Analytical to Preparative HPLC Method Transfer

An easy way to scale up from UHPLC to preparative HPLC using focused gradients

Technical Overview

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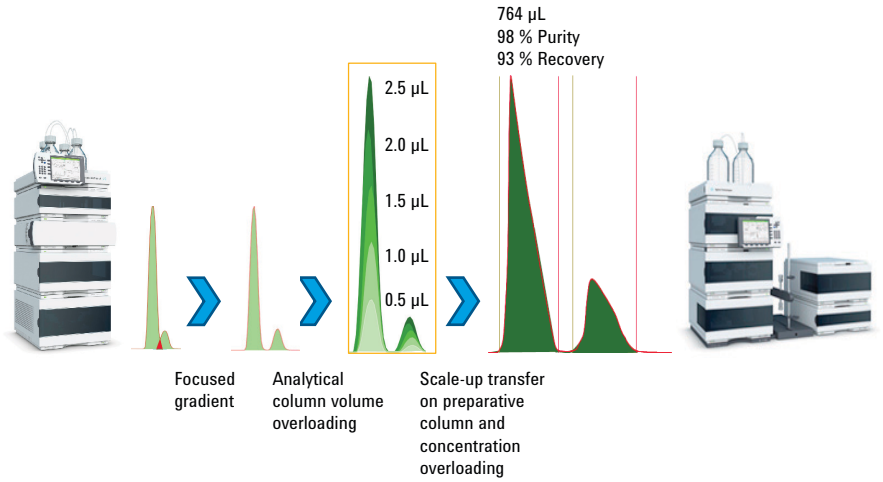
Abstract

Synthesizing novel compounds or isolating natural products can be a laborious and time-consuming process. After analyzing the precious sample on an analytical UHPLC system, the crucial step is to transfer the method to a preparative system with a minimal risk of losing valuable work or collecting impure compounds.

This Technical Overview describes a practical way to optimize the scale-up process for reversed-phase chromatography from an analytical UHPLC system to preparative LC systems using a focused gradient to increase the sample load on the preparative column to achieve optimum purity.



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Introduction

Generic gradients are suitable to cope with a large variety of sample types when there is no capacity or time to optimize the separation. Each gradient can be divided into four different steps. After the injection, an isocratic hold step can be applied to remove the injected solvent from the column and to improve resolution especially for polar compounds. The second step is a linear slope, which will be applied to separate effectively based on the chromatographic properties of the target compounds, followed by a purge phase. In the last step, the column is re-equilibrated at the initial solvent composition for the next sample analysis or purification run. To improve resolution around the target compound, the linear slope has to be modified.

In preparative chromatography, most often the goal is to isolate, efficiently, a large amount of one or a few target compounds out of a crude mixture. Ideally, the chromatographic resolution around the target peak and the column load are increased without significantly increasing the separation runtime. From the crude sample, an optimized method can be generated with the goal to extend the resolution between the target peak (green peak, Figures 1 and 2) and its neighbor compounds.

A basic approach to generate optimized preparative methods can be to divide the linear generic gradient method into time slices (described in the Technical Note 5991-3070EN[1]). This approach generates a set of preparative methods that can be used in any further sample purification simply by identifying the time slice where the target peak elutes.

In this Technical Overview, an approach with focused gradients on target peak was used. This approach generates then a unique and dedicated method to a concerned target peak which has the advantage to increase the resolution better than the time slices method.

All optimization steps occur on the analytical system. After obtaining the first chromatographic information of the crude mixture by using a generic gradient, the resolution is optimized by flattening the slope followed by a loading study to determine the maximum column load before scaling up to the preparative column dimensions.

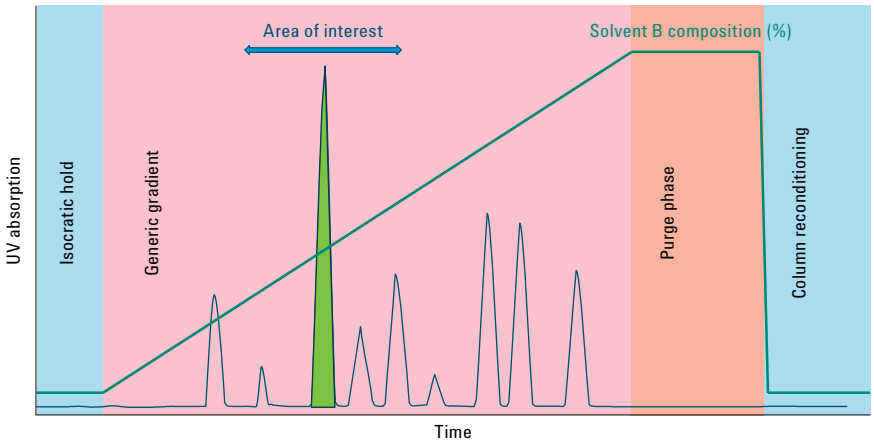

Figure 1. Schematic view of a generic gradient with the desired product peak (highlighted in green).

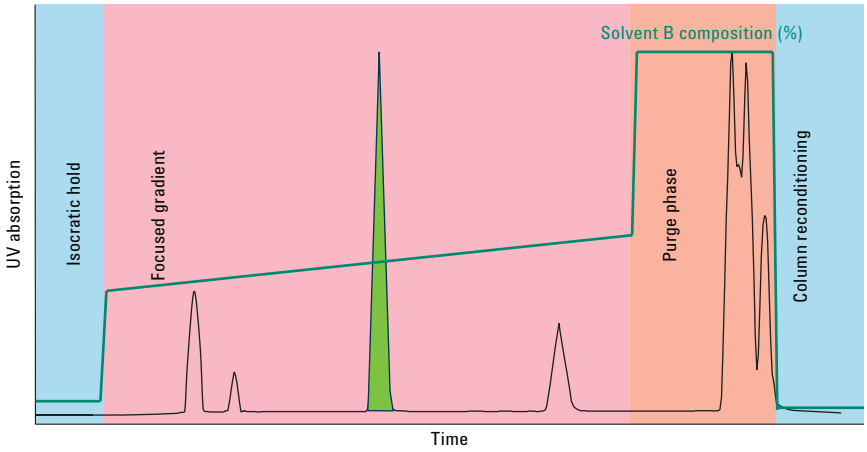

Figure 2. Schematic view of a focused gradient.

Experimental

Instrumentation

Analytical System

Agilent 1290 Infinity Binary LC:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler with Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316B)
- Agilent 1290 Infinity Diode-Array Detector, including a Max-Light standard cartridge cell with 10-mm path length, V(σ) 1 µL (G4212A)

Preparative System

Agilent 1260 Infinity Preparative-scale Preparative System:

- Agilent 1260 Infinity Preparative Binary Pumps (G1361A, G1391A)
- Agilent 1260 Infinity Preparative Autosampler (G2260A)
- Agilent 1260 Infinity Multiple Wavelength Detector (G1365D) equipped with a Quartz flow cell 0.06-mm path length for MWD (G1365D#026)
- Column organizer module (G1383A)
- Agilent 1260 Infinity Fraction Collector PS (G1364B)

Columns

- Agilent ZORBAX RRHD SB-C18, 2.1 × 50 mm, 1.8 µm (857700-902)
- Agilent ZORBAX SB-C18, Prep HT Cartridge 21.2 × 150 mm, 5 µm (870150-902) with end fittings (820400-901)

Software

Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems, Rev. C.01.05 [36]

Solvents and Samples

Solvent A
Water + 0.1 % formic acid

Solvent B
Acetonitrile + 0.1 % formic acid

Purification mixture for preparative runs
O-acetyl salicylic acid (0.07 g/mL)
Salicylic acid (0.175 g/mL) in DMSO:acetonitrile 3:1

Purification mixture for analytical runs (dilution by 50 in water:acetonitrile 1:2)
O-acetyl salicylic acid (1.4 µg/µL),
Salicylic acid (3.5 µg/µL)

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak)

Results and Discussion

A multistep process has been developed that ensures consistent purification results. The first step is only done once for a purification system. The further steps are to be repeated for each sample.

Step 1

Determination of dwell volume and column volume

The scale-up process starts with a characterization of all void volumes from the analytical and the preparative systems.

The total system void volume (dwell volume, column volume, and extra column volumes caused by the flow path after the column) has to be determined first on the analytical and the preparative LC system.

These void volumes generate a delay between the programmed gradient and the effective gradient (Figure 3). To determine the effective solvent composition at a certain retention time of a compound, for example, the percentage of organic solvent, in this example corresponding to 50 %, needs to be corrected due to the dwell volume (153 µL for the 1290 Infinity Binary LC) and the column volume (147 µL for this ZORBAX RRHD 2.1 × 50 mm, 1.8 µm column). For the desired compound shown, Figure 3, the effective elution composition, or elution point of the sample corresponds to 30 % of solvent B.

If the dwell volume of the system is not known, it can be measured using the following procedure:

1. Prepare Solvent A:
   25 % water/75 % acetonitrile.
2. Prepare Solvent B:
   25 % water/74 % acetonitrile/
   1 % acetone.

On the analytical system/flow path

3. Run a linear gradient from 5–95 % B in 10 minutes at the usual flow rate used on the analytical column (for example 1 mL/min) at 270 nm detection wavelength.
4. Replace the column by a zero dead volume connection.
5. Run the same gradient at the same flow rate as in the previous run at 270 nm detection wavelength.
6. Calculate the time differences at 50 % of the maximum absorption, between the programmed gradient and the obtained signal with a bypass (t_{Dwell}), and between the signals obtained with bypass and with column (t_{Column}).
7. The Dwell volume is then:
   $V_{Dwell} = t_{Dwell} \times Flow$,
   and the column volume is:
   $V_{Column} = t_{Column} \times Flow$.

On the preparative flow path/system

8. Repeat Steps 3 to 7 on the preparative flow path using the typical preparative flow rate (for example, 25 mL/min for a 21.2 mm id preparative column).

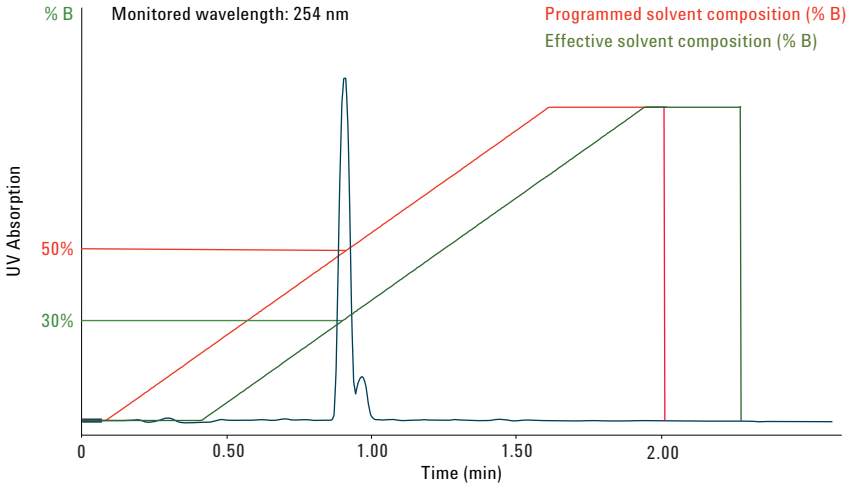

Figure 3. Sample mixture with the programmed solvent composition (red), and the effective gradient composition (blue) after consideration of the system void volume.

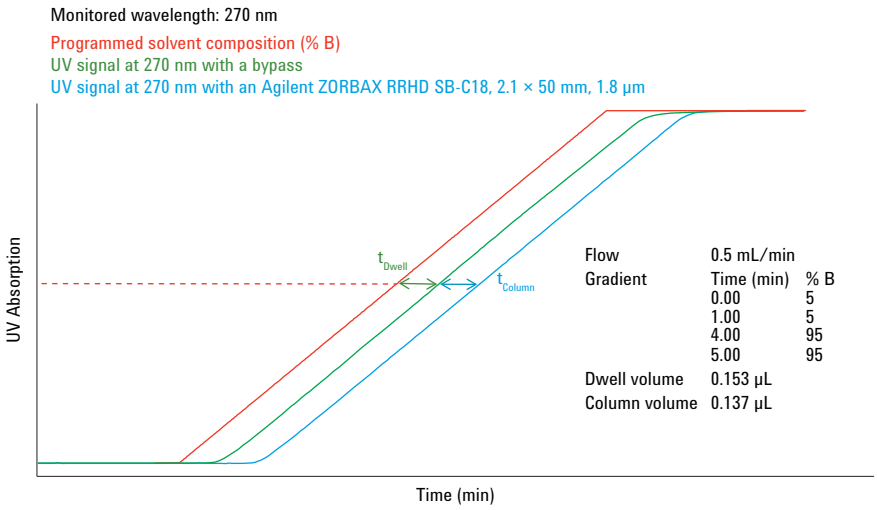

Figure 4. Characterization of the analytical system: Overlays of the programmed solvent composition (red), UV signal of the tracer with a bypass (blue) and the signal of the tracer with column (green).

Steps 2 and 3

Analyzing the crude mixture by a generic gradient and generation of a focused gradient

The virtual elution point is defined as the percentage of solvent B determined at which the elution of a compound occurs in the applied gradient and by taking into consideration the void volume of the system.

After the elution point of a target compound has been calculated, an area around this peak is defined where a linear gradient with a flat slope will be applied. Table 1 gives typical slopes which can be used for the focused gradients:

The elution point of the target peak in this example has been identified at 30 % (Figure 3). A new linear gradient around the elution point from 25 % to 35 % organic solvent with a slope of 20 %/min can be applied to improve the resolution of the target peak.

The example presented in Figure 5 shows the target peak (main peak on both analytical chromatograms) with a non-baseline separated neighbor compound using a generic gradient (upper chromatogram). The lower chromatogram shows a baseline separation after the focused gradient has been applied. The runtime has been reduced by a factor of two.

Table 1. Typical slopes for focused gradients.

Column lengths	50	100	150	250
Slope (%B/min)	10-20	5-10	3-6	2-4

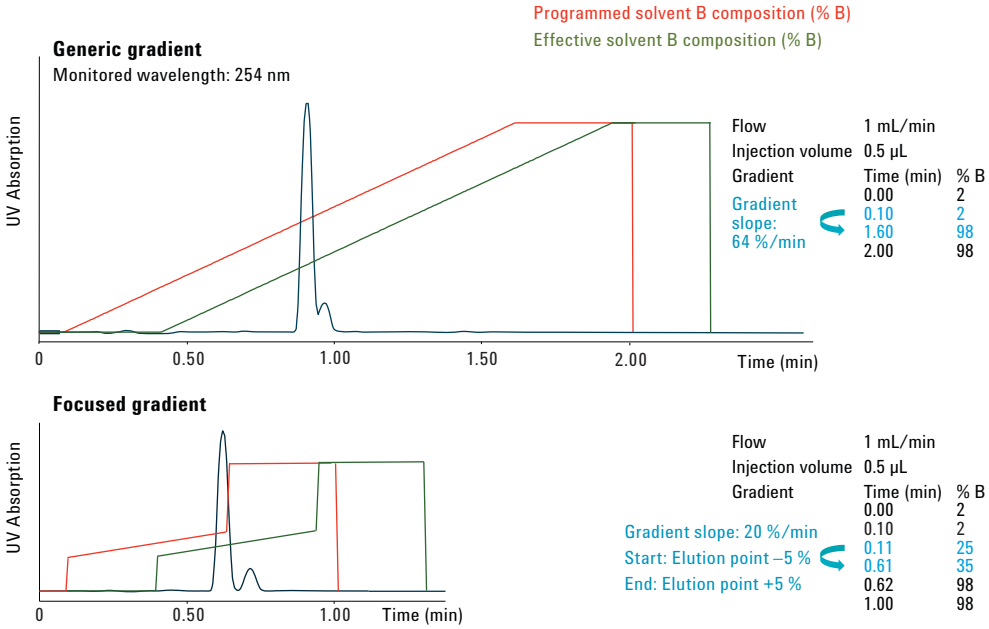

Figure 5. Initial generic gradient (above), and focused gradient on target peak (below).

Step 4

Loading study

After identification of the optimal method, a loading study was performed to determine the maximum column load without losing the chromatographic resolution. The maximum injection volume on the analytical column was determined by increasing the injection volume stepwise (Figure 6).

In this example, the maximum injection volume was experimentally determined to be 2.5 µL on the ZORBAX SB C-18, 2.1 mm column, corresponding to a pure target compound loading mass of 133 mg on the preparative column (a scale-up from 0.5 µL of injection volume would allow purifying 26.6 mg of pure target compound).

Step 5

Application of the method transfer formulas[2]

The method transfer formulas between HPLC and UHPLC systems are well known from literature. It is an equivalent process when performing a sample analysis of the crude mixture on a UHPLC column and transfer the results to a much larger column dimension for purification. For this method transfer, the goal was to retain the resolution and the column load from the analytical run. A method transfer without complicated formulas can also be performed, but the gradient duration and the injection volumes are parameters that will change the resolution of the target peak from the other substances and might cause collection of impure fractions.

For this purpose, the method transfer formulas could be applied between the analytical and preparative systems. The Table 2 describes the two system characteristics which will be applied on the method transfers.

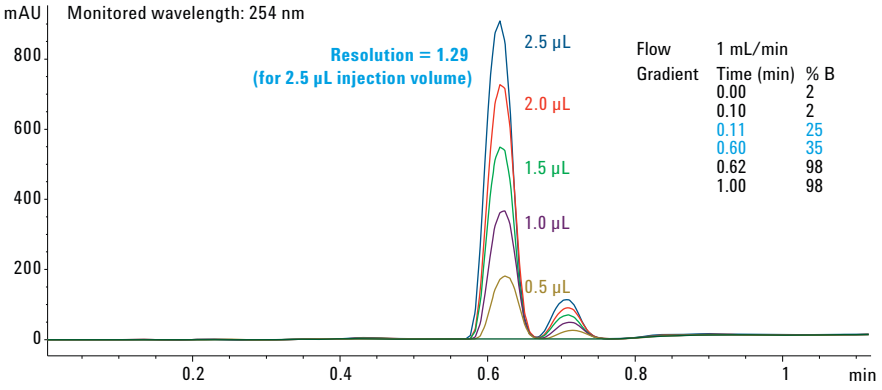

Figure 6. Column overloading study: The maximum loading has been determined here by an increment of 0.5 µL per injection.

Flow rate calculation: $$Flow_{PREP} = Flow_{ANA} \times \left(\frac{D_{PREP}}{D_{ANA}}\right)^2 \times \left(\frac{D_{part,\,ANA}}{D_{part,\,PREP}}\right) \quad (a)$$

Isocratic hold: $$T_{ini,\,PREP} = \left(T_{ini,\,ANA} + \frac{Dwell_{ANA}}{Flow_{ANA}}\right) \times \left(\frac{L_{PREP,\,col}}{L_{ANA,\,col}}\right) \times \left(\frac{D_{part,\,PREP}}{D_{part,\,ANA}}\right) - \frac{Dwell_{PREP}}{Flow_{PREP}} \quad b)$$

Segment duration: $$T_{grad,\,PREP} = T_{grad,\,ANA} \times \left(\frac{L_{PREP,\,col}}{L_{ANA,\,col}}\right) \times \left(\frac{D_{part,\,PREP}}{D_{part,\,ANA}}\right) \quad (c)$$

Injection volume: $$V_{inj,\,PREP} = V_{inj,\,ANA} \times \left(\frac{L_{PREP,\,col} \times D_{PREP,\,col}^2}{L_{ANA,\,col} \times D_{ANA,\,col}^2}\right) \quad (d)$$

Figure 7. Scale-up formulas.

Table 2. System characteristics and methods from UHPLC to HPLC preparative system.

Prepative flow path	Analytic flow path
System characteristics	
$Dwell_{PREP}$ = 3.25 mL	$Dwell_{ANA}$ = 0.153 mL
Column characteristics	
$D_{col,\,PREP}$ = 21.2 mm	$D_{col,\,ANA}$ = 2.1 mm
$L_{col,\,PREP}$ = 150 mm	$L_{col,\,ANA}$ = 50 mm
$D_{part,\,PREP}$ = 5 µm	$D_{part,\,ANA}$ = 1.8 µm
Gradient	
Obtained from scale-up methods	
2.96 min isocratic hold at 2 %	0.10 min gradient from 2 %
6.12 min gradient from 25 to 35 %	0.60 min gradient from 25 to 35 %
4.89 min purge segment at 98 %	0.40 min purge segment at 98 %
Flow rate: 25 mL/min	Flow rate: 1 mL/min
Injection volume: 764 µL	Injection volume: 2.5 µL

The scale-up from the analytical method to the preparative system was accomplished by applying formulas b, c, and d to the analytical and preparative systems (Figure 7).

A method transfer from the gradient on a UHPLC (1.8 µm) column to a preparative (5 µm) column was done by applying formulas b, c, and d using a flow rate of 25 mL/min on the target LC system.

The injection volume was scaled up to 764 µL, which is equivalent to 187 mg of sample mixture on the preparative column.

By applying the method transfer described in this Technical Overview, the target compound was purified on the preparative flow path (Figure 8), and a purity of 98 % and a recovery of 93 % was obtained. The resolution was retained during the scale-up transfer, and a 47 % reduction of solvent consumption was achieved.

Table 3. Scale-up process.

No.	Step
1	Determination of dwell volume and column volume for the system
2	Sample analysis to determine the elution point of the target compound
3	Creation of a focused gradient to increase resolution for the target compound
4	Determine the maximum column loadability
5	Scaling up to a preparative column using the available formula
6	Preparative injection and fraction collection

Note: The first step is performed only once for a system to characterize its void volumes.

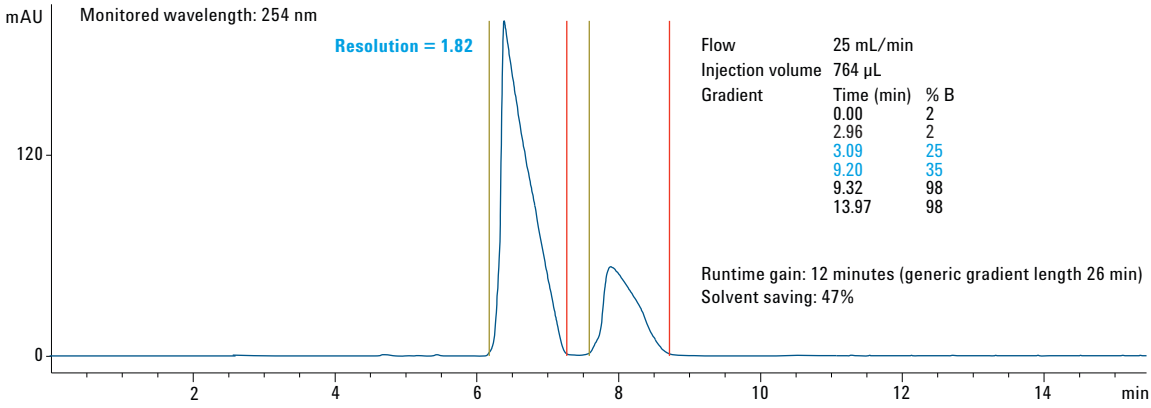

Figure 8. Preparative chromatogram.

Conclusion

A scale-up from a 2.1-mm id column on a UHPLC system to a 1260 Infinity Preparative scale system equipped with a 21.2-mm id column was successfully developed.

For all scale-up situations, a correct method transfer was required to keep the resolution constant. This ensured maximum purity and recovery from the precious sample.

The steps in Table 3 summarize the process.

References

1. Peptides Purification on the Agilent 218 Bio-inert Binary Purification System, Agilent Technical Overview, 5991-3070EN.

2. Guidelines for the use of UHPLC Instruments. Requirements for UHPLC instruments, method development in UHPLC and method transfer from regular HPLC to UHPLC, Dr. Davy Guillarme, Prof. Jean-Luc Veuthey, Freeware download: http://www.unige.ch/sciences/pharm/fanal/lcap/telechargement.htm

3. U. Huber and R.E. Majors, "Principles in preparative HPLC", Agilent Technologies Primer, Publication Number 5989-6639EN, **2007**.

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Published in the USA, September 1, 2013
5991-2013EN

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