Novel Approach to Optimization of a High-Throughput Semipreparative LC/MS System

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Automated semipreparative LC/MS systems are now well established commercially and commonly used for purification of early stage drug discovery compounds. A number of vendors have instruments on the market that are capable of reliably purifying compounds with good water/acetonitrile solubility. However, these systems often fail when the sample has poor solubility, extreme polarity, and/or poor ionization. Even in cases when substantial optimization has been done prior to purification, a certain percent of failures to recover the desired product is unavoidable. In the past, when the majority of samples run on LC/MS semipreparative systems were large combinatorial libraries, some losses in this high throughput mode were acceptable. However, now that more chemistry laboratories are making smaller more focused libraries with higher purity requirements, reliability and recovery are more crucial. This paper describes modifications made to customize an MS-triggered semipreparative LC/MS system in order to ensure improved reliability and recovery of products from traditional medicinal chemistry as well as combinatorial libraries.

Introduction

Automated semipreparative LC/MS systems are now well-established commercially and commonly used for purification of early stage drug discovery compounds.[1−9] A number of vendors[10−13] have instruments on the market that are capable of reliably purifying compounds with good water/acetonitrile solubility. However, these systems often fail when the sample has poor solubility, extreme polarity, and/or poor ionization. Even in cases where substantial optimization has been done prior to purification, a certain percent of failures to recover the desired product is unavoidable. In the past, when the majority of samples run on LC/MS semipreparative systems were large combinatorial libraries, some losses in this high throughput mode were acceptable. However, now that more chemistry laboratories are making smaller focused libraries[14] with higher purity requirements, reliability and recovery are more crucial. This paper describes modifications made to customize an MS-triggered semipreparative LC/MS system in order to ensure improved reliability and recovery of products from traditional medicinal chemistry as well as combinatorial libraries.

Experimental Section

Figure 1 depicts the system configuration as originally set up by the vendor.

The semipreparative LC/MS system consists of a Waters 2767 sample manager (Milford, MA), a Waters 2565 binary module gradient system, three Waters 515 pumps—makeup flow, at-column dilution,[15] and column regeneration—a Waters column fluidics organizer (CFO),[10] an Acurate by Dionex/LC Packings 1:1000 splitter (Sunnyvale, CA), a Waters 2996 photodiode array detector (referred to as prep PDA) with a semipreparative flow cell (path length 3 mm), and a Waters ZQ single quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) source.

To support a high speed analoging team's library syntheses, a high-throughput purification system was required that could routinely purify 96 and up to 384 member libraries. The following protocols were established for the system. Samples were dissolved in 2.5 mL 1:1 MeOH:DMSO, submitted in 24 well plates (Whatman, Uniplate, 24 wells, 10 mL, Clifton, NJ). The entire volume was injected, and the purified product was collected in 20 mL tared scintillation vials. Fractions were evaporated using a Genevac HT-12 evaporator, using their standard HPLC fraction method.

The time for each run was reduced and optimized to 8 min. A dual column system was used to allow purification to be conducted on one column while the second column was reconditioned thereby reducing run time. Generic gradients were employed to minimize development time for each library. The XTerra Prep MS C18 column (Waters, Milford, MA) 5 μm, 19 × 50 mm, 2−12 pH range was

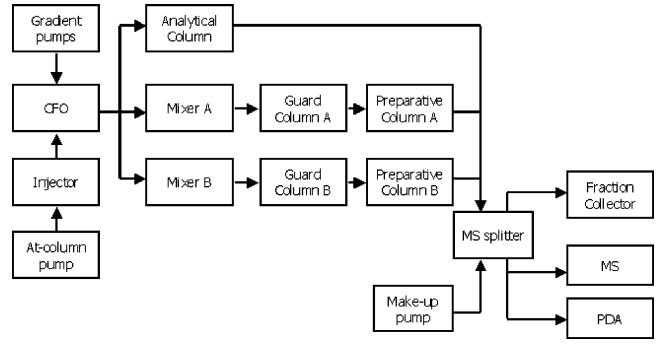

Figure 1. Original configuration of semipreparative LC/MS system.

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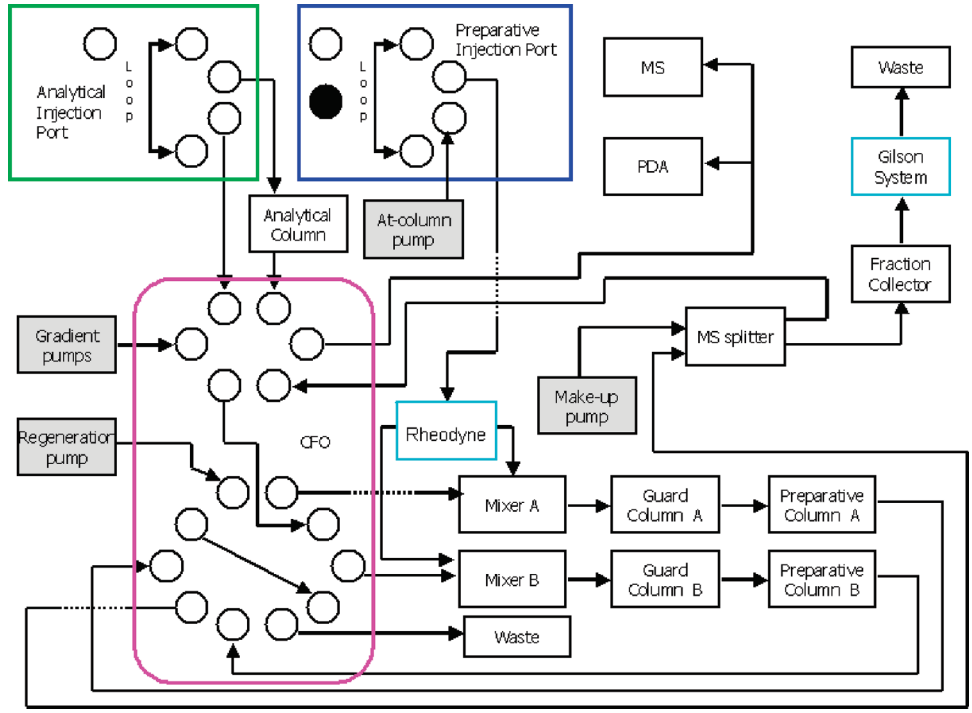

Figure 2. Customized configuration of semipreparative LC/MS system.

identified as a robust and universal column. At-column dilution[15] was adopted so samples could be dissolved in strong solvents (e.g., DMSO) for loading on the column while minimizing the effect on the chromatography.

In addressing the bottleneck of postpurification fraction handling, commercial solutions were found to be inadequate. One-for-one collection was available from the system software, MassLynx v 4.0 SP2. However, using this option to collect just one vial per injection increased the risk of losing compounds. For example, if the molecular ion is detected prior to the main peak, the system will not collect the main peak when it elutes. To prevent compound loss, a system had to be developed that would collect the required compound without having to collect multiple fractions. To ensure that all samples were collected, it was decided to monitor the waste stream from the main LC/MS and to collect any remaining detected peaks. The waste collection option provided by the manufacturer was inadequate to perform this task. The actual manifold to collect the waste was a logistical challenge due to its physical dimensions and the number of compounds purified daily in our lab. Also, it did not have an option to collect waste based on UV activity. So the decision was made to customize a UV-triggered collection system to ensure the robustness and versatility of the overall purification process. Later, as the system evolved an evaporative light scattering detector (ELSD) was added as an alternative to UV-triggered collection for compounds with poor UV absorbance. Also, a Waters Atlantis T3 column, 5 μm, 19 $\times$ 50 mm, was evaluated and identified as a more versatile column for retention and separation of compounds with a wide range of polarities vs the original XTerra Prep MS C18 column.

Two major modifications were made to the original semipreparative LC/MS system setup. They are represented schematically in Figure 2.

First, a 2-position, 10-port Rheodyne valve under MassLynx software control was added between the injection port and mixer. This configuration allows mixing to occur directly before the column thereby eliminating the possibility of compound precipitating in the Rheodyne valve of the CFO.

Second, the waste line from the fraction collector of the main LC/MS was connected to a Gilson system (Madison, WI). This consisted of 119 UV detector (referred to as waste collection UV) and 215 liquid handler used for fraction collection controlled by Unipoint software v 3.30. The Gilson 215 fraction collector was configured with 5 Gilson 208 racks filled with 70 16 $\times$ 100 mm culture tubes (VWR, West Chester, PA) each.

The columns used were XTerra Prep MS C18, 5 μm, 19 $\times$ 50 mm, with matching XTerra MS C18 5 μm, 19 $\times$ 10 mm precolumn (Waters, Milford, MA), and Atlantis T3, 5 μm, 19 $\times$ 50 mm, with a matching Atlantis T3, 5 μm, 19 $\times$ 10 mm precolumn (Waters, Milford, MA). The organic phase was HPLC-grade acetonitrile, and the aqueous phase was 50 mM ammonium acetate. Makeup flow was 100% methanol, and at-column dilution was typically 100% acetonitrile. The at-column dilution pump was set to 2.5 mL/min (10% of the total flow), and the Waters 2565 binary module gradient system was set to 22.5 mL/min. The generic gradient was 5−95% acetonitrile in aqueous 50 mM ammonium acetate over 6.5 min with an overall cycle time of 9 min. This was modified to accommodate for differences in the polarity of compounds and to improve resolution. This LC/MS purification system was controlled using MassLynx v 4.0 SP2 and FractionLynx Collection Control v 4.0 SP2 software packages.

The main LC/MS and waste collection UV systems run independently and do not communicate directly with each other. To facilitate this, a Gilson control method was written for the waste collection system that mirrored the length of

Time, min.	Device(s)	Command
0.00	Detector 16	Autozero Channels
0.02	Detector 16	Set Single Wavelength Wave
0.04	Fraction Collector	Set Peak Width and Peak Sensitivity 2 min., 2 % Full Scale
0.06	Fraction Collector	Set Collection and Travel Depths 73 mm, 64 mm
0.08	Fraction Collector	Set Fraction by Time Inside a Peak 0.5 min.
0.10	Fraction Collector	Set Fraction Site Fraction
0.12	Data Channels	Start Chromatogram Channels
0.14	Fraction Collector	Start Collection
9.90	Data Channels	Stop Chromatogram Channels
10.00	Fraction Collector	Stop Collection

Figure 3. Unipoint control method.

the method on the LC/MS system. An operation file for the system would contain the startup control method in line 1, the required number of fraction collection control methods, and a shutdown control method in the last line.

The startup control method homes the Gilson 215 liquid handler, initializes the UV detector, and then tells the system to wait. The operator can then initiate purification on the Waters system and immediately cancel the wait instruction on the Gilson. At this point, as both systems are running identical length methods, the systems are synchronized. One could choose to use a contact closure to synchronize the two, but in our case, it did not appear to be a significant advantage.

A fraction collection control method (Figure 3) schedules events for the Gilson 215 liquid handler and Gilson 119 UV. The same control method can be used for every injection. As a result the operations file can be generated rapidly by copying this line and simply pasting in the same number of injections on the main purification system. The control method has the collection wavelength defined as a variable to allow different wavelengths to be entered for each injection if necessary.

Finally a shutdown control method homes the Gilson 215 liquid handler and switches off the 119 UV detector.

The ELSD was configured similarly to the UV detector within the control method. This secondary detection was set up using a new data channel within the Unipoint software; in order to interface the two, a Gilson 506C system interface box was incorporated to allow up to 1 V of input data. Two different control methods were established on the waste collection system to provide versatile analyte detection. The Unipoint control method can trigger fraction collection solely by ELSD in the same manner as mentioned above for UV detector. The waste collection system can be set up to collect either using a UV/ELSD trigger or by time.

Results

The customized semipreparative LC/PDA/MS and the UV/ELSD monitored waste collection[19] allows the user a direct comparison of the quality of the MS-triggered purification collection as well as secondary UV/ELSD-triggered collection of any significant peaks remaining in the effluent.

Ideally, desired product collected in one tube from primary MS triggered collection is reflected in the waste collection UV trace as a missing peak compared to the prep PDA trace as shown in Figure 4. The green highlighted region in the top two chromatograms represents the desired product with molecular weight 453.3 *m*/*z* collected by the MS-triggered

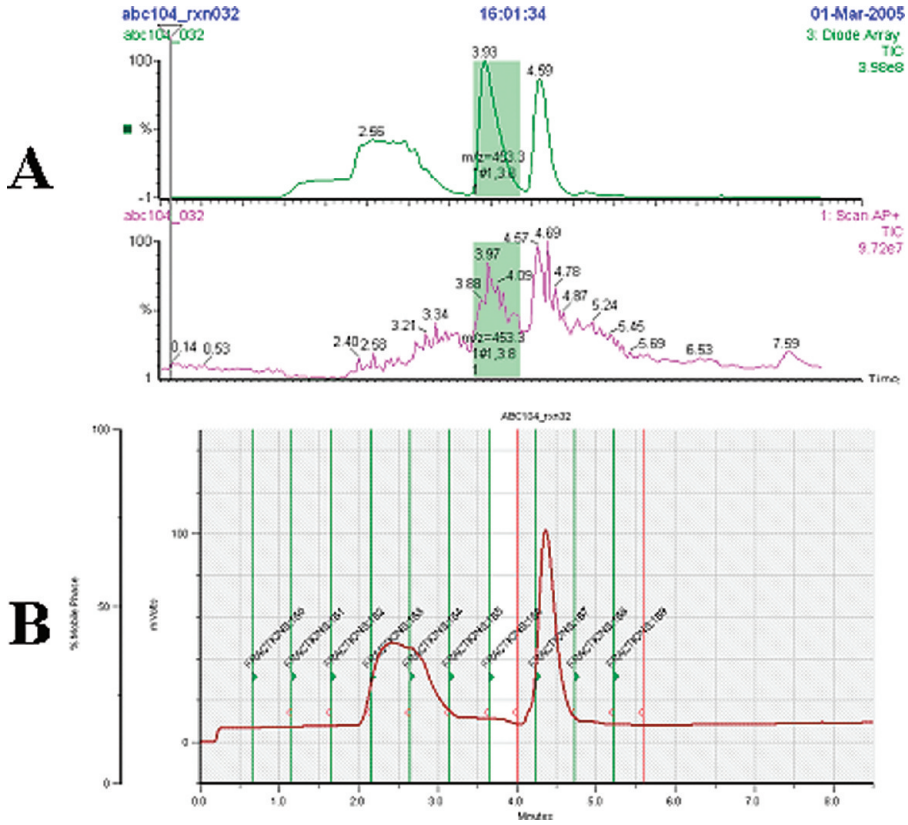

Figure 4. Chromatograms from MS-triggered collection (A) and waste collection UV trace (B) of the purification of a compound with *m*/*z* 453.3. The product peak at 3.93 min in prep PDA trace (A) is absent from the waste collection UV trace (B).

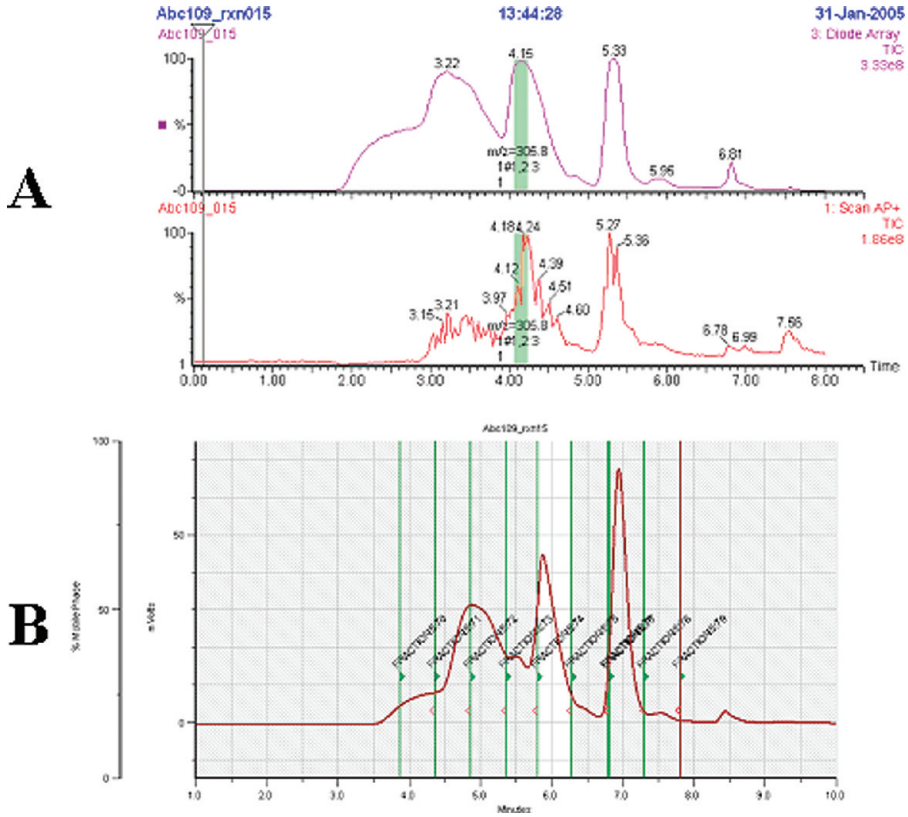

Figure 5. Chromatograms from the MS-triggered collection (A) and UV-triggered waste collection (B) of the purification of a compound with *m*/*z* 305.8. A product peak at 4.15 min in prep PDA trace is partially collected by the MS-triggered system (A), and the fraction containing the remainder of the peak is highlighted on the waste collection UV chromatogram (B).

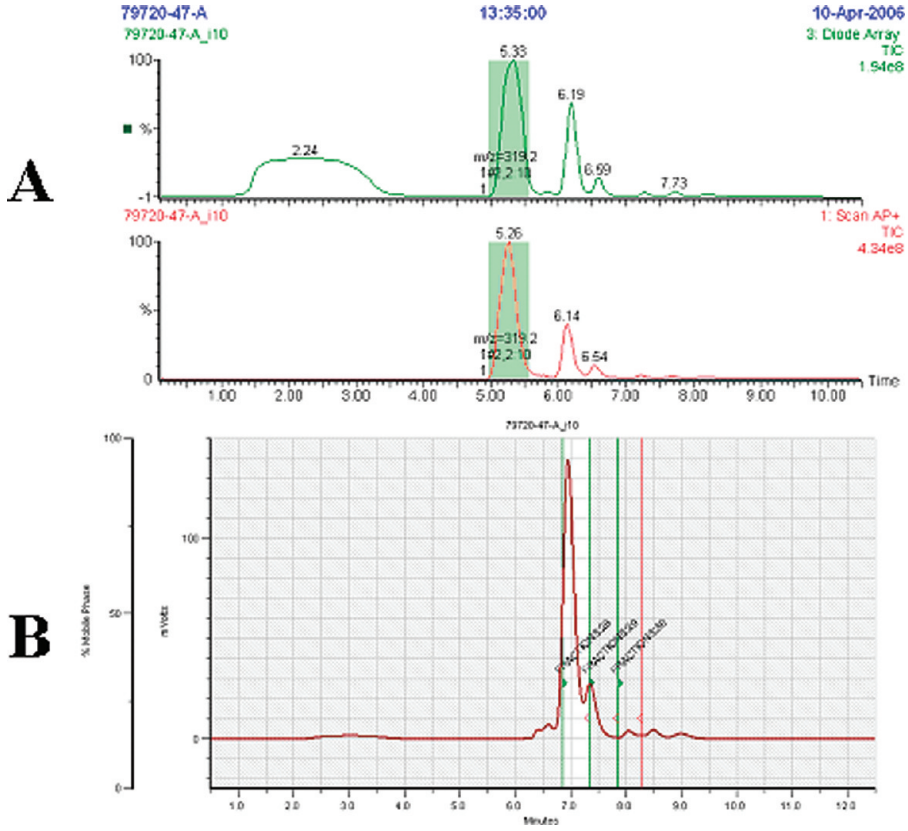

Figure 6. Chromatograms from the MS-triggered collection (A) and UV-triggered waste collection (B) of the purification of a compound with *m*/*z* 319.2. A product peak at 5.33 min is highlighted in green collected by MS-triggered system (A). A starting material peak at 6.19 min in prep PDA trace (A) is collected by the waste monitoring system—fraction 28 highlighted in the waste collection UV chromatogram (B).

Table 1. Recovery and Purity Assessment of 23 Library Compounds

reaction	molecular weight of product, Da	crude reaction mixture purity, %	MS fraction, mg	UV fraction, mg	comparison of recovery, % difference	combined fractions average purity, %
A	251.33	43.7	27.5	29.0	3.5	93
B	275.35	51.2	23.0	21.5	4.6	100
C	225.29	49.0	25.9	27.0	2.8	100
D	211.26	48.8	21.9	23.2	3.8	100
E	229.25	90.2	39.9	42.0	3.4	100
F	241.29	57.1	27.0	25.0	5.2	99
G	225.29	33.9	13.5	16.2	11.8	96
H	279.26	61.7	27.7	29.0	3.0	100
I	315.41	66.0	32.4	30.2	4.7	92
J	245.71	56.6	26.5	26.0	1.3	92
K	243.28	70.2	33.2	31.9	2.7	100
L	304.19	40.0	20.4	19.9	1.7	100
M	315.41	63.4	25.6	24.3	3.5	100
N	289.38	36.5	21.4	20.9	1.6	98
O	239.32	69.2	43.2	42.9	0.5	100
P	237.30	67.8	33.1	32.9	0.4	98
Q	225.29	61.1	33.9	34.2	0.6	96
R	241.29	41.5	54.0	56.1	2.5	92
S	229.25	68.5	44.0	43.7	0.5	100
T	245.71	85.5	40.1	41.2	1.8	98
U	225.29	87.6	42.0	43.0	1.6	100
V	237.30	56.9	26.3	25.0	3.4	100

system. The bottom chromatogram from UV-triggered system shows almost complete disappearance of the product peak while the other two peaks on each side of it still remain.

In cases where desired product is only partially collected, a poor recovery can be prevented by correlating the prep PDA and the waste collection UV traces to identify and recover the remainder of the peak. An example of this is shown in Figure 5. In this example, the ionization level across the peak was unexpectedly weaker than the trigger threshold, causing only part of the peak to be collected. With the UV-triggered waste collection in place, the material not collected in the MS-triggered fraction was easily identified and recovered from the UV-collected waste fractions.

An additional advantage that has been realized using this "tandem" collection system is starting material and multiple product recovery. An example of this is shown in Figure 6. Here, the product highlighted in green is collected by the MS-triggered system, while the second major peak—a starting material in this case—is collected and easily recovered from the UV/ELSD-triggered system.

To further demonstrate the "near zero percent loss" system, purification of a focused, 23 compound library is described. For comparison purposes, four injections of each sample were made. Two injections were triggered by the main MS-triggered system, and the waste backup collection triggered the other two injections. In order to force trigger fraction collection on the backup system, the sample list was deliberately populated with incorrect molecular weights.

Prepurification UV purities (Table 1) of the samples were estimated by integrating all of the peaks in the crude reaction mixture. No workup was done prior to samples being submitted for purification. Postpurification QC analysis of the combined MS- and UV-triggered fractions shows that 4 out of the 23 compounds had purities between 92 and 93% and the other 19 compounds had purities above 95%. The four that had purities below the 95% mark were due to insufficient resolution between products and other components of the reaction. On average, the final purity was 98% for the 23 compounds purified. Comparison of sample recoveries from the MS- and UV-triggered systems shows that the average difference between the two is 2.9%. No samples were lost.

Even with the enhancements realized with the tandem waste collection, it should be recognized that there are still rare, but possible, scenarios where a sample can be lost. Two of these are: if collection threshold on the waste monitoring system is set too high or if hardware/software errors occur unexpectedly. With this caveat, since the implementation of waste monitoring and collection, unattended and overnight runs have become routine in our lab, with no loss of compounds.

Conclusion

The robustness and flexibility of our customized prep LC/MS system are greatly enhanced by the addition of the UV-triggered waste collection system. A number of benefits using this tandem collection system have been realized:

- successful implementation of one-for-one collection
- near 0% loss of compounds
- starting material recovery
- multiple product recovery without system reconfiguration
- better recoveries for broad, tailing peaks that cannot fit in one vial
- problem-free overnight runs
- constant monitoring of the waste to ensure robust system performance
- recovery of samples with poor ionization

As has been the case for many laboratories in the past several years, the application of purification in our lab has shifted from high-throughput for library purification, to providing more flexible and rigorous separations of small focused libraries as well as individual compounds. In this setting, the implementation of waste monitoring and collection continues to be extremely beneficial.

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