptide in 50/50 ACN/water (v/v) was spiked with different amounts of sodium (as sodium chloride). The mixture was analyzed via direct injection into the mass spectrometer operated in positive ESI mode.

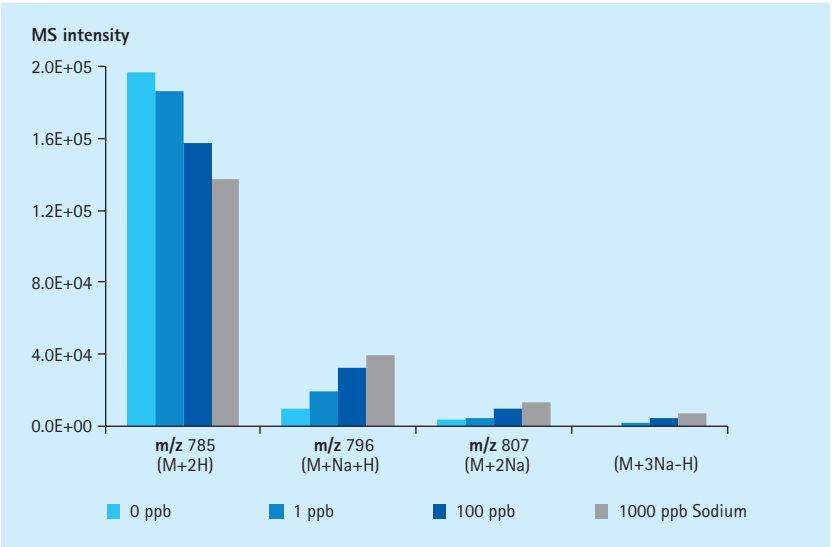
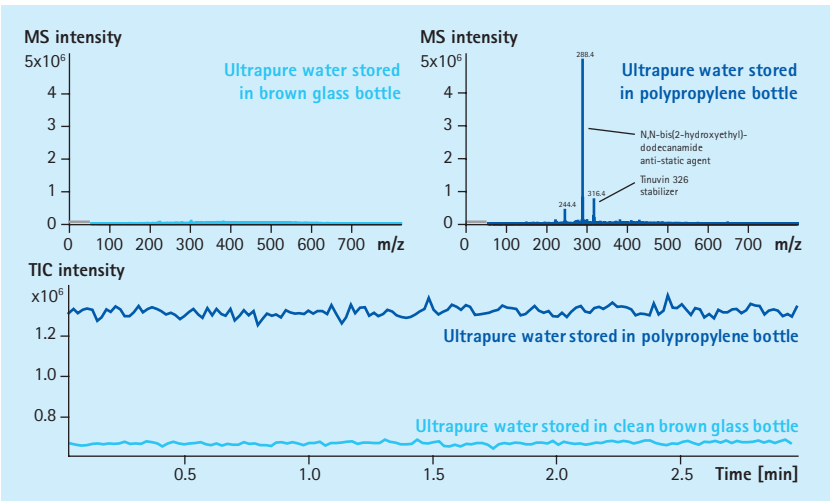


Figure 11
Mass spectra of two Ultrapure water samples stored in brown glass and polypropylene bottles, respectively (top), and TICs of the same samples (bottom). The analyses were performed via direct injection of the solvents into the MS operated in positive ESI mode.

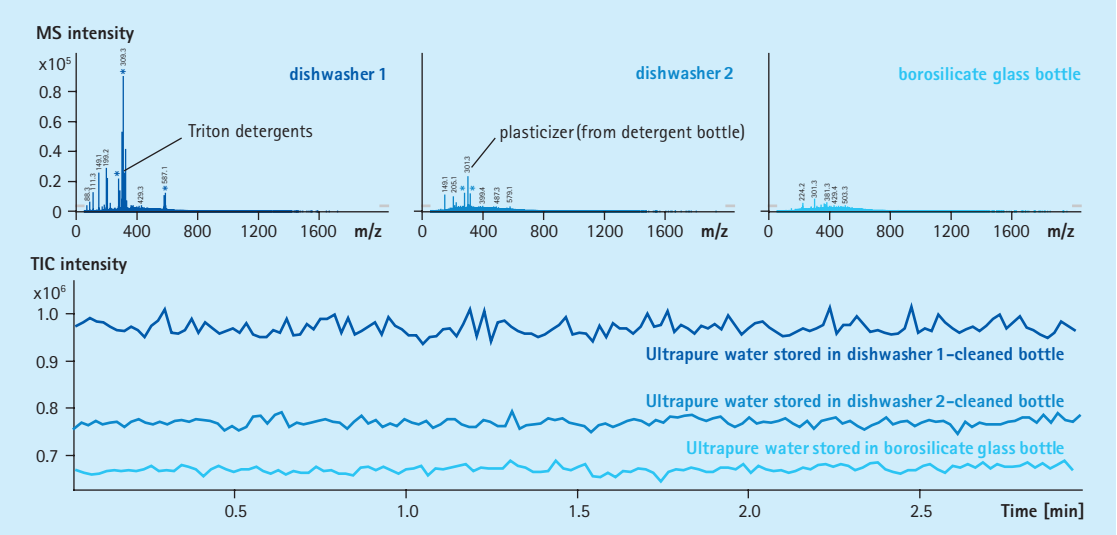


Equipment cleaning

Dishwashers are standard laboratory equipment, but they are operated utilizing chemicals such as strong bases and surfactants. The former lead to a dissolution of silica and alkali from glassware and cause the formation of adducts $[M+X]^+$ with analytes, while traces of the latter remain on the glass surface after the cleaning process and decrease MS sensitivity by increasing background noise. The effect of dishwashing is illustrated in Figures 12 and 13.

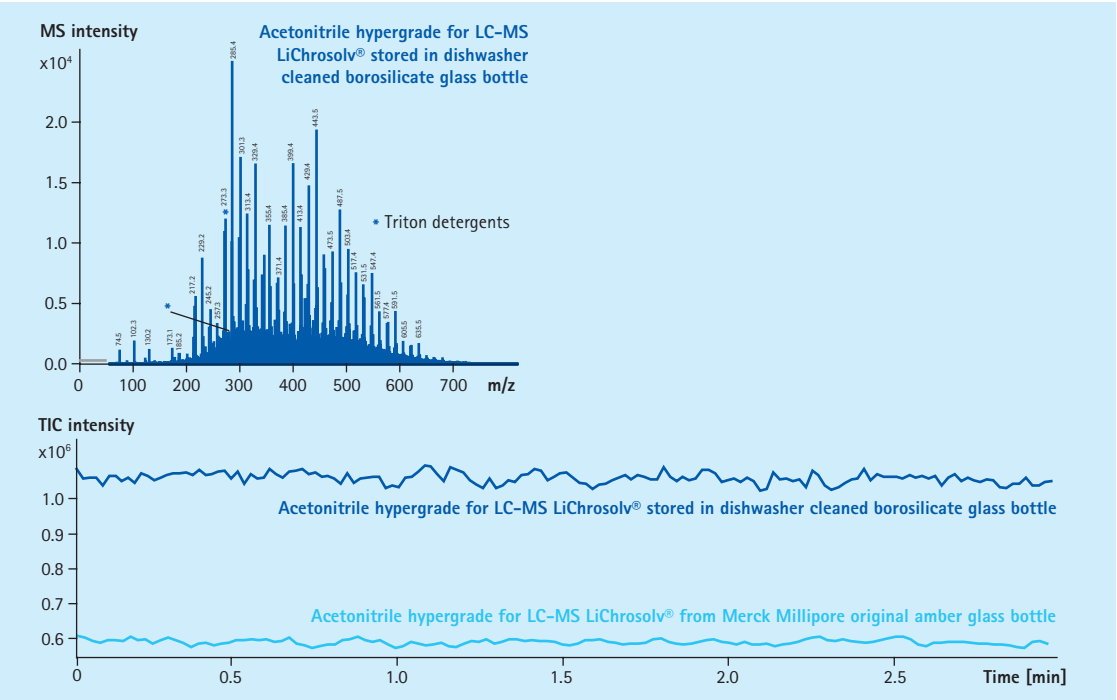
The easiest way to avoid dishwashing is "cleaning" of all equipment via simple evaporation of both solvents and additives. All chemicals dedicated to the application in LC-MS are volatile, therefore this procedure is straightforward as long as chemicals are highly pure and microbial growth can be eliminated. In case of equipment contamination flushing with LiChrosolv® or Ultrapure water or organic hypergrade solvents has to be performed to achieve sustainable cleaning.

Figure 12



Comparison of MS spectra (top) and TICs (bottom) of Ultrapure water stored in bottles cleaned in two different dishwashers and stored in a borosilicate glass bottle cleaned via flushing with MS grade water and acetonitrile. The mixture was analyzed via direct injection into the mass spectrometer operated in positive ESI mode.

Figure 13



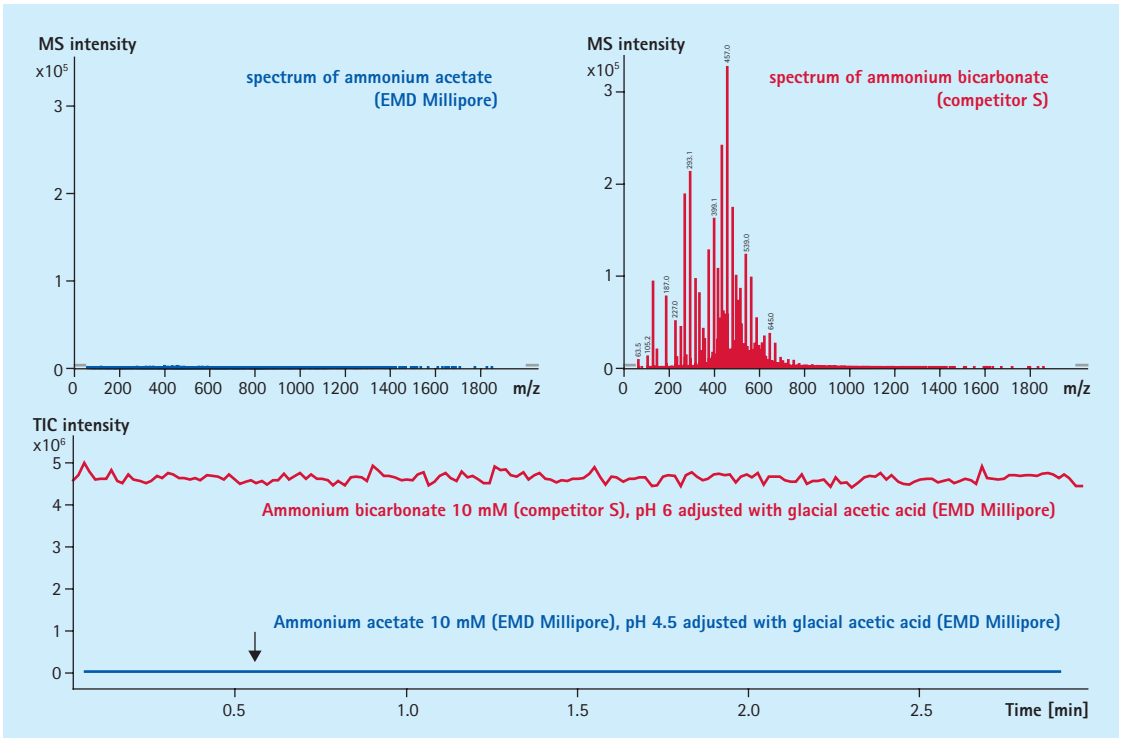
MS spectrum (top) and TICs (bottom) of acetonitrile hypergrade for LC-MS LiChrosolv® stored the original amber glass bottle (bottom, TIC only) and after decanting into a dishwasher cleaned borosilicate glass bottle (spectrum and TIC). MS detection: positive ESI-MS, direct injection.

Buffers

Buffers for MS use have always to be prepared utilizing the purest salt and acid / base quality available. If possible, avoid working with ammonium bicarbonate buffer.
The salt is normally highly contaminated, see comparison with ammonium acetate (Figure 14).

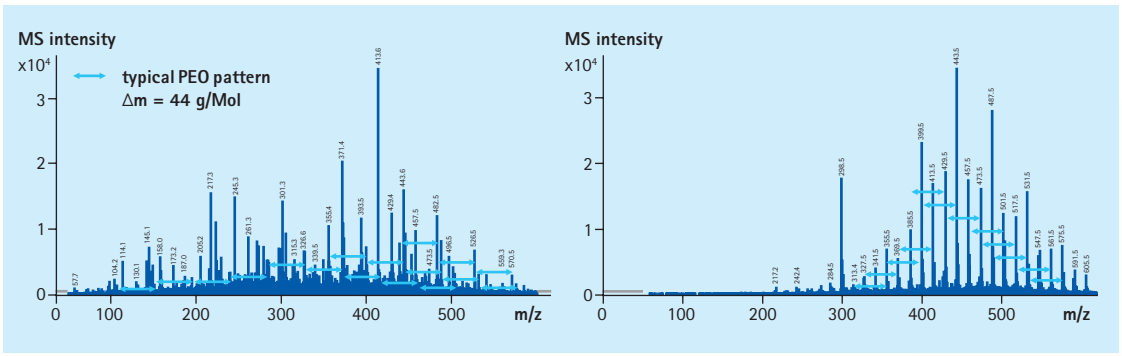
The pH of buffers is generally adjusted via a titration with the respective acid or base and monitored with a pH electrode. The unavoidable contamination of the buffer solution with alkali ions from the pH electrode can be decreased by utilizing a miniaturized system available from several suppliers. Other than standard equipment with a diameter of approximately 10 mm, the diameter of miniaturized electrodes is only 3 mm. The influence of eluent alkali content on the complexity of an MS spectrum is shown in Figure 15.

Figure 14



Comparison of MS spectra (top) and TICs (bottom) of the two buffer systems ammonium bicarbonate and ammonium acetate. Both mixtures were prepared utilizing ultrapure water and the same acetic acid source and were analyzed via direct injection into the mass spectrometer operated in positive ESI mode. Note the enormously high MS background noise when utilizing ammonium bicarbonate as a buffer.

Figure 15



MS of a polyethylene oxide mixture including sodium and potassium adducts (left) in comparison to a spectrum of a clean polyethylene oxide sample (right): Adduct formation leads to a complex spectrum and decreasing target peak intensity (in the m/z range 400 – 500). In both spectra $[M+Na]^+$ and $[M+K]^+$ peaks are unassigned. MS detection: positive ESI-MS, direct injection.

Solvent purity and MS sensitivity

Acetonitrile, methanol and water are commonly used to perform LC-MS analyses. The performance of MS instruments regarding resolution and sensitivity has tremendously improved in recent years. Also the regulatory requirements to reach the limits of detection of various analytes have become increasingly challenging. These prerequisites raise the purity requirements for solvents and reagent water. To perform reliable measurements the absence of contamination sources within the LC-MS system is highly important. Thus, laboratories involved in mass spectrometry analysis need to ensure that the solvents and reagents they use are of consistently high purity and comply with the stringent requirements of MS instrumentation. The mobile phase plays a major role in this regard as organic trace impurities in solvents can cause elevated background noise and ion suppression, result in adducts with the target molecules or affect analysis by interfering with analyte signals.

These effects altogether not only compromise analyses, complicate data interpretation and add the risk of repeating experiments, but also decrease column life time and maintenance intervals of analytical instruments. A minimization can be achieved by using high-purity LC-MS specified solvents. LC-MS grade solvents from EMD Millipore meet all the requirements of the user concerning sensitivity and robustness.

Water

The aqueous mobile phase is the most frequently used solvent in an LC-MS laboratory and therefore plays an important and also critical role in LC separations. It is not only utilized as a single solvent, but also for the preparation of buffers or blanks and in sample preparation. Hence, water of poor quality (that is, not specified to be used in LC-MS applications) must not be utilized in high sensitivity MS experiments. In addition, it has to be taken into account that ultrapure water has relatively aggressive characteristics and can leach contaminants out of, e.g., plastic surfaces.

These impurities are detected by the mass spectrometer in the shape of ghost peaks and elevated baselines. Bottled water of LC-MS grade or freshly produced ultrapure water from water purification systems is offered for use in LC-MS experiments and to answer the specific needs of each laboratory. Independent of the type of water source the quality and MS suitability is guaranteed. The choice between LiChrosolv® LC-MS bottled water and ultrapure water from purification systems usually depends on the volume of water consumed by the laboratory per day.

Bottled water for LC-MS

Table 4

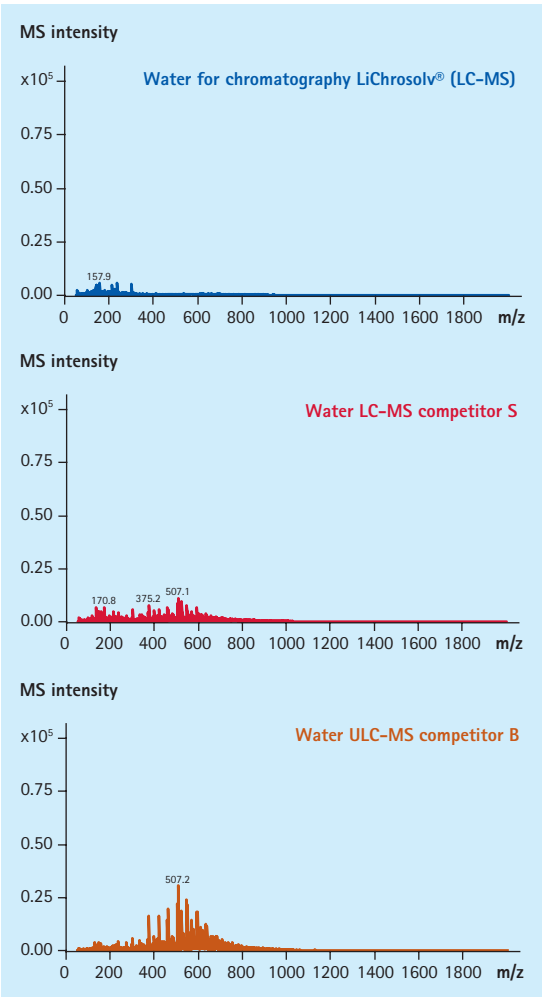
Water sample	Specification
For chromatography **LiChrosolv®** (LC-MS)	≤1 ppb
Competitor S (LC-MS)	complies
Competitor B (ULC/MS)	≤50 ppb

Specification values for different water samples.

For the analysis of LC-MS grade water direct infusion experiments utilizing a syringe pump have been performed to visualize trace impurities. In the mass spectra the basic noise signal of different bottled water samples is shown (Table 4 and Figure 16).

The water was directly injected into the MS via a syringe pump. Water for chromatography LiChrosolv® from EMD Millipore shows only few signals in the low mass range. In contrast the LC-MS water from competitor S without a designated specification value displays some higher contamination signals in the mass range from m/z 50 – 800. Ultra high performance liquid chromatography (UHPLC) MS grade water from competitor B contains quite a high level of organic contaminants, as indicated by the intense signal distribution pattern.

Figure 16



MS conditions	
System	Bruker Esquire 3000+ ion trap MS
Detection	Pos. ESI-MS, m/z range 50 – 2000
Flow rate	0.2 mL/min via syringe pump
Temperature	25°C
Sample	Three different water qualities as indicated in the graphics

Mass spectra displaying the results of the direct infusion of different water qualities from EMD Millipore and two competitors into the MS.

Organic solvents

LC-MS suitability test of different acetonitrile qualities

LC-MS grade acetonitrile from EMD Millipore is specified via the reserpine test. In detail, every batch of organic solvent is spiked with reserpine at a final concentration of 2 ppb (ng/mL) for ESI and APCI in positive mode; in negative mode the concentration is 20 ppb. The sample is then introduced into the MS interface free of contamination using a syringe pump and analyzed (flow injection analysis mass spectrometry: FIA-MS) via monitoring of the TIC over three minutes. Specification is met as long as the signal intensity of any contaminant does not exceed the reserpine signal intensity.

The mass spectra of four different acetonitrile grades clearly show the variation in the intensity of the reserpine signal ($[M+H]^+$ = 609) as well as the extent of the background signals (Figure 20). The differences in the intensity of the reserpine signal are caused by ion suppression. This effect occurs due to interfering trace contaminants that can be present in acetonitrile.

The acetonitrile hypergrade for LC-MS LiChrosolv® from EMD Millipore displays the highest signal intensity. This result also connects with the lowest reserpine value specification in comparison to all competitors (Table 6). Acetonitrile gradient grade for liquid chromatography LiChrosolv® specified for liquid chromatography does not possess sufficient suitability for LC-MS analysis. This can be anticipated from the distinct signal suppression. The LC-MS acetonitrile without a designated specification value from competitor S shows only a low reserpine signal intensity. UHPLC-MS grade acetonitrile from competitor B does not indicate special qualities. The background signals of this acetonitrile grade are very high ("black hump").

The described effects concerning the LC-MS suitability evaluating the reserpine test can also be observed in the gradient comparison of different acetonitrile grades (see page 10 "tips and tricks"). There it is shown that LC-MS grade acetonitrile leads to smoother baselines.

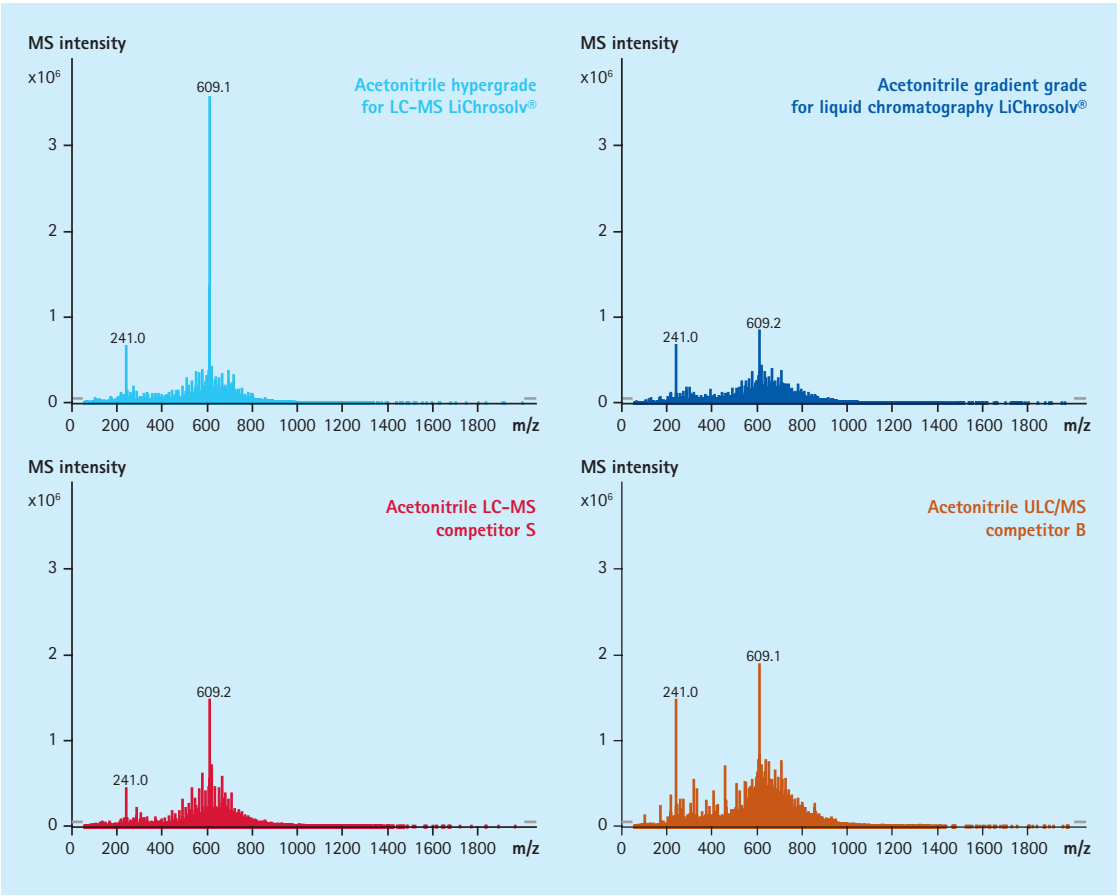
Table 6

Acetonitrile sample	Intensity of reserpine signal	Specification
Hypergrade for LC-MS **LiChrosolv®**	3.6 x 10^6	≤2 ppb
Gradient grade for liquid chromatography **LiChrosolv®**	0.8 x 10^6	n/a
Competitor S (LC-MS)	1.5 x 10^6	complies
Competitor B (ULC/MS)	1.9 x 10^6	≤50 ppb

Reserpine signal intensity and specification values for different acetonitrile samples.

Solvent purity and MS sensitivity

Figure 20

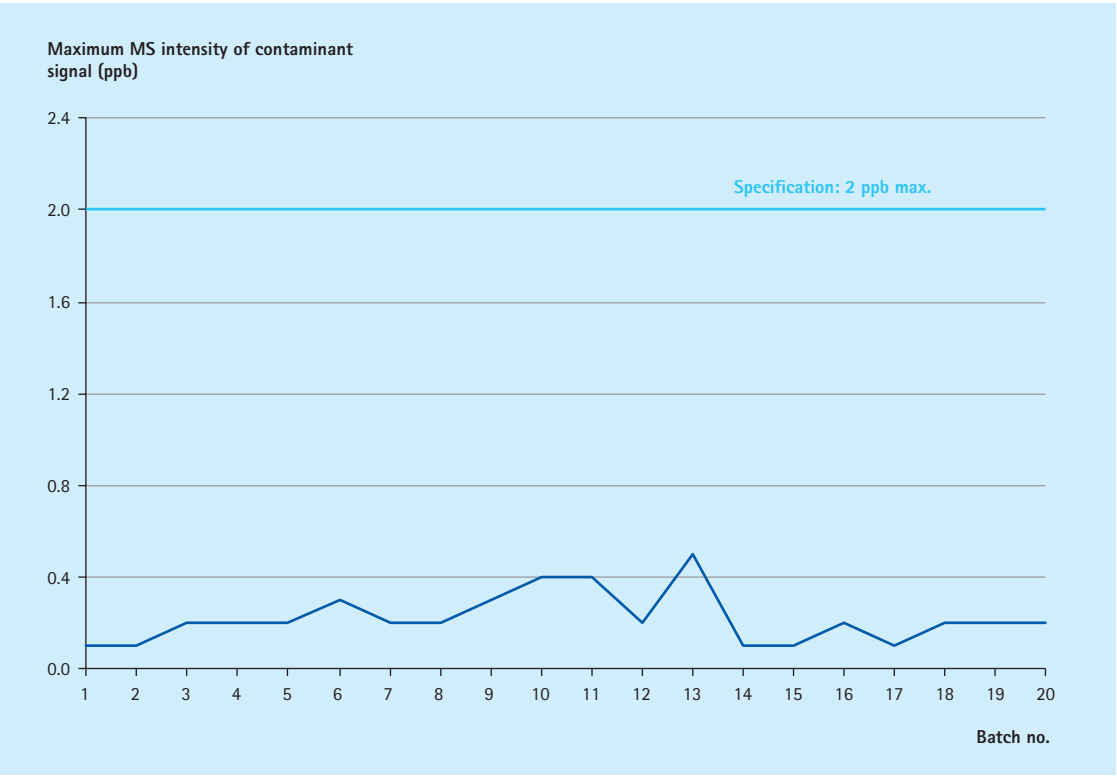


MS conditions	
System	Bruker Esquire 3000+ ion trap MS
Detection	Pos. ESI-MS, m/z range 50 – 2000
Flow rate	0.2 mL/min via syringe pump
Temperature	25°C
Sample	Reserpine (m/z 609.1), internal standard (m/z 241.0)

Mass spectra displaying the results of the reserpine test of different acetonitrile qualities from EMD Millipore and two alternative competitors.

Batch-to-batch reproducibility

Figure 21



Progression of the intensity of the contaminant signal in quality control experiments of acetonitrile hypergrade for LC-MS LiChrosolv® in 20 consecutive batches. **For experimental conditions see figure 16.**

The robustness of the analytical system is the most important factor in achieving reproducible results. For LC-MS the mobile phase plays a major role in this regard because of organic trace impurities that can interfere with target analytes. Each LC-MS grade solvent batch is accurately tested using a dedicated mass spectrometer for LC-MS grade solvents and reagents at our quality control laboratory. Figure 21 shows the batch-to-batch consistency of the specification value for twenty consecutively produced batches of LC-MS grade acetonitrile. The detected values for the trace impurities in acetonitrile are clearly below the specification limit and the deviation between the values is very small.

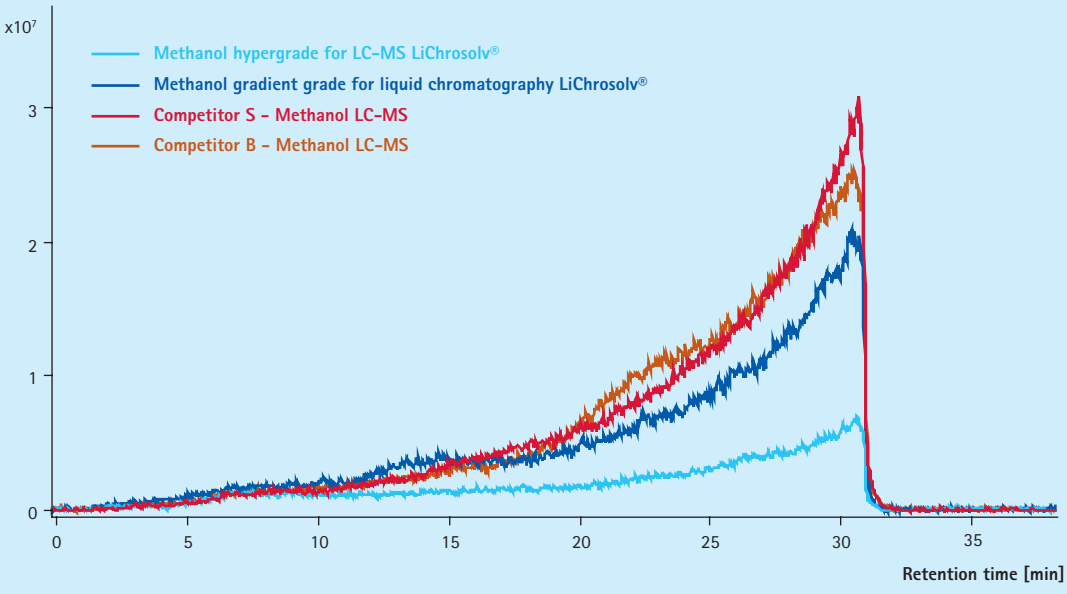
Benefits of LC-MS grade acetonitrile, methanol and water

- low impurity background → little signal suppression
- low level of contaminants → no ghost peaks and smooth baselines
- lowest specification values → high analyte signal intensities
- high batch-to-batch consistency → good reproducibility of results

LC-MS gradient test of different methanol qualities

The course of the baseline highly influences the chromatographic resolution and peak shape. Preferably flat baselines are targeted in liquid chromatography. The mass spectrometer as a universal detector reacts especially sensitively to organic trace impurities in the mobile phase. The ionization of these impurities leads to an increase of the background noise. Here a typical reversed phase gradient without a chromatography column is shown comparing various methanol grades via their TICs (Figure 22). An increasing methanol content of the mobile phase leads to a slope at the end of each gradient program; the characteristic of this slope can directly be correlated to the purity of the organic solvent. The LC-MS grade methanol from EMD Millipore leads to a smooth and comparably flat baseline, while gradient grade methanol specified for liquid chromatography shows an uneven progression and a slope increased with rising methanol content. LC-MS grade methanol from competitors S and B are less suitable than gradient grade methanol because of the steep rise of the baseline.

Figure 22



Chromatographic conditions	
System	Bruker Esquire 3000+ ion trap MS
Detection	Pos. ESI-MS, m/z range 50 – 2000
Flow rate	1 mL/min
Mobile phase	A: Water (**VWR Cat No. EM1.15333.1000**) B: Various methanol qualities as indicated in the graphics
Gradient	0 min 98 % A, 30 min 0 % A, 38 min 98 % A
Temperature	25°C

Comparison of LC-MS blank baselines (TIC) of different methanol qualities from EMD Millipore and two competitors.

The use of LC-MS grade solvents allows reliable and reproducible results without artifacts such as signal suppression or background noise due to a very low level of organic trace impurities. These quality characteristics of EMD Millipore solvents save cost and time intensive repetition of analyses, decrease system contamination and extend maintenance intervals.

Dirty sample analysis

The analysis of samples with high matrix load requires tedious and time-consuming sample preparation steps. For cost-effective investigations sample handling has to be kept as short as possible and combined with robust LC columns displaying a high matrix tolerance and long lifetime. Two fast and simple sample preparation protocols were developed for the analysis of different food samples, and quick LC separation was performed with monolithic silica columns. In a second set of experiments TLC-MS was utilized for the determination of analytes in complex sample matrices without applying any sample preparation step.

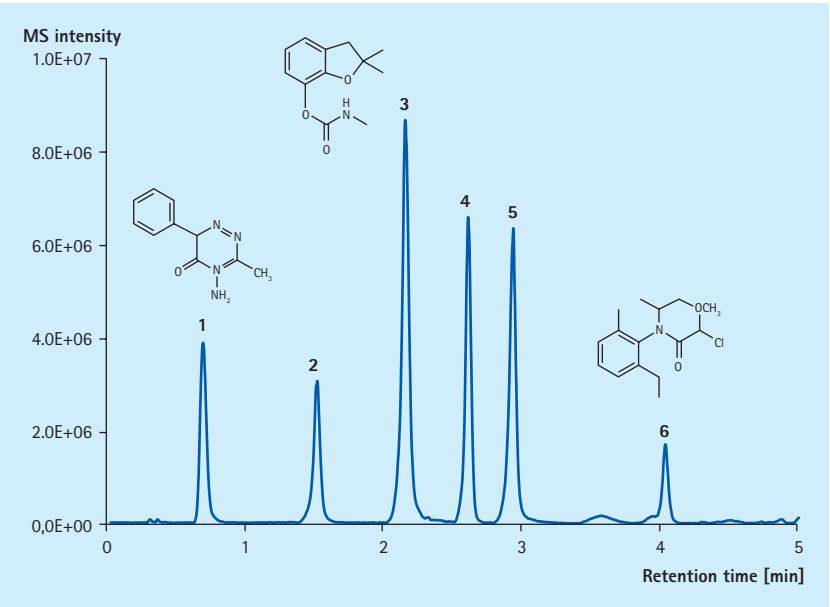


Figure 23 *LC-MS analysis of a mixture of six pesticides in porcine kidney.*

Monolithic silica columns

The first example describes the analysis of a set of six pesticides in a complex porcine kidney matrix (Figure 23). Pesticides utilized to grow crops can easily enter the food chain (via plants and animals) and become ingested by humans. A proper method for fast and reliable analysis of trace contaminants without tedious sample preparation is therefore desirable. Here, the spiked kidney homogenizate first underwent a typical solid-phase extraction (SPE) procedure utilizing a LiChrolut® RP-18e cartridge. Then the extract was separated on a short 2 mm i.d. monolithic silica column enabling fast gradient runs, and peaks were identified via mass spectrometry. Within only four minutes all six pesticides eluted and showed baseline separated peaks. The clean chromatogram displays the absence of any matrix compound and reveals the exceptional performance of the applied SPE process.

Chromatographic conditions	
Column	Chromolith® FastGradient RP-18 endcapped 50-2 mm (**VWR Cat. No. 97007-930**)
Injection volume	1 µL
System	Bruker Esquire 6000plus
Detection	Pos. ESI-MS, m/z range 190 – 370, base peak chromatogram (BPC)
Flow rate	0.6 mL/min
Mobile phase	A: Ultrapure water from water purification system + 0.1 % formic acid (**VWR Cat. No. EM1.00264.2500**) B: Acetonitrile (**VWR Cat. No. EM1.00029.2500**) + 0.1 % formic acid
Gradient	0 min 80 % A, 6 min 20 % A, 7 min 20 % A
Temperature	25°C
Sample	Spike 1 g of porcine kidney with 2 mL of stock solution (**1** Metamitron 159 mg/L, **2** Carbetamid 487 mg/L, **3** Carbofuran 548 mg/L, **4** Isoproturon 159 mg/L, **5** Metazachlor 796 mg/L, **6** Metolachlor 395 mg/L). Mix with 10 mL of ACN/water 20/80 (v/v). Homogenize and centrifuge, remove ACN. Transfer to preconditioned LiChrolut® RP-18 E (40 – 63 µm) 500 mg 3 mL standard PP-tube (**VWR Cat. No. 48219-220**), elute with ACN. Add water, remove ACN, filter through a syringe filter driven unit PTFE 0.45 µm. Dilute 1:100 with ACN and inject.

Dirty sample analysis

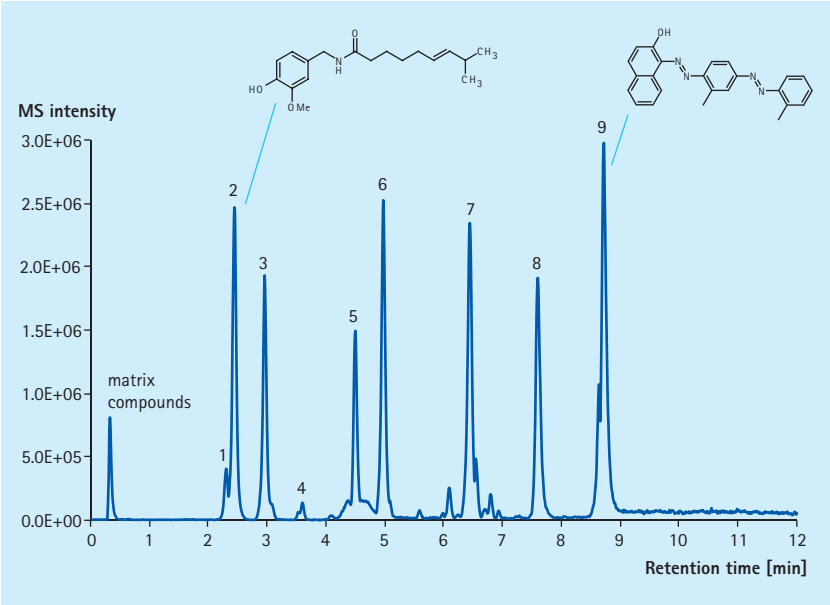


Figure 24 *LC-MS analysis of carcinogenic colorants in hot chili sauce*

The second method describes the sample preparation and analysis of five carcinogenic colorants in a hot sauce matrix (Figure 24). Products containing chili or tomato are often colored artificially to increase the attractiveness (e.g., of hot sauces, pesto, chili powder, curcuma and herb mixtures). Colorants used can be carcinogenic azo dyes such as Sudan or Para Red banned in the European Union, which makes a fast and reproducible analysis method including sample preparation necessary. A liquid-liquid extraction of hot chili sauce was performed and the extract spiked with Sudan type dyes was analyzed using a gradient run on a short monolithic silica column. All nine analytes (dyes and capsaicinoids) were separated from remaining matrix components and detected via MS, with only the peak pair nordihydrocapsaicin and capsaicin not being baseline separated. The 50-2 mm monolithic silica column is the ideal choice for fast gradient run liquid chromatography and the applied low flow rates make it the perfect pick for MS detection. Analysis of dirty samples such as food or tissue can be performed on this robust column type without the need for a guard column or tedious and complex sample preparation procedures.

Chromatographic conditions	
Column	Chromolith® FastGradient RP-18 endcapped 50-2 mm (**VWR Cat. No. 97007-930**)
Injection volume	2 µL
System	Bruker Esquire 6000plus
Detection	Pos. ESI-MS, m/z range 100 – 700, BPC
Flow rate	0.4 mL/min
Mobile phase	A: Ultrapure water from water purification system + 0.1 % formic acid (**VWR Cat. No. EM1.00264.2500**) B: Acetonitrile (**VWR Cat. No. EM1.00029.2500**) + 0.1 % formic acid
Gradient	0 min 35 % B, 8 min 95 % B, 11 min 95 % B
Temperature	25°C
Sample	Soxhlet extract approx. 10 g of a hot chili sauce during six hours with ethanol 96 %. (**1** Nordihydrocapsaicin, **2** Capsaicin, **3** Dihydrocapsaicin, **4** Homodihydrocapsaicin, **5** Para Red, **6** Sudan I, **7** Sudan II, **8** Sudan III, **9** Sudan IV). Filter solution using a syringe filter and dilute in a ratio of 1:1000 with ACN/water 50/50 (v/v). Combine 3 mL of sauce extract with 1 mL of colorant stock solution (10 mg each of Para Red, Sudan I, Sudan II, Sudan III and Sudan IV in 500 mL acetonitrile), make up with ACN to 13 mL. Concentrations of the colorants in the final mixture are: Sudan I 1.80 ng/mL, Sudan II 2.55 ng/mL, Sudan III 1.54 ng/mL, Sudan IV 1.49 ng/mL, Para Red 1.50 ng/mL.

Thin layer chromatography (TLC)

One unique domain of TLC is the separation of samples with high matrix content without the need for any sample preparation. In combination with a powerful detection technique such as mass spectrometry a fast and cost-saving setup can be realized. To support this development and to exploit the full potential of a mass spectrometer, special MS-grade TLC plates offering high sensitivity and low background signals for constantly high-quality MS measurements are desirable.

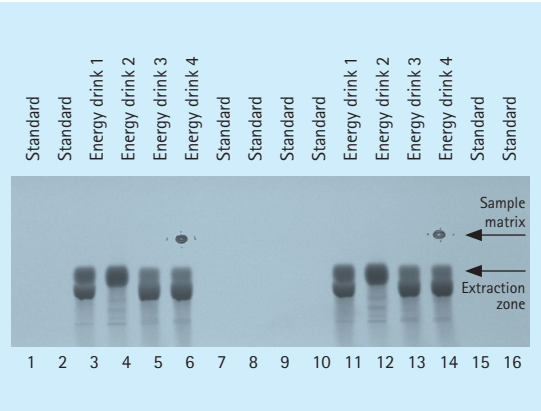
Two methods were developed for the quantification of caffeine and paracetamol using MS-grade high performance thin layer chromatography (HPTLC) Silica gel 60 plates and the elution based TLC–MS Interface from CAMAG (Figure 25) coupled with the expression compact mass spectrometer (CMS) from Advion (Figure 26). Because of the high matrix tolerance of TLC, no complicated sample preparation procedures were needed. The analysis of caffeine in energy drinks can be done by direct application of the energy drink samples on the plate; main matrix component of this sample is saccharose.

Figure 25
TLC-MS Interface (CAMAG, Switzerland) for the direct extraction of TLC / HPTLC plates.

Figure 26
Compact mass spectrometer expression CMS (Advion, USA).

The image in Figure 27 displays the excellent separation of caffeine from the sample matrix components. The broad matrix spots were made visible via staining of the plate with *p*-anisaldehyde and sulfuric acid after the complete analysis process consisting of separation, extraction and identification / quantification. The result of the parallel separation of 16 energy drink samples and standards can be seen under UV irradiation (Figure 28).

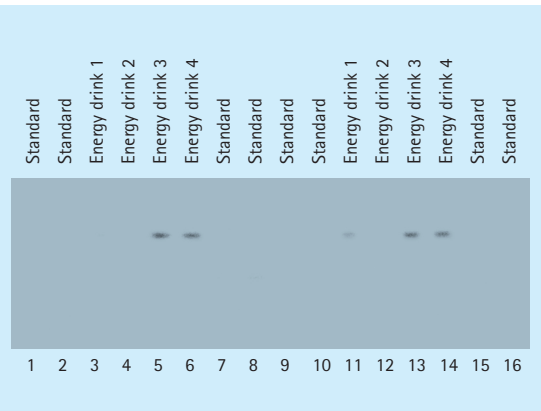
Figure 27



Chromatographic conditions	
Plate	HPTLC Silica gel 60 F_{254} MS-grade, 20 x 10 cm **(VWR Cat. No. 10755-322)**
Application volume	0.5 – 3 µL
Detection	Visible Staining with *p*-anisaldehyde **(VWR Cat. No. EM8.22314.1000)** /sulphuric acid **(VWR Cat. No. EM1.00713.2500)** reagent
Migration distance	5 cm
Migration time	50 min
Mobile phase	2-Propanol **(VWR Cat. No. EM1.01040.4000)** / *n*-heptane **(VWR Cat. No. EM1.04390.2500)** / water **(VWR Cat. No. EM1.15333.2500)** 7:3:1 (v:v:v)
Sample	No sample preparation, direct application of energy drinks
Sample application	ATS4 sample applicator (CAMAG) 6 mm bandwise

High matrix load of energy drinks made visible via staining of a plate with p-anisaldehyde / sulfuric acid after the whole analysis process (separation, extraction, identification / quantification). The image was taken under ambient light conditions. Several large sample matrix spots were clearly separated from the caffeine spot. In the extraction zone two large extracted areas are visible (samples 6 and 14).

Figure 28



Chromatographic conditions	
System	TLC – MS interface (CAMAG) coupled with expression CMS (Advion)
Detection	Pos. ESI-MS, m/z range 100 – 500
Extraction solvent	Acetonitrile **(VWR Cat. No. EM1.00029.2500)** / water **(VWR Cat. No. EM1.15333.2500)** 95:5 (v:v) + 0.1 % formic acid **(VWR Cat. No. EM1.00264.2500)**
Extraction flow	0.1 mL/min
For all other conditions see figure 27.	

Image of a TLC plate under UV irradiation (254 nm) after separation of 16 different caffeine samples (energy drinks and standards). The samples were applied on an HPTLC silica gel 60 F_{254} MS-grade 25 glass plate 20 x 10 cm and separated in parallel utilizing 2-propanol/n-heptane/water 7:3:1 (v:v:v) as a mobile phase in 50 minutes.

The application of standard solutions together with different samples on one and the same plate enables a precise quantification. The caffeine content of the analyzed energy drinks is in the range from 0.13 mg/mL to 0.41 mg/mL (Table 7).

Table 7

Track	Sample	Concentration	Applied volume
1 / 9	Caffeine standard	0.10 mg/mL	0.5 µL
2 / 10	Caffeine standard	0.10 mg/mL	1.0 µL
3 / 11	Energy drink 1	0.17 mg/mL	0.5 µL
4 / 12	Energy drink 2	0.13 mg/mL	0.5 µL
5 / 13	Energy drink 3	0.41 mg/mL	0.5 µL
6 / 14	Energy drink 4	0.40 mg/mL	0.5 µL
7 / 15	Caffeine standard	0.10 mg/mL	2.0 µL
8 / 16	Caffeine standard	0.10 mg/mL	3.0 µL

Sequence table for the quantification of caffeine in energy drinks including sample information, concentration and applied volume.

As a second example, the paracetamol content of three different formulations (suppositories, syrup, pills) and of several standards was analyzed (Figure 29).

Figure 29

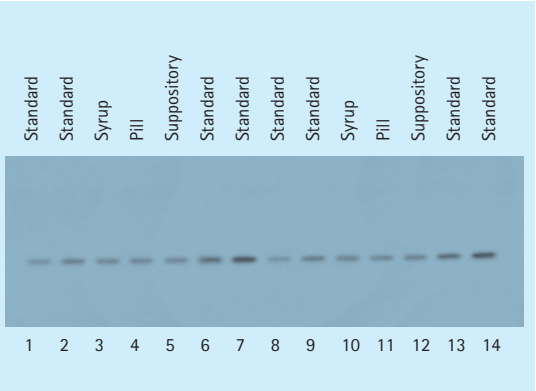


Image of a TLC plate under UV irradiation (254 nm) after separation of 14 paracetamol samples (three different formulations and standards).

Chromatographic conditions	
Plate	HPTLC Silica gel 60 F_{254} MS-grade, 20 x 10 cm (**VWR Cat. No. 10755-322**)
Application volume	0.3 – 1 µL
System	TLC – MS interface (CAMAG) coupled with expression CMS (Advion)
Detection	Pos. ESI-MS, m/z range 100 – 300 UV (254 nm)
Migration distance	5 cm
Migration time	9 min
Mobile Phase	Acetone (**VWR Cat. No. EM1.00020.4000**) / toluene (**VWR Cat. No. EM1.08327.1000**) 1:1 (v:v) + 0.1 % acetic acid (**VWR Cat. No. EM1.00063.2500**)
Extraction solvent	Acetonitrile (**VWR Cat. No. EM1.00029.1000**) / water (**VWR Cat. No. EM1.15333.2500**) 95:5 (v:v) + 0.1 % formic acid (**VWR Cat. No. EM1.00264.2500**)
Extraction flow	0.1 mL/min
Samples	One suppository containing 125 mg Paracetamol is diluted in 125 mL ethanol at 36°C; 0.2 mL paracetamol pain syrup containing 4 % Paracetamol is diluted in 7.8 mL methanol (**VWR Cat. No. EM1.06035.2500**); one pill containing 500 mg paracetamol is diluted in 500 mL methanol and filtered with a 0.45 µm syringe filter. All sample solutions were applied directly after dissolution.
Sample application	ATS4 sample applicator (CAMAG) 6 mm bandwise

Dirty sample analysis

Straightforward MS identification was performed with the combination of a TLC-MS interface coupled with an expression CMS mass spectrometer. The analysis of a caffeine spot derived from the separation of an energy drink revealed two signals with m/z 195.12 and 236.06. These were attributed to the protonated molecular ion of caffeine $[M+H]^+$ and the acetonitrile adduct $[M+ACN+H]^+$, respectively. Paracetamol from a syrup formulation was identified via the signals with m/z 152.12 $[M+H]^+$ and 193.18 $[M+ACN+H]^+$ (Figure 30).

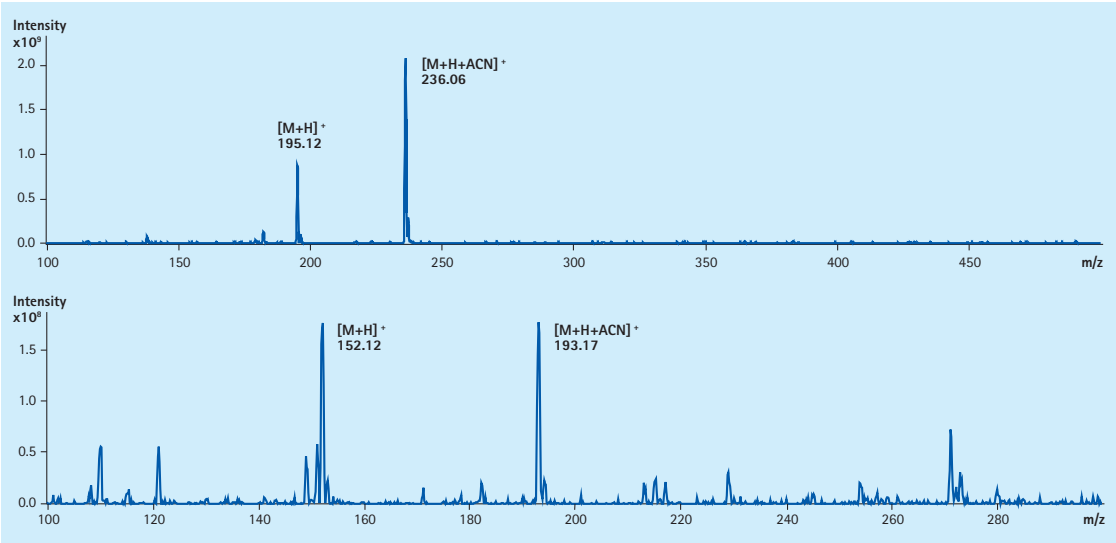


Figure 30 *MS spectra of an energy drink sample (top) and a paracetamol formulation (bottom).* **For conditions see figures 27 – 29.**

Thin layer chromatography can be performed without any classical tedious sample preparation procedure. In combination with the option of a parallel analysis of many samples low costs per sample and short analysis times can be realized. A hyphenation to mass spectroscopic detection adds additional value by generating data about molecular masses and detailed structural parameters of target molecules and is by far more sensitive in the wide majority of applications than classical detectors such as UV or fluorescence.

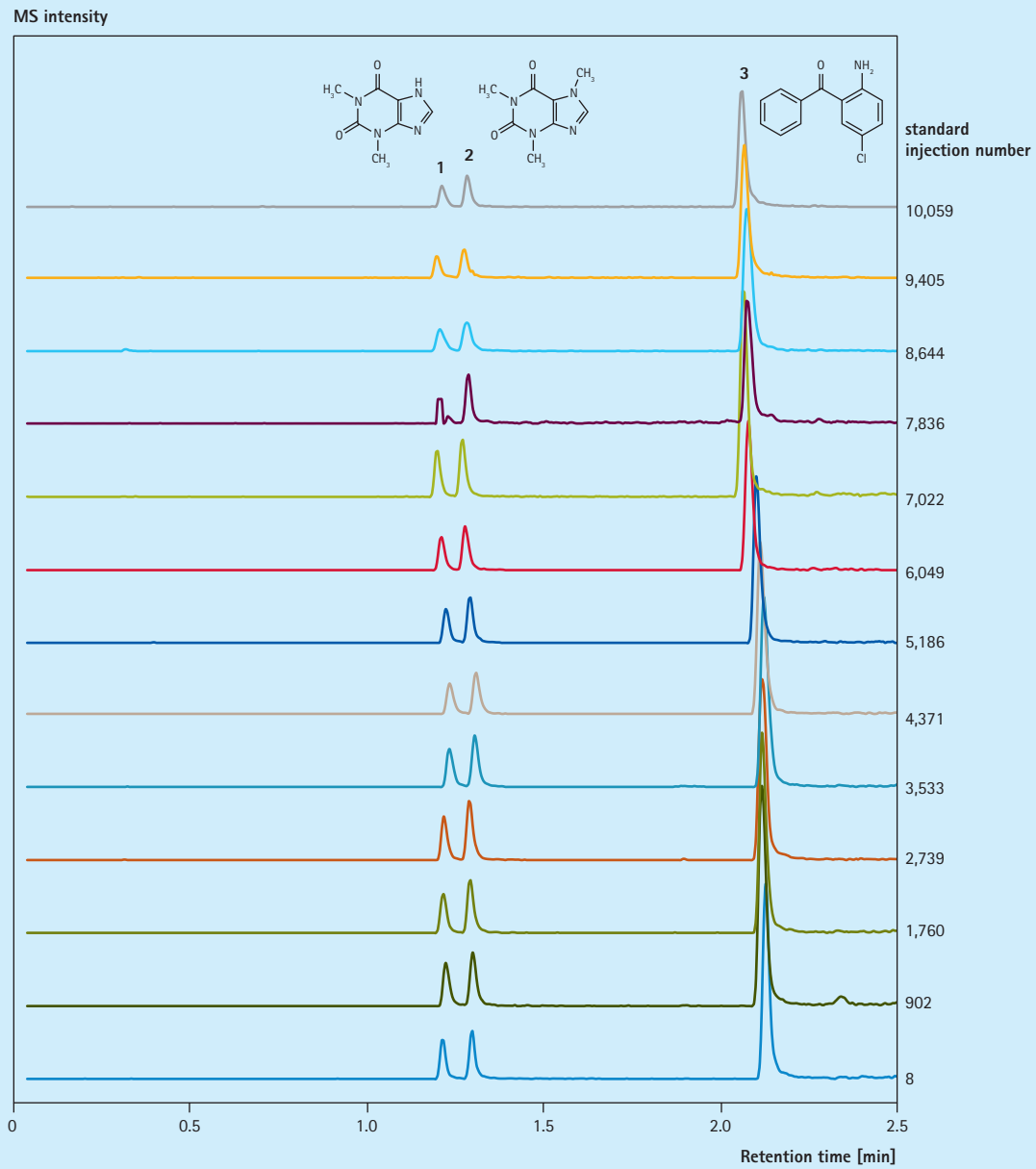
TLC plates

Column robustness and lifetime

Exceptional robustness or lifetime of a column – described as number of injections – directly affects the economic situation in a laboratory in a positive way. But there is also an indirect effect: Understanding robustness as high matrix tolerance decreases tedious sample preparation steps, speeds up all processes and allows for fast and simple HPLC analyses.

Two examples display the utilization of Chromolith® columns in a pharma R&D as well as in a high throughput screening (HTS) everyday lab environment. In pharma R&D small molecules (products or intermediates) synthesized during drug development have to be analyzed via fast and robust methods without shutdown periods caused by column failure. Typical run times for these experiments are in the range of 2 – 3 minutes. In combination with standard HPLC equipment, columns showing low backpressures are desirable to perform these quick runs. In this example an LC-MS system is utilized for separation and detection. The performance of the setup is checked for consistency by the injection of a standard mixture of theophylline, caffeine and 2-amino-5-chlorobenzophenone every workday morning. In this environment the monolithic silica column allows for more than 10,000 chromatographic runs on standard HPLC equipment within approximately half a year (Figure 31) before it is replaced routinely. All test runs performed during this period display consistent peak shape and retention as well as chromatographic resolution and performance without any increase in column backpressure.

Figure 31



Chromatographic conditions	
Column	Chromolith® Performance RP-18 endcapped 100-3 mm (**VWR Cat. No. 82031-440**)
Injection volume	1 µL
System	Agilent 1100 HPLC and Waters single quadrupole MS
Detection	Pos. API-MS, m/z range 85 – 800
Flow rate	0.4 mL/min (MS; HPLC flow 2 mL/min, post-column split)
Mobile phase	A: Ultrapure water from water purification system + 0.05 % formic acid (**VWR Cat. No. EM1.00264.2500**) B: Acetonitrile (**VWR Cat. No. EM1.00029.1000**) + 0.04 % formic acid
Gradient	0 min 99 % A, 1.8 min 0 % A, 2.5 min 0 % A
Temperature	25°C
Sample	**1** theophylline, **2** caffeine, **3** 2-amino-5-chlorobenzophenone (1 mmol each) dissolved in methanol / water 50:50 (v:v).

Pharma R&D small molecule analysis, results of everyday standard runs testing the performance of the utilized column.

HTS is utilized to screen pharma libraries containing numerous small molecules for their suitability as an active ingredient. In a similar way as for pharma R&D, fast separations on standard HPLC equipment is an important issue in HTS that can best be addressed by columns showing low backpressures. Robust and reliable column technology is an inalienable prerequisite to avoid any system failure and shutdown. In this example an HTS system with four columns as well as four evaporative light scattering detectors (ELSD) and UV-MS detectors operated in a parallel setup is used. The performance of this setup is checked for consistency by the injection of a standard (Sarizotan) every 50 runs.

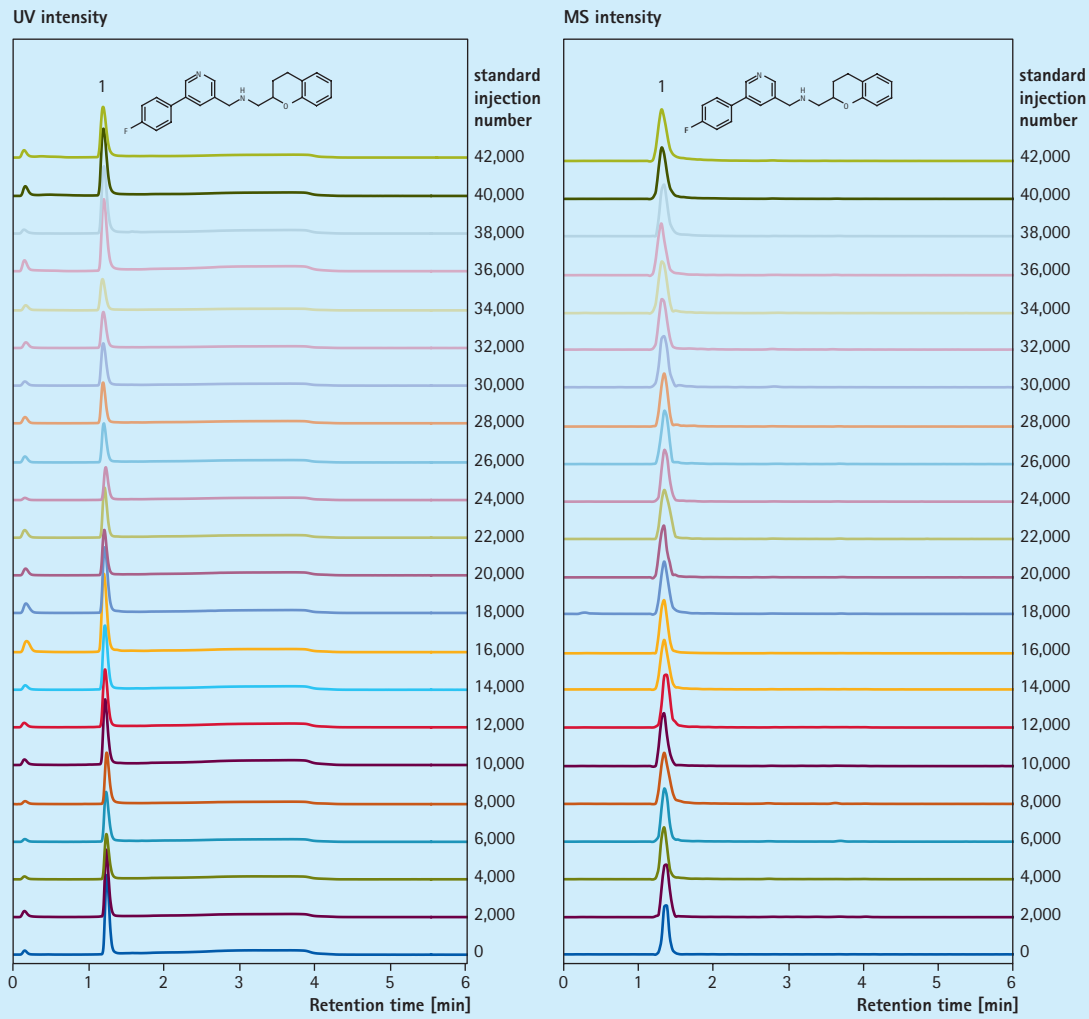
Within one year more than 40,000 fast chromatographic runs were performed on each of the four monolithic silica columns (Figure 32). During this period all relevant chromatographic criteria (performance, peak shape, retention time) remained constant and no degradation of the column was observed.

Extreme robustness of Chromolith® columns

The two application examples display the extreme robustness of Chromolith® columns in a lab environment, where time of analysis has to be low and speed, reproducibility and a long column lifetime are required. Chromolith® delivers these features on standard HPLC systems without the need for upgrading to high-priced LC solutions.

Chromolith® columns

Figure 32



Chromatographic conditions	
Column	Chromolith® Flash RP-18 endcapped 25-2 mm (**VWR Cat. No. EM1.52014.0001**)
Injection volume	5 µL
System	Waters 2777 sample manager, 2488 MUX-UV detector, 4 2420 ELS detector, ZQ-MUX
Detection	Left: pos. ESI-MS, m/z range 160 – 1000, base peak chromatogram (BPC); right: UV 254 nm, ELSD
Flow rate	0.8 mL/min
Mobile phase	A: Ultrapure water from water purification system + 0.1 % formic acid (**VWR Cat. No. EM1.00264.2500**) B: Acetonitrile (**VWR Cat. No. EM1.00029.1000**) + 0.1 % formic acid
Gradient	0 min 95 % A, 1.7 min 0 % A, 3 min 0 % A, 3.01 min 100 % A, 6.25 min 95 % A
Temperature	25°C
Sample	Sarizotan (1 mmol) dissolved in dimethyl sulfoxide

High throughput screening experiment, results of the control runs testing column performance.

Sensitivity and column selection

Samples of, e.g., biological origin are often only available in small amounts and the concentration of analytes is low. Typical analytes are proteins or digested proteins, peptides and numerous types of metabolites. Under this precondition a setup consisting of both highly sensitive separation and detection techniques is necessary for proper identification of the target molecules. The sensitivity of a separation is influenced by the internal diameter (i.d.) of the column used, e.g. when changing from a 4.6 mm i.d. column to a 0.1 mm i.d. capillary column, sensitivity increases by a factor of approximately 2000. Hence, a combination of chromatography utilizing capillaries coupled to mass spectrometry is the best combination for high sensitivity analysis.

Two gradient separation methods were developed describing the analysis of a tryptic digest of cytochrome c and of a peptide mixture on nano-LC equipment. The chromatographic separation was performed on a small i.d. analytical monolithic silica capillary column directly coupled to the source of a mass spectrometer.

The amino acid sequence of cytochrome c from cattle (bos taurus) was analyzed and a set of nine tryptic fragments was identified within a run time of approximately 10 minutes (Table 8 and Figure 33). Utilization of a monolithic silica capillary column and a two-step gradient enabled the elution of narrow peaks and baseline separation of all peptides except for the critical peak pair No. 5/6. The amino acid sequence of all fragments was identified via the obtained MS data and a library search.

In a second experiment a mixture of five peptides was analyzed utilizing a monolithic silica capillary column and gradient elution conditions (Figure 34). The analysis of all five compounds was achieved within less than seven minutes, including the baseline separation of the critical peaks of Met-enkephalin, angiotensin II and Leu-enkephalin.

In combination with mass spectrometry detection the use of monolithic silica capillary HPLC columns offers maximum sensitivity analyses of low amounts of biological samples at overall low backpressure and minimized solvent consumption. Due to the rigidity and robustness of the fritless column bed no tedious sample preparation is necessary and clogging can be minimized.

Table 8

Peak no.	Amino acid sequence	Retention time (min)	Detected mass m/z (g/mol)
1	YIPGTK	2.21	339.7
2	IFVQK	2.70	317.7
3	KTGQAPGFSYTDANK	5.05	528.9
*	unidentified compounds	5.98 / 6.07	n.a.
4	TGPNLHGLFGR	6.95	390.2
5	GEREDLIAYLKK	7.20	478.9
6	MIFAGIK	7.28	390.2
7	EDLIAYLK	8.24	482.8
8	IFVQKCAQCHTVEK	9.33	545.3
9	GITWGEETLMEYLENPKK	10.37	713.4

Peak number, amino acid sequence, retention time and detected molar mass of fragments obtained after tryptic digestion of cytochrome c.

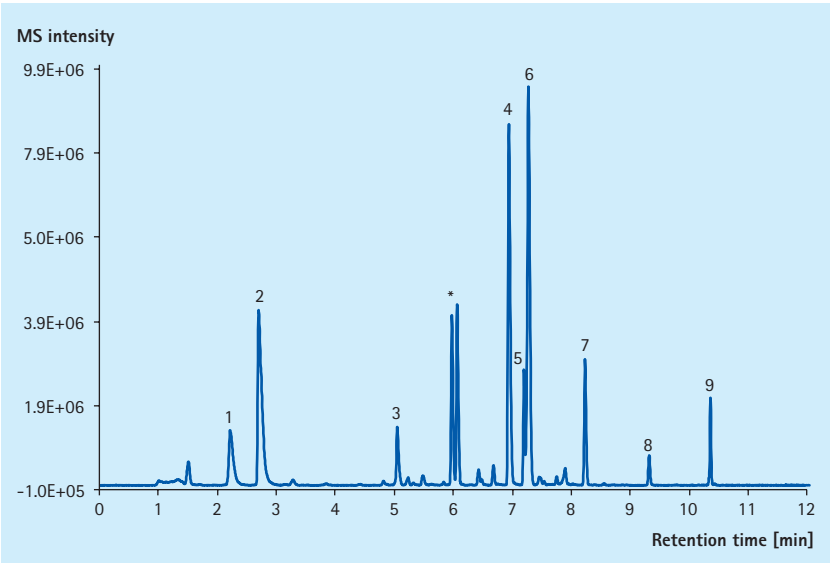


Figure 33 *LC-MS analysis of a tryptic digest of cytochrome c utilizing a monolithic silica capillary column.*

Chromatographic conditions	
Column	Chromolith® CapRod® RP-18 endcapped 150-0.1 mm capillary column (**VWR Cat. No. EM1.50402.0001**)
Injection volume	1 nL
System	Bruker Esquire 6000plus
Detection	Pos. ESI-MS, m/z range 300 – 750, BPC
Flow rate	3.5 µL/min
Mobile phase	A: Ultrapure water from water purification system + 0.1 % formic acid (**VWR Cat. No. EM1.00264.2500**) B: Acetonitrile (**VWR Cat. No. EM1.00029.1000**) + 0.1 % formic acid
Gradient	0 min 95 % A, 7 min 75 % A, 10 min 30 % A
Temperature	25°C
Sample	Lyophilized tryptic digest of cytochrome c (from cattle, bos taurus) resuspended in ACN/water 5/95 (v/v)

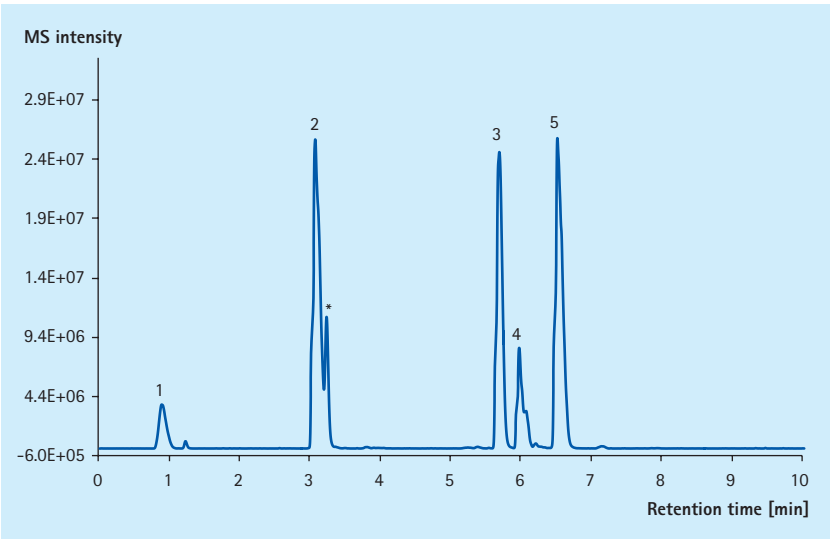


Figure 34 *LC-MS analysis of a mixture of five peptides on a monolithic silica capillary column.*

Chromatographic conditions	
Column	Chromolith® CapRod® RP-18 endcapped 150-0.1 mm capillary column (**VWR Cat. No. EM1.50402.0001**)
Injection volume	1 nL
System	Bruker Esquire 6000plus
Detection	Pos. ESI-MS, m/z range 300 – 750, BPC
Flow rate	3.5 µL/min
Mobile phase	A: Ultrapure water from water purification system + 0.1 % formic acid (**VWR Cat. No. EM1.00264.2500**) B: Acetonitrile (**VWR Cat. No. EM1.00029.1000**) + 0.1 % formic acid
Gradient	0 min 99 % A, 5 min 85 % A
Temperature	25°C
Sample	Lyophilized peptides: **1** Gly-Try, **2** Val-Tyr-Val, **3** Met-enkephalin, **4** Angiotensin II, **5** Leu-enkephalin, resuspended in water.

Speed and sensitivity in GC-MS

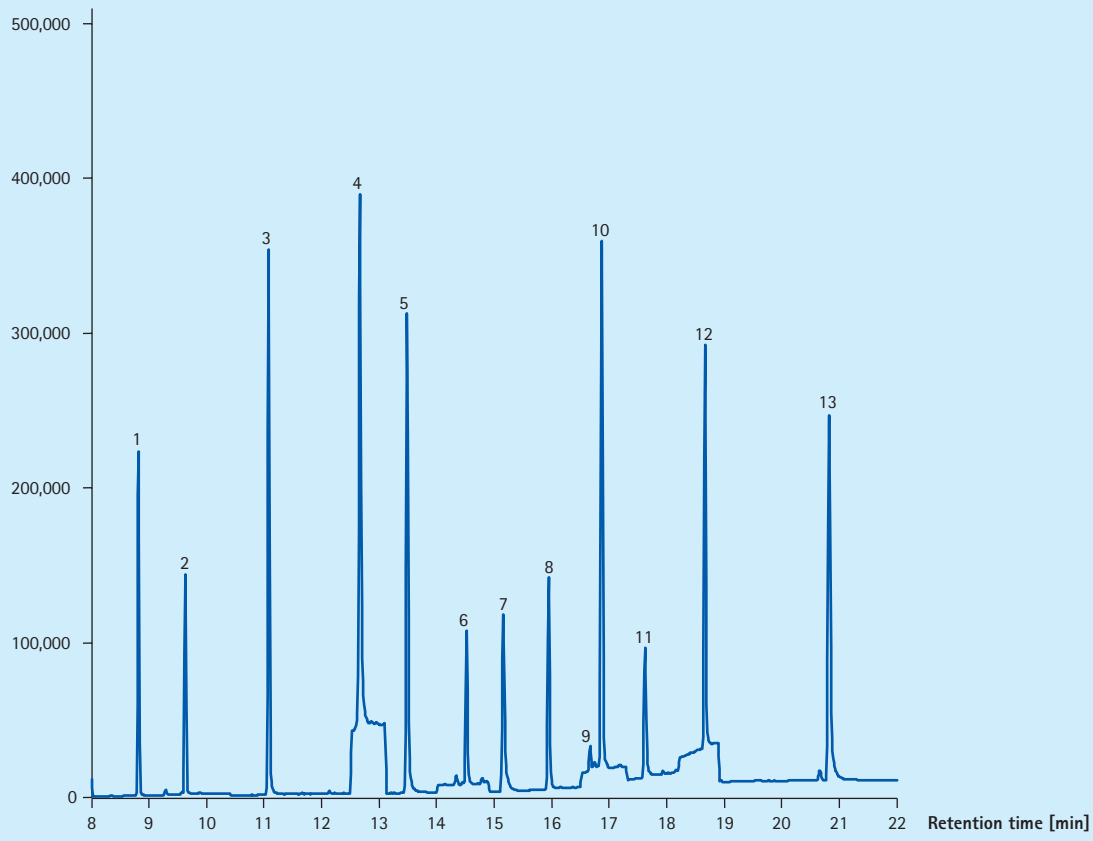
All juice producers need to check the marketability of their juices by analyzing, e.g., contaminations such as pesticides. Strict regulations about maximum residue levels (MRL) are valid nationally and / or internationally. Today approximately 500 different pesticides are detectable in juices.

Classical pesticide residue analysis is still done utilizing GC-EC detection, e.g. according to the US Environmental Protection Agency (EPA) 508 method. Sample preparation is based on liquid-liquid extraction with SupraSolv® solvents n-hexane, ethyl acetate, dichloromethane or acetone. Here, a new and fast QuEChERS method was developed for the extraction of an apple juice spiked with a mixture of 13 pesticides. The sample preparation procedure is based on liquid-liquid extraction with EXtrelut® NT 20 pre-packed columns and dichloromethane as a solvent. The QuECHERS method reduces manual effort and solvent consumption, increases sample throughput, improves analytical safety, and extends the range of detectable pesticides.

Subsequent detection was performed via GC-MS. Figure 35 displays the separation of all 13 pesticides within a chromatographic runtime of approximately 21 minutes. One important requirement for this chromatographic analysis is a high purity of the solvent offering a clear baseline and excellent signal-to-noise ratio. In addition, a reliable batch-to-batch consistency of the solvent quality enables accurate and reproducible results and in turn saves time and cost.

All these features can be found combined in EMD Millipore solvents SupraSolv®.

Figure 35



Chromatographic conditions	
Column	30 m, 0.25 mm i.d., 0.25 µm ft
Injection volume	2 µL
System	Agilent 7890A, MSD 5975C, inert XL MSD
Carrier gas	Helium, constant flow
Sample	**1** Trifluralin, **2** Profluralin, **3** Pirimiphos-methyl, **4** Procymidon, **5** p,p'-DDE, **6** Trifloxystrobin, **7** Quinoxyfen, **8** Etoxazol, **9 + 10** lambda-Cyhalothrin, **11** Fenarimol, **12** Halfenprox, **13** Azoxystrobin; Liquid-liquid-extraction with EXtrelut® NT 20 (**VWR Cat. No. EM1.15096.0001**) as eluting solvent.

GC-MS chromatogram (TIC) of apple juice spiked with a mixture of 13 pesticides.

Bioanalysis and MS

The term bioanalysis traditionally refers to the measurement of small molecules in biological fluids but this discipline has expanded substantially because of the increased interest in biopharmaceuticals such as proteins and peptides. Targeted analytes may be either of endogenous character (naturally occurring) or molecules being administered as drugs to humans (exogenous). Pharmaceutical companies, institutes, universities and hospitals have an interest in analytical methods for accurate and sensitive qualification and quantification of these substances and / or their metabolite(s). Thus, the development of reliable and sensitive methods using different sample preparation techniques and LC-MS detection is necessary.

The matrix of most of the analyzed samples of human origin is either blood (whole blood, plasma or serum) or urine, making a sample preparation step prior to analysis inevitable. This sample preparation procedure can be either a liquid-liquid extraction (LLE), protein precipitation / protein crash or solid phase extraction; in order to determine the recovery rate of an analyte, an internal standard has to be added to each sample.

EMD Millipore can provide useful tools

- **EXtrelut®** pre-packed columns for extraction of lipophilic compounds from aqueous solutions – for LLE workflows
- Solvents, acids, bases, salts – for protein precipitation
- LiChrolut® product range – for SPE

Depending on the character of the analytes, hydrophobic or hydrophilic stationary phases will be the item of choice. Hydrophobic C8 or C18 modified stationary phases are available, with a monolithic silica column (Chromolith®) being the best choice where proper sample preparation is unwanted / not suitable / not possible for various reasons. These columns provide long column lifetime, have very good matrix tolerability and no frits in the column body; thus matrix rich biological samples (salts, proteins, lipids etc.) can be handled without difficulty. However, when using MS detectors it can be beneficial to remove as many matrix components as possible to prevent ion-suppression and / or ion-enhancement of co-eluting analytes.

With cleaner samples, appropriate sample preparation, and where high separation efficiency / peak capacity is needed, a particulate column such as Purospher® STAR RP-18 endcapped with small particles will deliver best results.

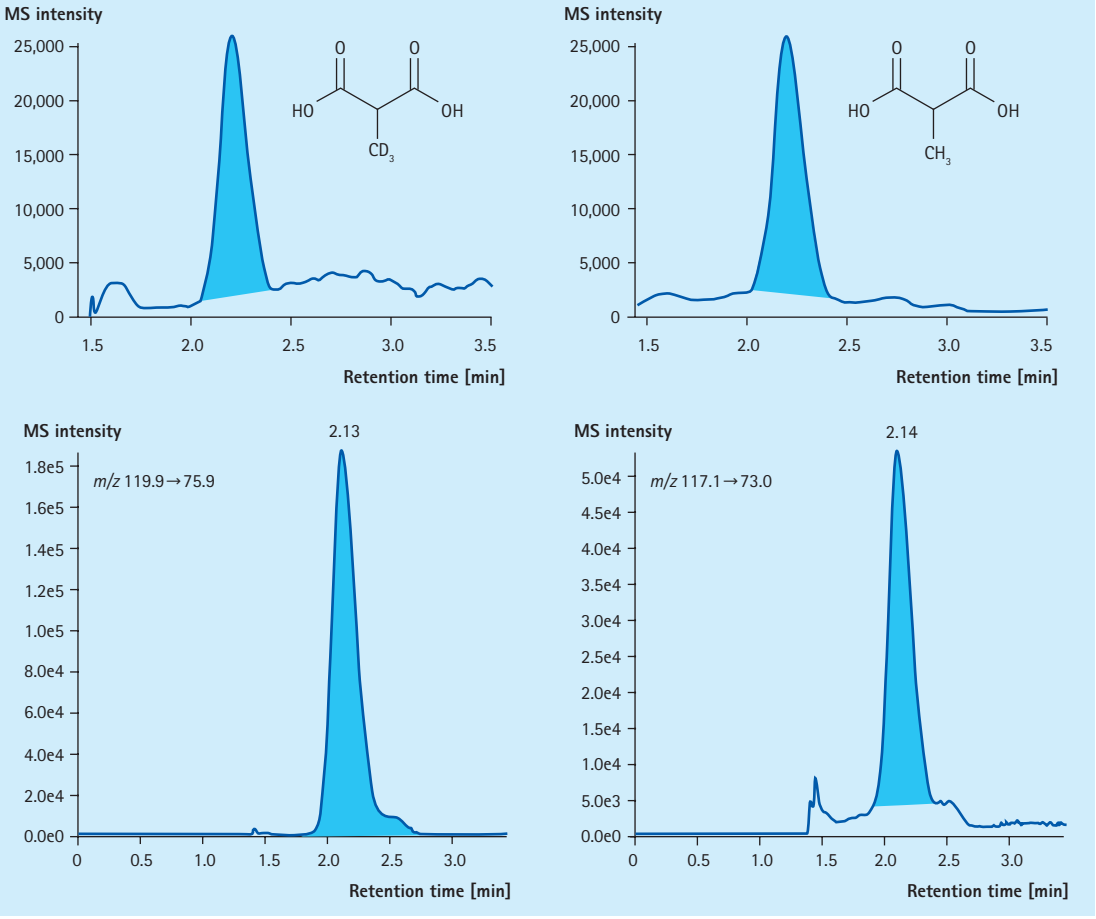
For polar hydrophilic molecules, i.e. the majority of the endogenous molecules, it is more appropriate to use hydrophilic interaction liquid chromatography (HILIC) in general, and bonded zwitterionic stationary phases such as SeQuant® ZIC®-HILIC/cHILIC in particular. Due to the applied solvents and additives HILIC combines perfectly with ESI-MS detection and, in comparison with reversed phase chromatography, a significant increase in sensitivity can be realized.

Two methods were developed utilizing ZIC®-HILIC and reversed phase chromatography for the MS qualification and quantification of the endogenous compounds methylmalonic acid (see page 40 – 41) and vitamin D2 and D3 (see page 42).

Analysis of methylmalonic acid (MMA) in human plasma

This example describes the analysis of methylmalonic acid (MMA) in human plasma via MS and MS/MS detection. The main problems to overcome in method development are related to the low physiological concentrations (100 – 500 nM) of MMA in plasma and the fact that MMA is a hydrophilic non-volatile compound. MMA shows poor retention on RP liquid chromatographic columns and the structural isomer succinic acid (SA) may interfere (ion suppression), since serum concentrations of SA are usually considerably higher than MMA.

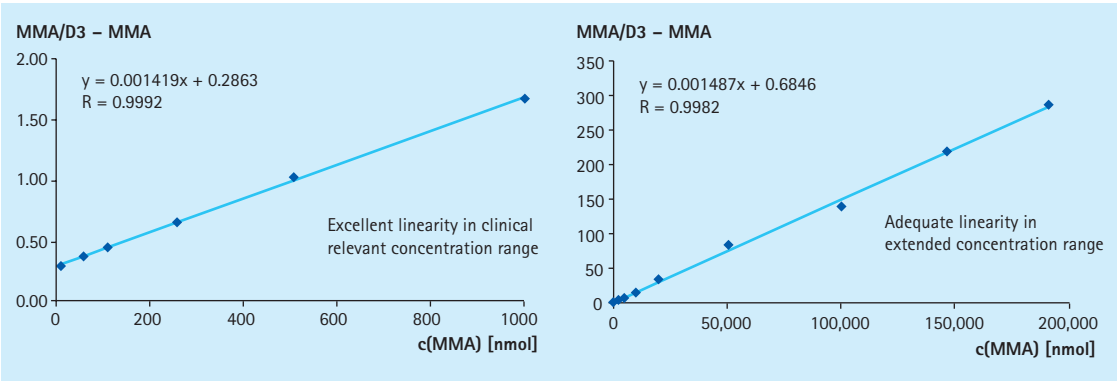
Figure 36



Chromatographic conditions	
Column	SeQuant® ZIC®-HILIC (3.5 µm,100 Å) PEEK 100 x 2.1 mm (**VWR Cat. No. 10144-126**)
Injection volume	4 µL
Detection	Neg. ESI-MS, SIM (LC-MS: m/z 117.2 and 120.2; LC-MS/MS: m/z 117.1→73.0; 117.1→55.1 and 119.9→75.9)
Flow rate	0.4 mL/min
Mobile phase	A: 100 mmol ammonium acetate pH 4.5 in Ultrapure water from water purification system, prepared from ammonia solution (**VWR Cat. No. EM1.05423.1000**) and acetic acid (**VWR Cat. No. EM1.00062.1011**) B: Acetonitrile (**VWR Cat. No. EM1.00029.1000**)
Gradient	0 min 80 % A, 3 min 80 % A, 3.01 min 55 % A (at 0.8 mL/min), 5 min 55 % A (at 0.8 mL/min)
Temperature	25°C
Sample	200 µL of human EDTA or citrate plasma is mixed with 800 µL of protein precipitation solution (mixture of 43 µL of a 196 nM/L D3-MMA stock solution and 250 µL concentrated acetic acid and make up to 50 mL with acetonitrile) and shaken for five minutes. After centrifugation at 6200 rpm for ten minutes at 15°C the supernatant was injected.

LC-MS (top) and LC-MS/MS (bottom) analysis of internal standard D3-methylmalonic acid (left) and methylmalonic acid (right) in human plasma. For the LC-MS/MS quantification experiments the limits of detection and quantitation were 5 and 15 nM, respectively.

Figure 37



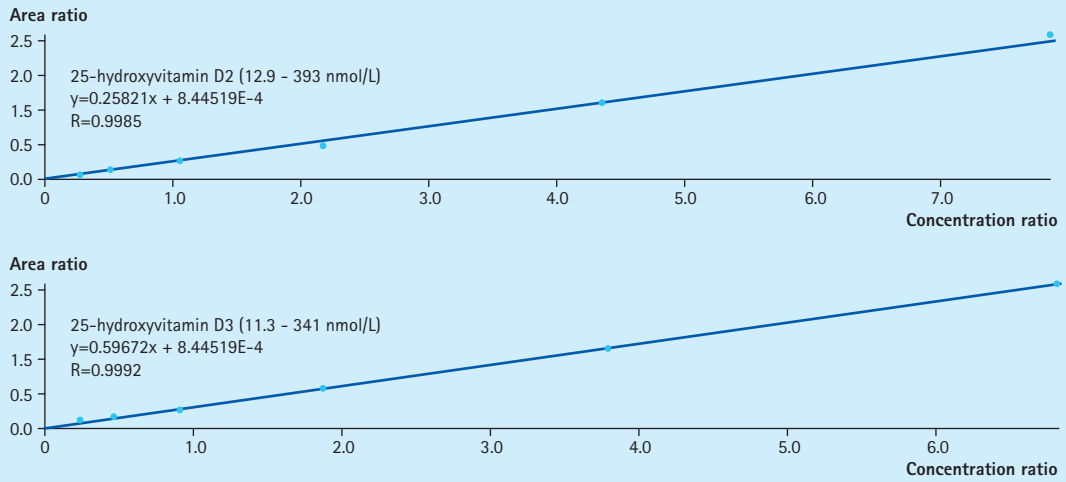
Calibration curves for the quantification of methylmalonic acid in plasma via LC-MS. Limit of detection: 30 nmol, limit of quantitation 90 nmol.
For experimental conditions see figure 36.

As a consequence, many labs use costly methods that require extraction and derivatization steps to yield MMA derivatives that are compatible with GC-MS techniques or reversed phase mode based liquid LC-MS/MS methods, where derivatives of MMA and SA may be differentiated due to different fragmentation patterns. Here a fast and low cost MMA quantification method was developed that combines HILIC with negative ESI-MS detection (Figures 36 and 37). A protein precipitation step including the addition of a deuterated internal MMA standard was used for sample preparation. The developed method allows for a fast and simple sample preparation and subsequent quantification at limits of detection as low as 30 nmol (LC-MS) and 5 nmol (LC-MS/MS).

Analysis and quantification of vitamin D2 / D3

As a second example a method for the analysis and quantification of vitamin D2 / D3 metabolites 25-hydroxy-vitamin D2 / D3 in serum and plasma using reversed phase LC-MS / MS is presented. Samples were prepared via simple protein precipitation, and the two internal standards 2H3-25-OH vitamin D2 / D3 were utilized. As it is not possible to completely eliminate the endogenous levels of 25-OH Vitamin D2 / D3 in human serum / plasma, deuterated 2H6-25-OH Vitamin D2 / D3 are needed. Standard curves were created from standard samples where the ratio of the deuterated 25-OH vitamin D2 / D3 and the two internal standards 2H3-25-OH vitamin D2 / D3 is plotted against the ratio of the concentrations of the same (Figure 38). The result of the analysis of two patient samples is displayed in Figure 39.

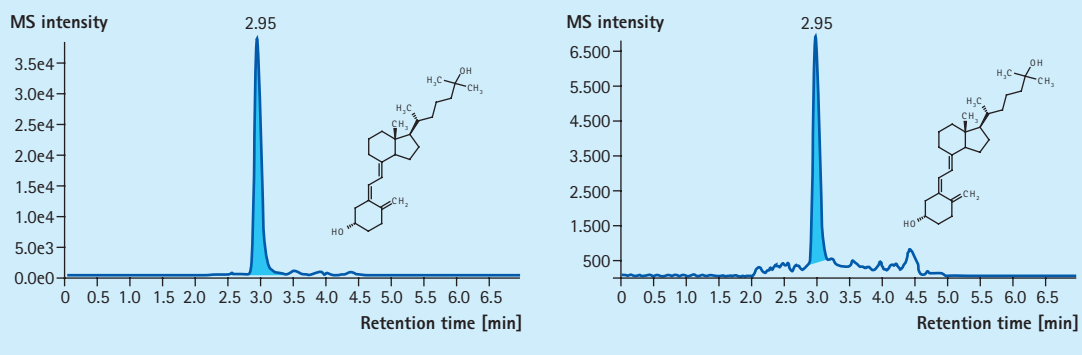
Figure 38



Chromatographic conditions	
Column	Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 100-2.1 (**VWR Cat. No. 97021-983**)
Injection volume	10 µL
Detection	APCI-MS/MS, MRM transitions: m/z 419.3/355.1, 416.3/358.1 (vitamin D2) and m/z 407.3/159.0, 404.3/162.0 (vitamin D3)
Flow rate	0.4 – 0.5 mL/min
Mobile phase	A: Ultrapure water from water purification system B: Methanol LiChrosolv® (**VWR Cat. No. EM1.06035.2500**)
Gradient	0 min 15 % A, 4.50 min 15 % A (both at 0.4 mL/min) 4.51 min 0 % A, 5.53 min 0 % A (both at 0.5 mL/min) 5.55 min 15 % A, 6.5 min 15 % A (both at 0.4 mL/min)
Temperature	50°C
Sample	Patient plasma and serum prepared with commercially available vitamin D kit.

Calibration curves for the quantification of 25-hydroxyvitamin D2 (top) and D3 (bottom) in serum / plasma via LC-MS/MS.

Figure 39



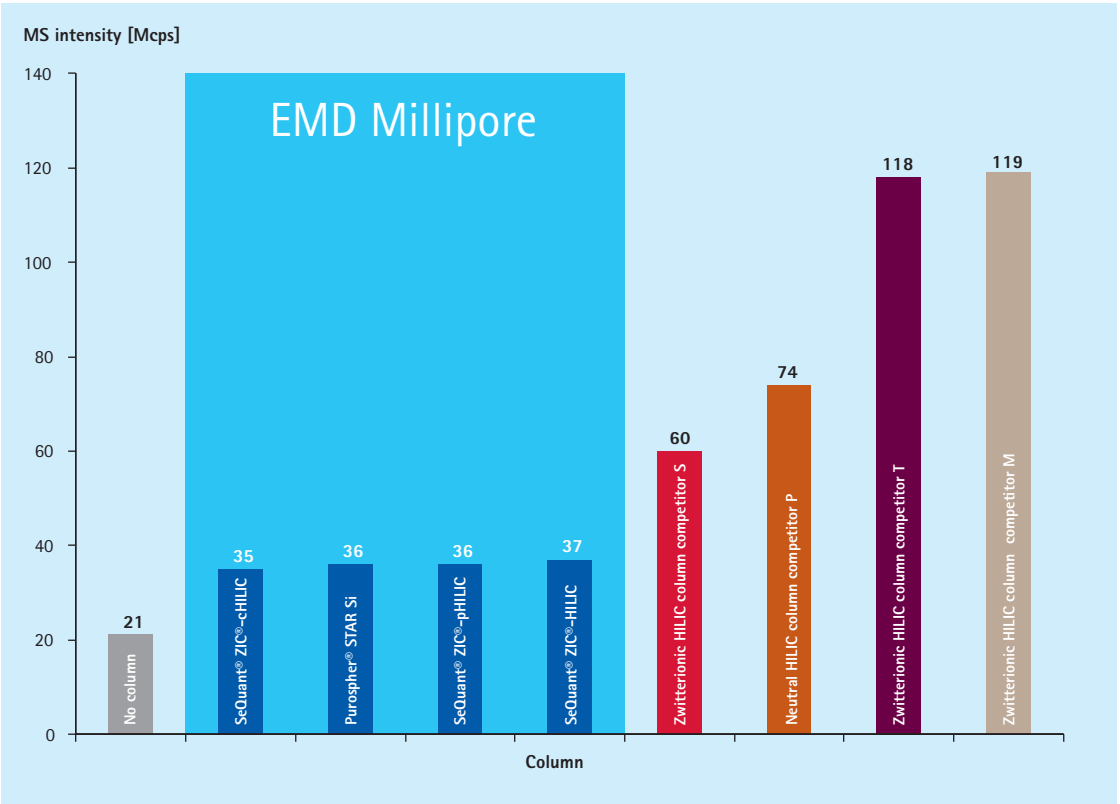
Quantification of the 25-hydroxyvitamin D3 content in two patient serum samples utilizing LC-MS/MS. The vitamin content was 55.7 (left) and 10.0 nmol/L (right), respectively. No 25-hydroxyvitamin D2 was found in these samples. **For experimental conditions see figure 38.**

HILIC column bleeding

All modern particle packed columns from EMD Millipore are based on high purity, low acidity silica that provide excellent peak shape and exhibit low column bleed, the latter a nuisance and major source of background signal in LC-MS analyses. Phase bleed occurs when the bonded phase detaches from the column during the analysis and may cause background noise and signal suppression and interfere with quantification, while increasing MS instrument wear and service costs. Phase bleed may originate from hydrolysis of the bonded phase at low mobile phase pH or due to unsuitable ligand binding. Column bleed prevents sensitive methods being developed.

In Figure 40, the results of column bleeding studies of several commercially available HILIC HPLC columns is shown. Many columns – including several marketed as "zwitterionic" – displayed 70 – 240 % higher column bleed than the EMD Millipore ZIC®-cHILIC columns. A number of bioanalytical LC-MS methods, for both hydrophobic and hydrophilic molecules have recently been worked out. All methods illustrate that when using EMD Millipore particle packed HPLC columns together with MS or MS/MS detection it is easy to develop robust bioanalytical methods where the low column bleed makes it easy to meet the method goals in terms of sensitivity.

Figure 40



Column bleeding as measured by mass spectrometry intensity in the m/z range 20 – 2000 using an eluent with 80 % acetonitrile in neutral ammonium acetate buffer and 100 x 2.1 mm columns at 50°C and 0.1 mL/min. Average of 3 measurements, each during 6 min. All columns were equilibrated 1 – 2 hours before measurement until baseline had stabilized.

List of abbreviations

Abbreviation	Description
ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BPC	base peak chromatogram
CI	chemical ionization
EI	electron ionization
ESI	electrospray ionization
ELSD	evaporative light scattering detector
FIA	flow injection analysis
GC	gas chromatography
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HTS	high throughput screening
ICP	inductively coupled plasma
i.d.	internal diameter
LC	liquid chromatography
LLE	liquid-liquid extraction
MALDI	matrix-assisted laser desorption ionization
MMA	methylmalonic acid
MS	mass spectrometry
SA	succinic acid
SPE	solid phase extraction
TFA	trifluoroacetic acid
TIC	total ion current
TLC	thin layer chromatography
TOC	total organic carbon
UV	ultraviolet
UHPLC	ultra high performance liquid chromatography
ZIC	zwitterionic

Ordering information

Product group	Product name	VWR Cat. No.
Chromolith® Columns		
	Chromolith® CapRod® RP-18 endcapped 150-0.1 mm capillary column	**EM1.50402.0001**
	Chromolith® FastGradient RP-18 endcapped 50-2 mm HPLC column	**97007-930**
	Chromolith® Flash RP-18 endcapped 25-2 mm HPLC column	**EM1.52014.0001**
	Chromolith® Performance RP-18 endcapped 100-3 mm HPLC column	**82031-440**
	Chromolith® Performance RP-18 endcapped 100-2 mm HPLC column	**97025-820**
Purospher® Columns		
	Purospher® STAR Phenyl (2 µm) Hibar® HR 50-2.1 mm UHPLC column	**10811-824**
	Purospher® STAR Phenyl (2 µm) Hibar® HR 100-2.1 mm UHPLC column	**10811-826**
	Purospher® STAR RP-8 endcapped (2 µm) Hibar® HR 50-2.1 mm UHPLC column	**10811-820**
	Purospher® STAR RP-8 endcapped (2 µm) Hibar® HR 100-2.1 mm UHPLC column	**10811-818**
	Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 50-2.1 mm UHPLC column	**97021-980**
	Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 100-2.1 mm UHPLC column	**97021-983**
	Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 150-2.1 mm UHPLC column	**EM1.50649.0001**
	Purospher® STAR RP-18 endcapped (3 µm) Hibar® HR 150-2.1 mm UHPLC column	**97021-986**
Sample Preparation		
	EXtrelut® NT 20 pre-packed columns for extraction of lipophilic compounds from aqueous solutions (20 mL sample)	**EM1.15096.0001**
	LiChrolut® RP-18 E (40 – 63 µm) 500 mg 3 mL standard PP-tubes 50 extraction tubes per package	**48219-220**
SeQuant® HILIC Columns		
	ZIC®-cHILIC (3 µm, 100 Å) PEEK 50 x 2.1 mm metal-free HPLC column	**52428-720**
	ZIC®-cHILIC (3 µm, 100 Å) PEEK 100 x 2.1 mm metal-free HPLC column	**52428-722**
	ZIC®-cHILIC (3 µm, 100 Å) PEEK 150 x 2.1 mm metal-free HPLC column	**52428-724**
	ZIC®-cHILIC (3 µm, 100 Å) 150 x 0.3 mm capillary HPLC column	**52428-742**
	ZIC®-cHILIC (3 µm, 100 Å) 150 x 1 mm capillary HPLC column	**52428-710**
	ZIC®-HILIC (3.5 µm, 100 Å) PEEK 50 x 2.1 mm metal-free HPLC column	**10144-124**
	ZIC®-HILIC (3.5 µm, 100 Å) PEEK 100 x 2.1 mm metal-free HPLC column	**10144-126**
	ZIC®-HILIC (3.5 µm, 100 Å) PEEK 150 x 2.1 mm metal-free HPLC column	**10144-128**
	ZIC®-HILIC (3.5 µm, 200 Å) PEEK 150 x 0.3 mm metal-free HPLC column	**10144-146**
	ZIC®-HILIC (3.5 µm, 200 Å) PEEK 150 x 1 mm metal-free HPLC column	**10144-148**
TLC / HPTLC MS-grade Plates		
	HPTLC silica gel 60 F_{254} MS-grade for MALDI 20 Aluminum sheets 5 x 7.5 cm	**10755-326**
	HPTLC silica gel 60 F_{254} MS-grade 25 glass plates 20 x 10 cm	**10755-322**
	HPTLC silica gel 60 RP-18 F_{254}s MS-grade 25 glass plates 20 x 10 cm	**10755-328**
	TLC silica gel 60 F_{254} MS-grade 25 glass plates 20 x 20 cm	**10755-320**

Product group	Product name	VWR Cat. No.
LiChrosolv® Solvents		
	Acetonitrile hypergrade for LC-MS LiChrosolv®	**EM1.00029.2500**
	Methanol hypergrade for LC-MS LiChrosolv®	**EM1.06035.2500**
	2-Propanol gradient grade for liquid chromatography LiChrosolv®	**EM1.01040.4000**
	Toluene for liquid chromatography LiChrosolv®	**EM1.08327.1000**
	Water for chromatography LiChrosolv® (LC-MS)	**EM1.15333.2500**
SupraSolv® GC-MS Solvents		
	Acetone for gas chromatography MS SupraSolv®	**EM1.00658.2500**
	Acetonitrile for gas chromatography MS SupraSolv®	**EM1.00665.2500**
	Cyclohexane for gas chromatography MS SupraSolv®	**EM1.00667.2500**
	Dichloromethane for gas chromatography MS SupraSolv®	**EM1.00668.2500**
	Ethyl acetate for gas chromatography MS SupraSolv®	**EM1.00789.2500**
	n-Hexane for gas chromatography MS SupraSolv®	**EM1.00795.2500**
	Methanol for gas chromatography MS SupraSolv®	**EM1.00837.2500**
	Toluene for gas chromatography MS SupraSolv®	**EM1.00849.2500**
Ultrapur® Inorganic Acids and Reagents		
	Hydrochloric acid 30 % Ultrapur®	**EM1.01514.0500**
	Hydrogen peroxide solution 31 % Ultrapur®	**EM1.06097.1000**
	Nitric acid 60 % Ultrapur®	**EM1.01518.1000**
	Sulfuric acid 96 % Ultrapur®	**EM1.01516.0250**
	Water Ultrapur®	**EM1.01262.1000**
Suprapur® Inorganic Acids and Bases		
	Acetic acid (glacial) 100 % Suprapur®	**EM1.00066.0250**
	Ammonia solution 25 % Suprapur®	**EM1.05428.1000**
	Formic acid 98 – 100 % Suprapur®	**EM1.11670.1000**
	Hydrochloric acid 30 % Suprapur®	**EM1.00318.1000**
Other Reagents		
	Acetic acid (glacial) 100 % anhydrous for analysis EMSURE® ACS, ISO, Reag. Ph Eur	**EM1.00063.2500**
	Ammonia solution 28 – 30 % for analysis EMSURE® ACS, Reag. Ph Eur	**EM1.05423.2500**
	Ammonium acetate for analysis EMSURE® ACS, Reag. Ph Eur	**EM1.01116.1000**
	Formic acid 98 – 100 % for analysis EMSURE® ACS, Reag. Ph Eur	**EM1.00264.1000**
	2-Propanol for analysis EMSURE® ACS, ISO, Reag. Ph Eur	**EM1.09634.2500**
ICP-MS Multi Element Standards		
	ICP Multi-element standard USP-I according to USP <232> oral dose As, Cd, Cu, Hg, Mo, Ni, Pb, V Certipur®	**EM5.05101.0100**
	ICP Multi-element standard USP-II As, Cd, Cu, Hg, Mo, Ni, Pb, V Certipur®	**EM5.05102.0100**
	ICP Multi-element standard USP-III 100 mg/L: Ir, Os, Pd, Pt, Rh, Ru Certipur®	**EM5.05103.0100**
	ICP Multi-element standard USP-IV 10 mg/L: Ir, Os, Pd, Pt, Rh, Ru Certipur®	**EM5.05104.0100**
	ICP Multi-element standard USP-V according to USP <2232> dietary supplements As, Cd, Hg, Pb Certipur®	**EM5.02232.0100**
	Multi-element standard VI, calibration in ICP MS	**EM1.10580.0100**
	Multi-element standard XXI, calibration in ICP MS	**EM1.09498.0001**

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