

# Strategies for Coupling of Nonvolatile Salt-Based HPLC to MS Detection for the Separation and Characterization of Related Substances in Biopharmaceuticals

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## On-Line LC-UV-SPE-MS

An important task in the pharmaceutical development process is the identification of impurities in drug substances and products. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has published guidelines on this topic. These guidelines require that impurities be identified when present above concentration limits of 0.05 and 0.10%, depending on the daily dose.<sup>1</sup>

The sensitivity and specificity of mass spectrometry (MS) are ideally suited for the identification of impurities. However, LC methods for separating pharmaceuticals that use mobile phases containing buffers are often incompatible with MS detection. Development of new LC methods is often expensive and time consuming. Validation with regulatory agencies is another barrier to implementing new methods. Solid-phase extraction (SPE) of buffers from the mobile phase after separation is a time- and cost-effective alternative to new method development.

Here, the authors describe an automated and hyphenated LC method for the identification of pharmaceutical compounds. An SPE desalting step is applied prior to sample introduction to the MS detector, removing buffers from the eluent stream. This allows current methods to be used with MS detection without new method development. On-line LC-UV-SPE-MS offers several advantages:

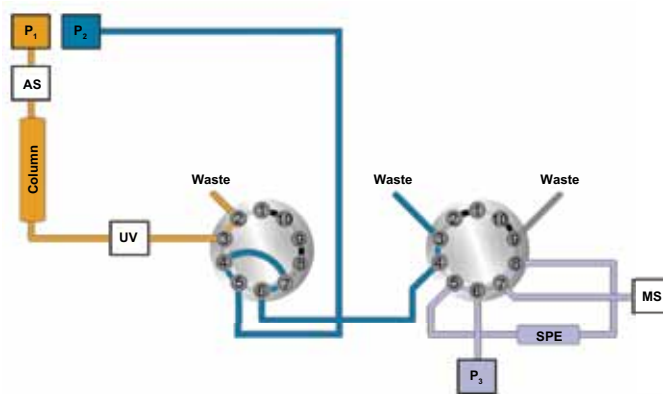
- Completely automated method facilitates method validation.
- Original (filed) HPLC method can be used.
- LC and MS sensitivity can be optimized independently.
- UV detection can be used for quantification of related compounds/impurities.
- SPE column can be used for the removal of interfering buffer salts and for preconcentration (multiple loadings).

The applicability of this system is demonstrated for recombinant human insulin and ribonuclease A, separated by reversed-phase and ion-exchange chromatography, respectively.

## System Configuration for On-Line LC-UV-SPE-MS

A Thermo Scientific Dionex UltiMate™ 3000 ×2 Dual-Gradient HPLC system comprising two integrated gradient pumps was used. Pump 1 was used for separation on the analytical column. Pump 2 was used to transfer the impurities to the SPE column and for desalting on the SPE column. An isocratic pump was used for elution of the impurities from the SPE column to the mass spectrometer. The configuration for on-line LC-UV-SPE-MS is shown in Figure 1. MS detection was performed with a Bruker HCTultra™ Ion Trap. Thermo Scientific Dionex Chromeleon™ Chromatography Data System software 6.8 was used to control the HPLC.

FIGURE 1. Configuration for on-line LC-UV-SPE-MS.



The experimental workflow includes the following steps:

- Separation of the analytes
- Trapping of related compound in loop
- Transfer of related compound to SPE column and removal of salts
- Elution of related compound to MS

The separation is monitored with UV detection. Selection of the related compound can be performed on retention time or by using a peak trigger based on the UV signal. The latter option has the advantage that retention time variation is accounted for.

The LC and SPE systems are linked via a two-position switching valve with or without an additional sample loop to trap the related compound. The concept of in-loop trapping (column → trapping loop → SPE column → MS) features some advantages compared to in-line trapping (column → SPE column → MS). The chromatographic method is independent of the SPE wash step and elution to the mass spectrometer. Also, the backpressure on the SPE column does not influence the LC performance (possible shift in retention times). In addition, this configuration maintains low backpressure on the UV flow cell as well as a low system pressure (see left valve, Figure 1). However, the loop dimensions must be carefully selected.

## USP HPLC Method for Related Compounds of Recombinant Insulin

Sample:	Recombinant human insulin 3.75 mg/mL in 0.01 M HCl	
Analytical Column:	Thermo Scientific Acclaim™ 120, C18, 3 μm, 4.6 × 150 mm	
SPE Column:	Oasis® 2.1 × 50 mm, 15 μm	
Solvents:	(A) 0.2 M sodium sulfate, pH 2.3/acetonitrile 82/18 (v/v %) (B) 0.2 M sodium sulfate, pH 2.3/acetonitrile 50/50 (v/v %)	
Gradient:	<i>Time (min)</i>	<i>% B</i>
	0	24.5
	36	24.5
	61	64.0
	67	64.0
	68	24.5
	91	24.5
Make-Up Liquid:	Water	
SPE Elution:	80/20 acetonitrile/H <sub>2</sub> O + 1% formic acid	

Flow Rates:	Analytical column: 1.00 mL/min Make-up: 0.50 mL/min Elution from SPE column: 0.05 mL/min
Detection:	UV: 214 nm, 280 nm MS: 300–2000 <i>m/z</i>

## Ion-Exchange HPLC Separation of Ribonuclease A

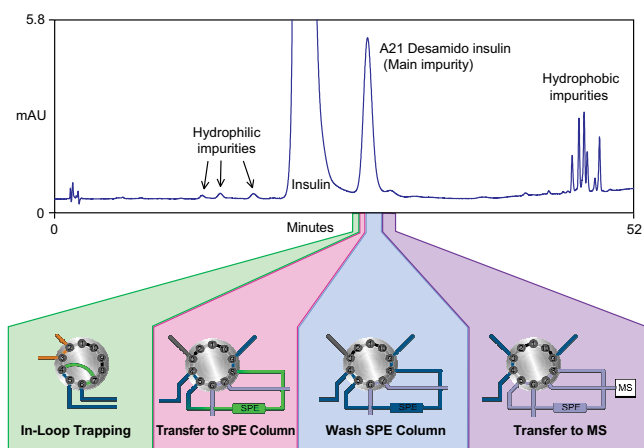
Sample:	Approximately 0.35 mg/mL Ribonuclease A (1.75 μg injected)
Analytical Column:	Thermo Scientific ProPac™ SCX-10, 2 × 250 mm
SPE Column:	Thermo Scientific Acclaim PepMap™ 300 C18, 5 μm, 1.0 × 15 mm
Solvents:	(A) 20 mM MES + 60 mM NaCl, pH 5.6 (B) 20 mM MES + 180 mM NaCl, pH 5.6
Gradient:	55–85% B in 50 min
SPE Elution:	80/20 acetonitrile/H <sub>2</sub> O + 1% formic acid
Flow Rates:	Analytical column: 0.20 mL/min Make-up flow rate: 0.50 mL/min Elution from SPE column: 0.05 mL/min

## Results and Discussion

### Reversed-Phase HPLC Separation of Related Compounds of Recombinant Insulin

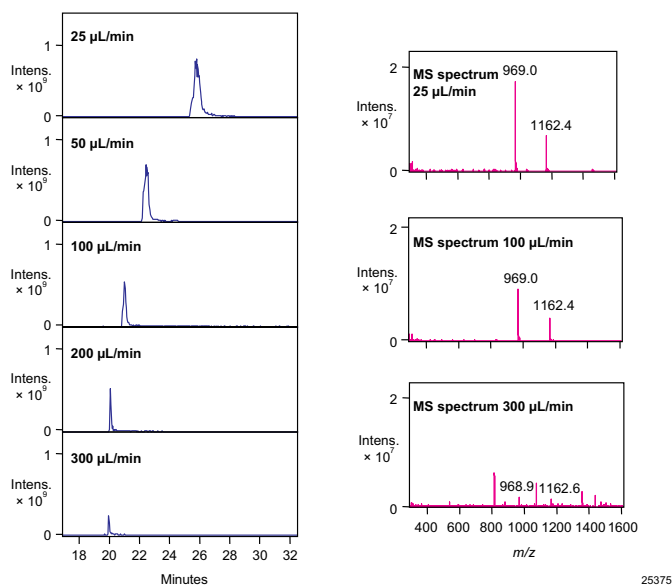
Human insulin, a 5.7 kDa polypeptide, is used to treat some forms of diabetes mellitus and is mainly produced through recombinant techniques. One of the release tests of human insulin is the quantification of related compounds. The United States Pharmacopeia (USP) has published a reversed-phase LC (RPLC) method for determining insulin and related impurities. A typical chromatogram is shown in Figure 2.

**FIGURE 2. Separation of human insulin and compounds according to the USP method.**



The influence of elution flow rate on MS detection of insulin and related compounds was studied. The resulting extracted ion chromatograms and MS spectra are shown in Figure 3. A significantly improved detection sensitivity was obtained with low elution flow rates. Depending of the amount of related compounds transferred to the SPE column, the flow rate can be adjusted to obtain sufficient MS signal with a reasonable analysis time. If the sensitivity is not sufficient applying this standard procedure, the related compound can be trapped on the SPE column multiple times before elution to the mass spectrometer.

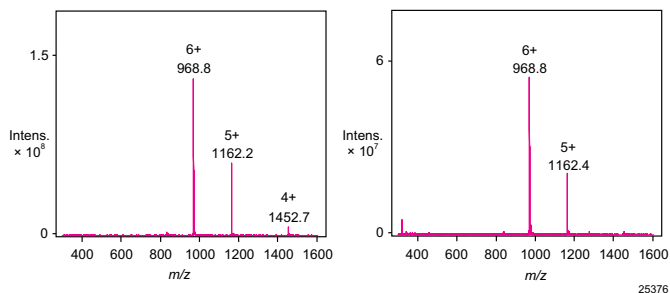
**FIGURE 3. Influence of SPE column elution flow rate on the mass spectrometer signal. Extracted ion chromatograms (EIC) of 969.0 m/z (left) and MS spectra summed across peak for 25, 100, and 300 µL/min elution flow rates (right) with 80/20 acetonitrile/H<sub>2</sub>O + 1% formic acid.**



The recovery of insulin was determined using a 0.1 mg/mL standard injected onto the SPE system with and without an analytical LC column installed. UV detection was applied to quantify the insulin peak. The recovery was 95%, which is sufficient for this qualitative method. The peak area precisions for insulin on the analytical column and SPE column were 0.10 and 3.5 %RSD, respectively.

One of the known modifications of insulin is deamidation of asparagine residues, forming an iso-aspartic acid residue. As a result of this modification the molar mass is increased by one unit. Insulin and A21 desamido insulin were trapped, desalted, and detected by MS. The resulting MS spectra are shown in Figure 4. The deconvoluted masses for insulin and desamido insulin were 5806.6 and 5807.3 Da, respectively.

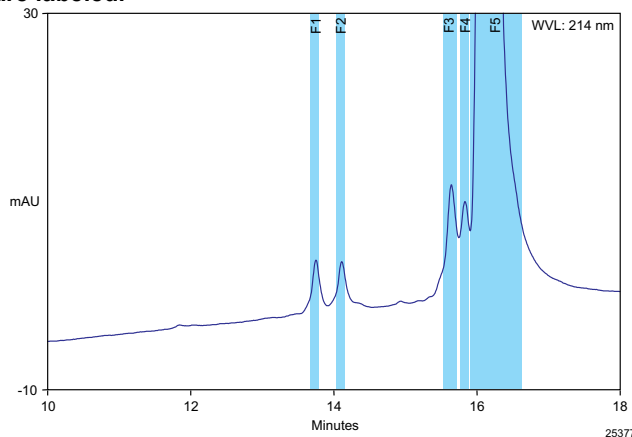
**FIGURE 4. MS spectra of insulin (left) and A21 desamido insulin (right).**



### Ion-Exchange HPLC Separation of Ribonuclease A and Variants

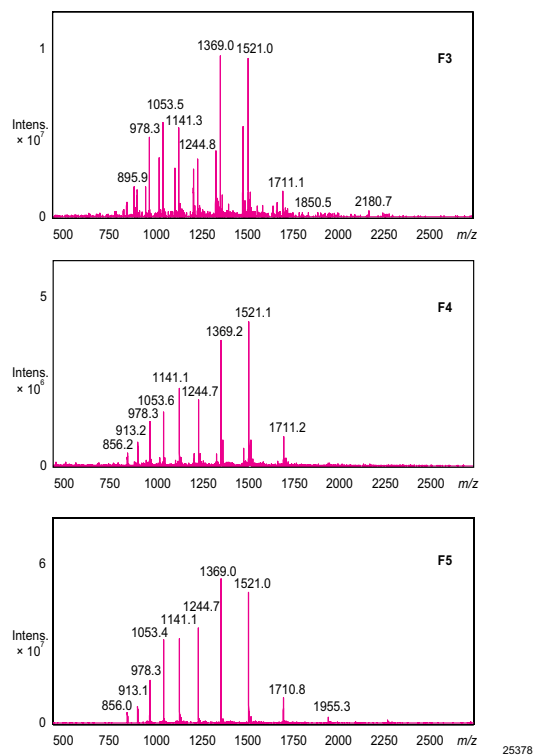
Ion-exchange chromatography is becoming more important as an LC technique for the characterization of biopharmaceutical proteins. For the separation of protein variants which often bear different charges, ion-exchange LC is often more selective than reversed-phase HPLC. The application of on-line LC-UV-SPE-MS for ion-exchange chromatography of intact proteins was tested with the analysis of ribonuclease A, a 124 residue, ~13.7 kDa protein. The chromatogram of ribonuclease A is shown in Figure 5.

**FIGURE 5. UV chromatogram of ribonuclease A on the ProPac SCX column. Collected fractions F1 through F5 are labeled.**



The MS spectra of selected protein variants and ribonuclease A are shown in Figure 6. One of the protein variants shows a mass difference of 98 Da with ribonuclease. This could be the result of an additional phosphorylation site.

**FIGURE 6. MS spectra of ribonuclease A (F5) and two variants eluting at 15.6 and 15.8 min in Figure 5 (F3 and F4). In all spectra, ribonuclease A was detected with a deconvoluted mass of Mw 13680.2. Protein variants of 13422.1 and 13777.7 Da were detected for LC fractions F3 and F4 with the aforementioned retention times.**



## Conclusion

On-Line LC-UV-SPE-MS is a useful technique for identification of impurities and product related compounds using nonMS compatible HPLC methods. The original (filed/validated) methods can readily be applied without the need for method redevelopment and revalidation.

The technique shows excellent performance for RPLC and ion-exchange LC methods and is applicable for (bio) pharmaceutical products (small molecules as well as therapeutic proteins) up to large intact proteins. The method is robust and shows good recovery and precision. In-loop analyte trapping triggered by UV signal provides the highest robustness and flexibility.

## Reference

1. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, *ICH Harmonised Tripartite Guideline Impurities In New Drug Substances Q3A(R2)*, Current Step 4 Version, 25 October 2006, [http://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Quality/Q3A\\_R2/Step4/Q3A\\_R2\\_Guideline.pdf](http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3A_R2/Step4/Q3A_R2_Guideline.pdf) (accessed Jun 27, 2011).

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