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Applikationsbericht

Characterizing and Monitoring Impurities in Lipid Nanoparticle Components Using the BioAccord™ LC-MS System with waters_connect™ Software

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Abstract

Lipid nanoparticles (LNP) have been used with high efficacy as delivery vehicles for nucleic-acid based therapeutics and vaccines. However, the components of these lipid shells can often contain impurities in addition to the core lipid components. These impurities must be rigorously screened for and characterized in order to ensure the safety of the formulated product. Impurity analysis is a critical aspect in both lipid raw material screening and LNP compositional analysis, and an instrument and informatics platform that can both detect and confidently identify low-level impurity peaks can substantially streamline the process. Here, the capability of the BioAccord System and waters_connect Software is demonstrated as a suitable solution that can streamline the analysis and identification of impurities in both lipid raw material and compositional analysis of LNPs.

Benefits

- A streamlined data acquisition and data analysis workflow for the identification and routine monitoring of lipid impurities using the BioAccord LC-MS System on the waters_connect informatics platform

- Integrated in-silico fragmentation tools on the waters_connect platform that enable elucidation and confirmation of lipid identities under Data Independent Acquisition mode using the BioAccord LC-MS System

Introduction

The development of lipid nanoparticles (LNPs) as delivery vehicles has enabled the success of mRNA-based vaccines and other nucleic acid-based therapeutics. These lipid shells serve to encapsulate and protect the nucleic acid payload, the active pharmaceutical ingredient (API), ensuring its safe and effective delivery into targeted cells. There are four main components that form the LNP, and they include 1) cholesterol (CHO) for fluidity, 2) a helper phospholipid distearoylphosphatidylcholine (DSPC), 3) a PEGylated lipid to generate a hydrophilic micelle outer surface, and 4) an ionizable lipid that is a primary driver of potency. When mixed at the correct ratio, these lipids self-assemble around the introduced genetic material to spontaneously form LNPs. Each of these lipid components can be synthesized in-house or procured from third-party vendors. Regardless of the source, rigorous analysis is critical to both raw material screening and LNP composition and impurities analysis. As such, analytical tools that can facilitate lipid and impurities characterization, identification, and monitoring applications are needed.

Typical LNP analyses use different platforms for upstream and downstream workflows. Optical workflows typically include an ultraviolet (UV) detector and/or a universal detector, such as an Evaporative Light Scattering Detector (ELSD) or a Charged Aerosol Detector (CAD), and are typically employed downstream in production monitoring and QC workflows due to their simplicity and ease of use. For upstream characterization workflows, LC coupled to mass spectrometry (MS) is the gold standard for sensitive, detailed analyses. However, deploying sophisticated MS instruments outside of core laboratories can be challenging. The BioAccord System was designed to overcome these limitations by being a benchtop Time-of-Flight (ToF) MS with a small footprint that is easy to operate and maintain.[1] This SmartMS-enabled system increases MS accessibility beyond core laboratories, including laboratories that lack MS expertise and/or are resource/budget-constrained. The BioAccord MS Platform offers high sensitivity and is engineered to deliver reproducible measurements with minimal tuning, enabling screening, and detecting of low-level impurities in LNP samples. Its ability to perform MS^E fragmentation, a Data Independent Acquisition mode, when used in conjunction with the integrated waters_connect Software facilitates identification and characterization of unknown impurities.

Here, we demonstrate how the BioAccord System on the waters_connect informatics platform enables an

efficient, streamlined workflow for confident lipid impurity analysis in both raw material screening and compositional analysis of LNPs.

Experimental

All lipids in this study were used exclusively for research and demonstration purposes and were purchased from commercially available sources. Stocks of each lipid were prepared in methanol at 1 mg/mL. Samples were diluted to the appropriate concentration at 90/10 methanol/water (v/v).

LC Conditions

LC system:	ACQUITY Premier Binary Solvent Manager System
Vials:	TruView™ Max Recovery Vials, (p/n: 186005662CV)
Vial caps:	Polyethylene Septumless Screw Cap, (p/n: 186004169)
Column(s):	ACQUITY Premier CSH Phenyl-Hexyl Column, 1.7 µm, 2.1 mm x 50 mm (p/n: 186009474)
Column temperature:	50 °C
Sample temperature:	8 °C
Injection volume:	3 µL
Flow rate:	0.400 mL/min

Mobile phase A:	0.1% formic acid (v/v) in water (LCMS grade)
Mobile phase B:	0.1% formic acid (v/v) in acetonitrile (LCMS grade)

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.400	40.0	60.0	Initial
6.00	0.400	10.0	90.0	6
8.00	0.400	10.0	90.0	6
8.50	0.400	40.0	60.0	6
12.00	0.400	40.0	60.0	6

MS Conditions

MS system:	BioAccord
Ionization mode:	ESI+
Acquisition range:	50–2000 *m/z*
Capillary voltage:	1.5 kV
Cone voltage:	30 V
Fragmentation cone voltage:	100–150 V
Desolvation temperature:	350 °C

Intelligent Data Capture (IDC):	On

Data Management

Data were acquired and processed using the waters_connect informatics platform with the integrated UNIFI App (version 3.1.0.16).

Results and Discussion

Before an LNP formulation can be released into the market, steps must be taken to screen for and appropriately characterize impurities to ensure both the safety and efficacy of the released product. Here, we demonstrate how impurity analyses can be streamlined using the BioAccord System together with the waters_connect informatics platform. Case studies are shown for using the workflow to characterize impurities while screening raw materials, as well as monitoring for impurities in compositional analyses.

Utilizing Built-In Predictive Fragment Ion Matching

In LNP analysis workflows, fragmentation spectra can help elucidate the identity of lipid nanoparticle components and their impurities. The BioAccord Instrument has the capability to perform MS^E data independent acquisition in which the mass analyzer cycles between low energy and high energy spectra, acquiring precursor and fragment ions for essentially all compounds across the chromatographic separation. The instrument uses a ramp to maximize the number of fragment ions generated for each precursor ion in the high energy spectra. While these spectra provide a wealth of information to help identify compounds, interpreting the spectra in a meaningful way can be time consuming and require notable expertise.

To facilitate interpretation of fragmentation spectra and aid in the identification of analyte molecules, the UNIFI App within the waters_connect Platform offers the capability to use in silico fragmentation of known structures to match fragment ions to predicted fragments of a given molecule. To use this feature, structure files can be imported into a scientific library as ".mol" files, as shown in Figure 1A. The in-silico fragmentation prediction feature can then be enabled by checking the box titled "Generate predicted fragments from structure" in the "Target by Mass" section of the analysis's processing method. This setting is indicated in Figure 1B. With this feature enabled, structures are displayed next to any fragment ions whose masses match a predicted structure of

the component in the high energy spectra. These structures expedite interpretation of spectra to rapidly confirm component identifications. Figures 1C and 1D show examples of matching in-silico fragment ions to ions in the high energy spectra for two example molecules, ionizable lipid SM-102 and cholesterol.

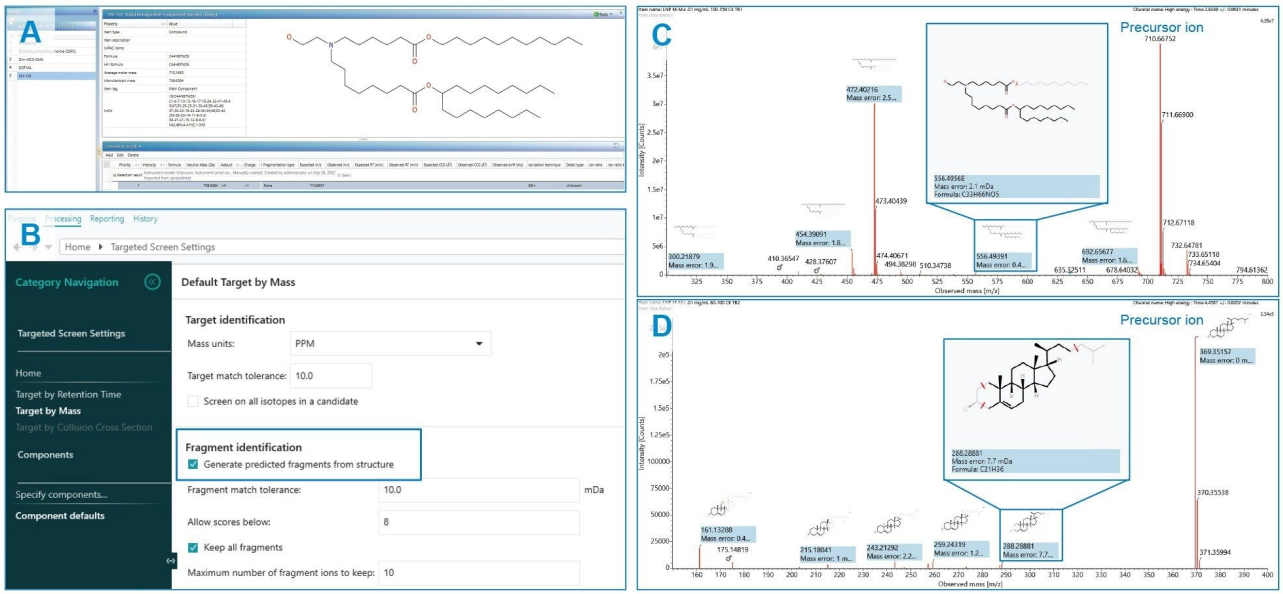

Figure 1. Steps for generating in-silico predicted structures for fragment ions in the UNIFI App, including (A) inclusion of structures as .mol files in the Scientific Library, (B) enabling fragment ion structure prediction in the analysis method, and resulting fragmentation spectra for (C) SM-102 and (D) cholesterol.

Impurity Characterization in Raw Material Screening

In addition to detecting impurity peaks in a component mixture, screening raw materials for impurities is critical to ensuring the final product is of an acceptable quality. It has been previously shown that materials from different vendors, or even different lots from the same vendor, can have unique impurity profiles.[2] The additional sensitivity and accurate mass information of both precursor and fragment ions improves both detection and elucidation of these impurities, expediting the workflow. An example is shown in Figure 2, where a sample of Dlin-MC3-DMA, a cationic ionizable lipid, was run on the BioAccord System. From examining the TIC, it is clear that there are numerous peaks in addition to the main peak at 3.38 min. To putatively assign possible identifications to these peaks, anticipated transformations of the main peak can be included in the search. Users can select from a list of common transformations, such as oxidation, reduction, or desaturation in the processing

method, as shown in Figure 2A. The resulting masses from each of the selected transformations are then included in the compound screening step in the UNIFI Workflow. There is also an option to localize the transformations using fragment spectra information. By searching for a list of anticipated transformations, the impurity peaks in the chromatogram were able to be identified based on accurate mass matching of precursor and fragment ions. The list of detected transformations of Dlin-MC3-DMA are shown in the component summary window of the review page (Figure 2B). Figure 2C shows the base peak ion (BPI) chromatogram of the Dlin-MC3-DMA screening, with the various transformations indicated. Figures 2D and 2E show the low energy and high energy spectra, respectively, for one of the oxidation peaks, providing both precursor and fragment ion *m/z* values.

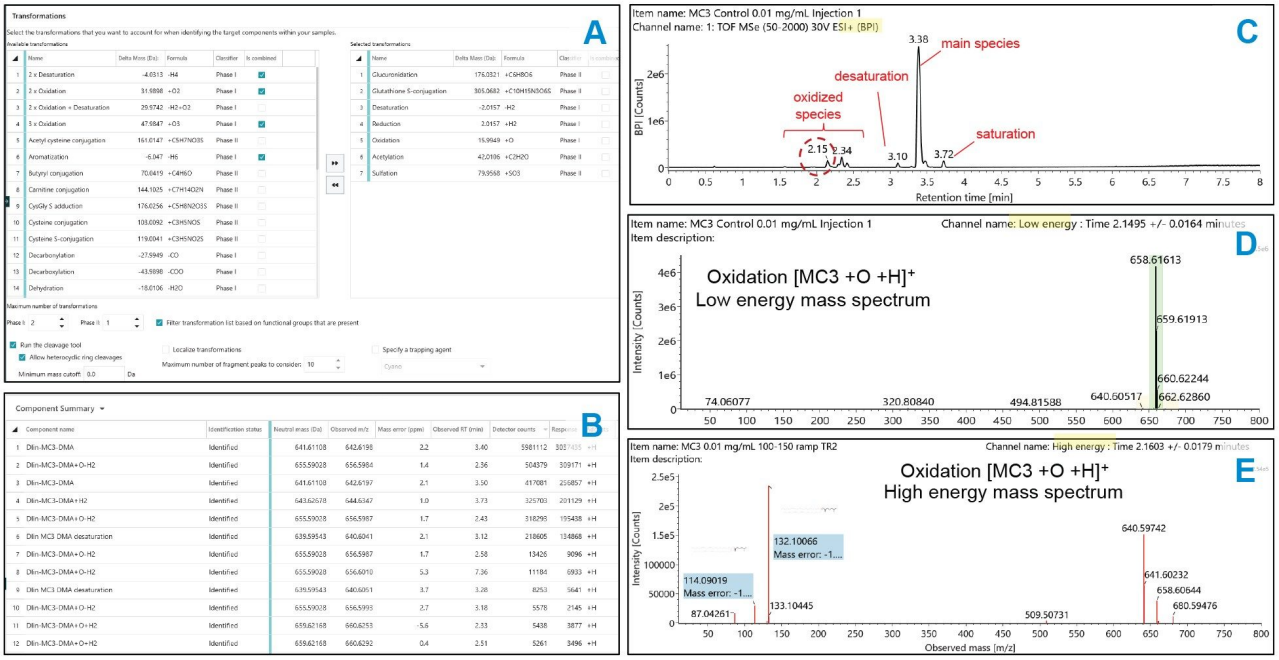

Figure 2. UNIFI Software results for raw material screening of ionizable lipid Dlin-MC3-DMA, including (A) selection of transformations to be monitored, (B) the resulting list of detected transformations, (C) the base peak ion chromatogram with identified impurities labeled, (D) low energy mass spectrum of the parent ion of an oxidation impurity in Dlin-MC3-DMA, and (E) high energy mass spectrum of the same oxidation impurity with fragment ions annotated.

For further elucidation of the structure of impurities, fragmentation spectra can be used to unambiguously

localize modifications on the molecule. As an example, oxidation of Dlin-MC3-DMA was investigated. There are two plausible types of oxidations that can be observed for the lipid: 1) amine oxidation on the polar headgroup, and 2) epoxidation of one of the double bonds. These structures were added to the scientific library and included in the component search. To improve profiling of oxidative peaks, the Dlin-MC3-DMA sample was subjected to oxidative stress with hydrogen peroxide, then run on the LC-BioAccord Platform. Reviewing the fragmentation spectra with the help from the in-silico fragmentation tool reveals the actual location of the oxidation, as shown in Figure 3. When the structure with amine oxidation was used to generate theoretical fragments, not only are there matching structures for nearly every observed fragment ion, but also there are key fragment ions that confirm the location of oxidation on the primary amine. These ions are labeled A, B, and C in the figure. When the double bond epoxidation structure is used to generate the fragment ions, there is no fragmentation that could lead to the *m/z* of A or B. Furthermore, the fatty acid chain fragment, labeled D, only matches when the oxygen is cleaved from the structure. These results confirm that oxidation is happening at the amine group.

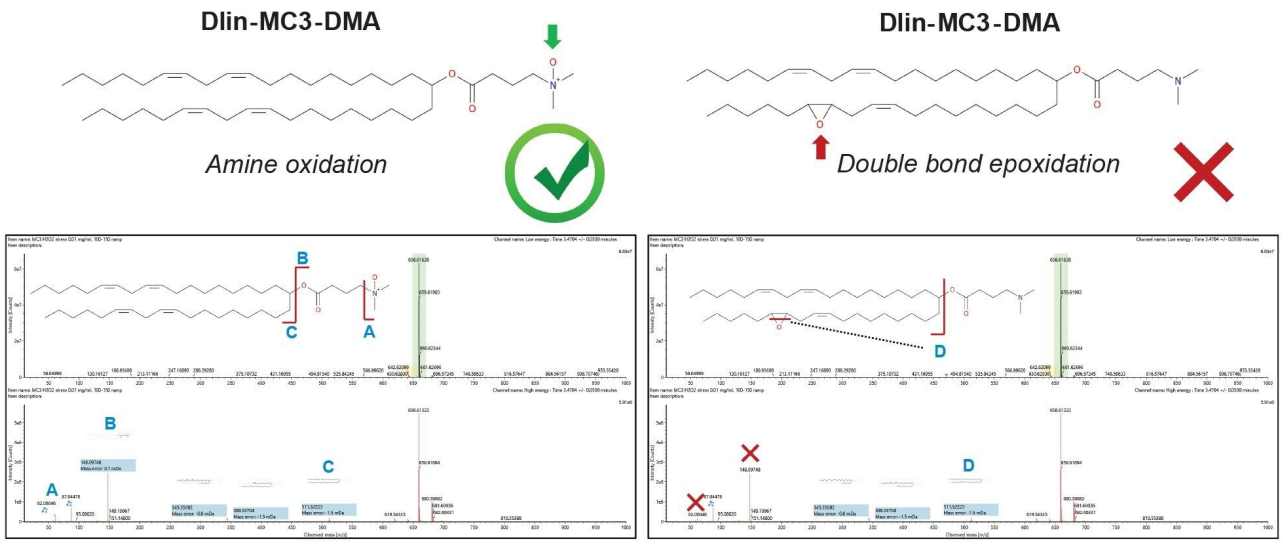

Figure 3. Localization of oxidation on the Dlin-MC3-DMA molecule. Two structures are shown with possible locations for the oxidation, including (left) oxidation of the amine group of the polar head group and (right) epoxidation of one of the double bonds in the fatty acid chain. Below each structure are the low energy (parent ion) and high energy (fragment ion) spectra with matching fragment ions labeled based on in-silico prediction using each structure. Key fragment ions indicative of the location of the oxidation are labeled as A, B, and C. The fragment ion labeled D is the same peak as C but corresponds to an unlikely fragmentation in the epoxidated structure.

Impurity Monitoring of LNP Mixtures

When monitoring these characterized impurities across multiple samples during compositional analysis, it is not only critical to ensure lipids are present at the proper ratio, but also that any low-abundance impurities can be reliably detected in the development and manufacturing of drug products. To facilitate tracking of impurity peaks across samples, item tags can be used to specify main components and known impurities previously characterized. These item tags can be added to the scientific library, enabling easy transfer across methods. To demonstrate the capability of the BioAccord System for detecting low-level impurities, a sample of four LNP components mixed at a ratio 1.0:0.42:0.22:0.11 for SM-102:CHO:DSPC:DMG-PEG 2000 was spiked with another ionizable lipid, DOTMA, at 0.1% the base peak intensity. Figure 4A shows the resulting TIC chromatogram for the mixture. While the DOTMA peak appears quite low compared to the other components in the mixture, it can still be detected, as evidenced in the component library (Figure 4B) where it has been “tagged” as a known impurity

(red box), as well as in the extracted ion chromatogram (Figure 4C). These tools enable known impurities to be rapidly monitored across numerous sample sets.

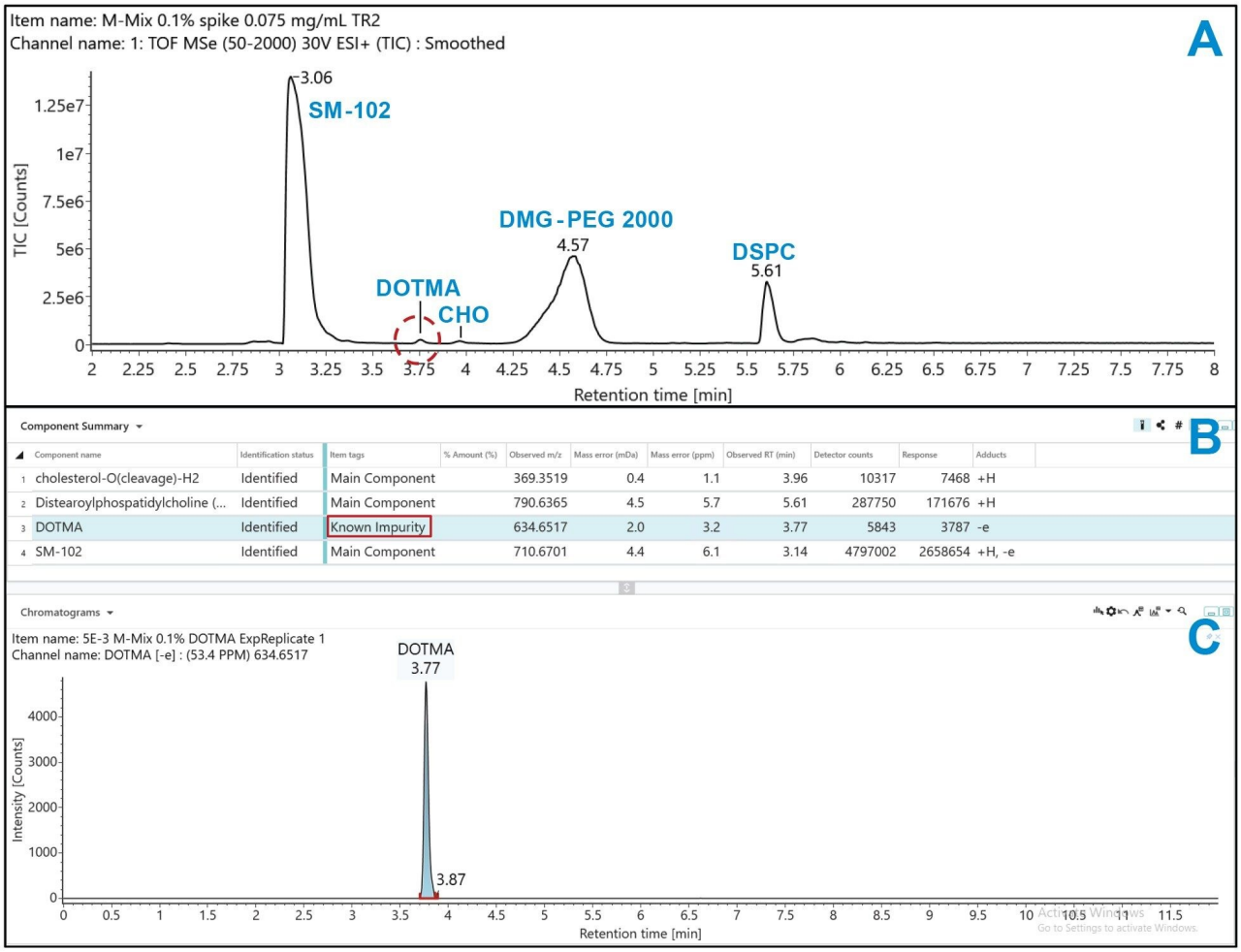

Figure 4. UNIFI Software results for the analysis of a mixture of lipid nanoparticle components, including ionizable lipid SM-102, cholesterol (CHO), PEGylated lipid DMG-PEG 2000, and distearoylphosphatidylcholine (DSPC), with another ionizable lipid, DOTMA, spiked in at 0.1% base peak intensity. (A) TIC chromatogram of the component mixture, (B) list of components with their type specified, and (C) extracted ion chromatogram of the DOTMA impurity peak.

Conclusion

Rigorous analysis of impurities throughout the LNP development and manufacturing process is necessary to ensuring the safety and efficacy of the released product. The BioAccord System enables detailed analyses for both raw material screening and compositional analysis to expedite detection and characterization of impurities in LNP samples. The accurate mass data of both precursor ions and fragment ions offers both sensitive detection and confident identification of unknown peaks in an LNP profile. The tools in the UNIFI App of the waters_connect platform streamline interrogation of the data to make good use of the wealth of information. The information obtained and methods developed can then be migrated downstream to monitoring workflows with single quadrupole MS or ELS detectors to help mitigate risk in the development and manufacturing of lipid nanoparticles.[2,3]

References

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2. Han D, DeLaney K, Alden BA, Birdsall RE, Yu Y. Lipid Nanoparticle Analysis: Leveraging MS to Reduce Risk. Waters Application Note, 720007716. 2022 Sept.
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