

Short Communication

Evaluation of Dissolution Release Profiles of Nicotine and Three Distinct Flavor Markers in Loose Moist Smokeless Tobacco Products *

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SUMMARY

This study describes the dissolution release profiles of nicotine and flavor markers from three loose moist smokeless tobacco (MST) products, same brand, each made with a distinct flavor. The US Pharmacopeia flow-through cell dissolution apparatus 4 (USP-4) was employed, following a previously published method that was validated to study the nicotine release from MST products. Herein, we expanded the scope of the analytical method by incorporating three flavor markers including methyl salicylate, ethyl salicylate, and glycyrrhizic acid to provide an understanding of the dissolution release profiles of not only nicotine but also of flavor markers. The dissolution release profiles of nicotine were found to be equivalent across all three tobacco products. In contrast, the release profiles of the studied flavor markers exhibited distinct differences, primarily influenced by their chemical properties, particularly polarity. Notably, glycyrrhizic acid demonstrated the most rapid release rate, while ethyl salicylate exhibited the slowest release rate. This study serves as a valuable resource for researchers, manufacturers, and regulatory bodies involved in the evaluation of MST products attributes and performance. [Contrib. Tob. Nicotine Res. 33 (2024) 157–163]

KEYWORDS

Smokeless tobacco; nicotine; flavors; dissolution, USP-4; release profile; method development; validation.

ZUSAMMENFASSUNG

In dieser Studie werden die Freisetzungprofile von Nikotin und Aromen aus drei losen, feuchten, rauchlosen Tabakprodukten (MST) derselben Marke beschrieben, die jeweils einen bestimmten Geschmack aufwiesen. Die US Pharmacopeia Durchflusszelle USP-4 wurde nach einer zuvor veröffentlichten und validierten Methode zur Untersuchung der Nikotinfreisetzung aus MST-Produkten eingesetzt. In diesem Bericht haben wir den Anwendungsbereich der Analysemethoden erweitert, indem wir die drei Geschmacksmarker Methylsalicylat, Ethylsalicylat und Glycyrrhizinsäure einbezogen haben, um ein Verständnis der Freisetzungprofile nicht nur von Nikotin, sondern auch von Geschmacksmarkern zu erhalten. Es wurde festgestellt, dass die Freisetzungprofile von Nikotin bei allen drei Tabakerzeugnissen gleich sind. Im Gegensatz dazu wiesen die Freisetzungprofile der untersuchten Aromastoffe deutliche Unterschiede auf, die in erster Linie durch ihre

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chemischen Eigenschaften, insbesondere ihre Polarität, beeinflusst wurden. Glycyrrhizinsäure zeigte die schnellste Freisetzungsrates, während Ethylsalicylat die langsamste Freisetzungsrates aufwies. Diese Studie ist ein wertvolles Hilfsmittel für Forscher, Hersteller und Aufsichtsbehörden, die mit der Bewertung der Eigenschaften und Leistung von MST-Produkten befasst sind. [Contrib. Tob. Nicotine Res. 33 (2024) 157–163]

RESUME

Cette étude décrit les profils de libération par dissolution de la nicotine et des marqueurs d'arôme de trois produits de tabac sans fumée humide en vrac (MST), de la même marque, chacun fabriqué avec un arôme distinct. L'appareil de dissolution cellulaire 4 (USP-4) de l'US Pharmacopeia a été utilisé, selon une méthode publiée précédemment et validée pour étudier la libération de nicotine à partir de produits de tabac sans fumée humide. Ici, nous avons élargi le champ d'application de la méthode analytique en incorporant trois marqueurs d'arôme, à savoir le salicylate de méthyle, le salicylate d'éthyle et l'acide glycyrrhizique, afin de mieux comprendre les profils de libération par dissolution non seulement de la nicotine, mais aussi des marqueurs d'arôme. Les profils de libération de la nicotine par dissolution se sont avérés équivalents pour les trois produits du tabac. En revanche, les profils de libération des marqueurs d'arôme étudiés présentaient des différences distinctes, principalement influencées par leurs propriétés chimiques, en particulier la polarité. En particulier, l'acide glycyrrhizique présentait la vitesse de libération la plus rapide, tandis que le salicylate d'éthyle présentait la vitesse de libération la plus lente. Cette étude constitue une ressource précieuse pour les chercheurs, les fabricants et les organismes de réglementation impliqués dans l'évaluation des attributs et des performances des produits MST. [Contrib. Tob. Nicotine Res. 33 (2024) 157–163]

INTRODUCTION

Smokeless tobacco products are a category of tobacco products that is consumed without being burned or smoked (1–4). Unlike traditional cigarettes or cigars, smokeless tobacco products are not ignited and inhaled. Instead, they are typically placed in the mouth, where they release nicotine and other chemicals that are absorbed through the oral mucosa (5–8). They are primarily made from processed tobacco leaves and contain nicotine, flavors, moisture, binders, preservatives, humectants, and pH adjusters (9). Loose moist smokeless tobacco (MST) products refer to a specific form of smokeless tobacco products that are loose rather than being pre-packaged in pouches or portions and are moist in texture (10–11). This type of smokeless tobacco is often used by placing a pinch or wad of the tobacco between the cheek and gum, where it is slowly chewed or sucked on.

The rate at which nicotine and flavors in the MST products dissolve into a medium such as saliva can impact the user's experience and exposure. While dissolution testing is not a direct measure of a user's exposure to the constituents

present in MST, it can be an effective tool to provide insight into the user's experience and exposure as well as to compare one product to another. Consequently, the development of dissolution methods to understand the release of nicotine and other constituents from MST products is becoming important from a regulatory perspective (12). While there are several well-established and standardized dissolution methods outlined in pharmacopoeias, the number of dissolution methods specifically developed for comparing MST products is limited (13–17). These methods utilize various analytical techniques and dissolution apparatuses, including USP-1 (basket), USP-2 (paddle), USP-4 (flow-through cell), etc., with a primary focus on evaluating the release of nicotine from smokeless tobacco products (14, 18–23). The USP-1 and USP-2 dissolution apparatuses were predominantly used for portioned or pouched MST products, while the USP-4 apparatus was employed to study the nicotine release profile of both pouched and loose MST products. For loose smokeless tobacco, the USP-4 apparatus offers distinct advantages. It can operate as an open system, allowing fresh artificial saliva to continuously pass through the cell containing the loose smokeless tobacco. This enables the adjustment of the flow rate of artificial saliva, ensuring the maintenance of sink conditions for poorly soluble compounds such as extended-release formulations and amphiphilic flavor markers. Additionally, the flow-through cell employed in the USP-4 apparatus, where loose tobacco is placed or embedded between glass beads, mimics the human practice of placing MST products between the lip and gum which easily prevents loose tobacco products from floating, when compared to the USP-1 and USP-2 apparatuses.

Recently, we have developed and validated a discriminatory and fit-for-purpose method to study the nicotine release profile from various traditional smokeless tobacco products (24). The method utilized a USP-4 flow-through cell apparatus and Ultra Performance Liquid Chromatography coupled to a Photodiode Array detector (UPLC-PDA) (24). This method accurately determined the nicotine released into artificial saliva from these products. Our previous findings indicate that the nicotine release profile depends on the form and cut of MST products. The method was then expanded to include oral tobacco-derived nicotine pouch products that do not contain tobacco leaf, specifically on![®] nicotine pouches (25). The dissolution release of nicotine from 35 on![®] nicotine pouch products across different nicotine levels was characterized and found to be equivalent using our established USP-4 method, showing product performance similarity across all on![®] nicotine pouch products (25).

In this study, we expand the scope of the above dissolution method for MST products to include three flavor markers (methyl salicylate, ethyl salicylate, and glycyrrhizic acid) along with nicotine. We characterized the dissolution release of the flavor markers and nicotine from three loose MST products, same brand, each made with a distinct flavor. These products will be named Product-A, -B, and -C in the manuscript. Product-A, -B, and -C contain methyl salicylate, ethyl salicylate, and glycyrrhizic acid, respectively. Our data revealed a similar release profile for nicotine across the three loose MST products. However, distinct

release profiles were observed for the flavor markers, which can be attributed to their physicochemical properties such as polarity and solubility in the artificial saliva used during dissolution.

MATERIALS AND METHODS

We conducted the dissolution testing using a USP-4 flow-through cell apparatus (SOTAX, Westborough, MA, USA) as per our established methodology and using 12 replicates for each MST product, following FDA guidance for industry (24, 26). Nicotine and the three flavor markers were quantified using Acquity I-Class Ultra Performance Liquid Chromatography coupled to a PDA detector (UPLC-PDA) (Waters, Milford, MA, USA). The UPLC system was equipped with a BEH C18 analytical column (2.1×100 mm, $1.7 \mu\text{m}$) and a BEH C18 VanGuard pre-column (2.1×5 mm, $1.7 \mu\text{m}$) (Waters, Milford, MA, USA). The artificial saliva was prepared following the recipe provided by the German Institute for Standardization (DIN) in their standard DIN V Test Method 53160-1 2002-10 (27). The collection of USP-4 fractions and preparation of UPLC solutions and standards were carried out according to our previously published report (24). The three loose MST products used in this study are of the same brand, but each product contains a distinct flavor marker including methyl salicylate, ethyl salicylate, and glycyrrhizic acid for Product-A, -B, -C, respectively. Methyl salicylate, ethyl salicylate, and glycyrrhizic acid standards were purchased from SPEX Certiprep (Metuchen, NJ, USA). The nicotine and flavors calibration standards were prepared at the following concentrations of 0.5, 2.0, 10.0, 20.0, 50.0, 100.0 ($\mu\text{g/mL}$) for each level 1 to 6, respectively. To ensure accurate results, individual ethyl salicylate and methyl salicylate stock solutions were purchased separately for the preparation of independent calibration curves. This is because commercially available ethyl salicylate stock solution may contain a small amount of methyl salicylate impurity, which can interfere with the methyl salicylate standard. The presence of this impurity in a combined solution of the analytes can alter the slope of the methyl salicylate curve, resulting in lower concentrations calculated in samples.

Briefly, the dissolution testing and fractions collection with artificial saliva was conducted using the USP-4 apparatus in an open loop and offline configuration, comprising seven flow-through cells, a cell holder with a water bath, a reservoir and pump for artificial saliva, and a fraction collection rack. The pump delivered a constant flow of artificial saliva (4 mL/min) through the flow-through cells (22.6 mm diameter). The cells were immersed in a water bath maintained at a temperature of $37 \pm 0.5^\circ\text{C}$. Each sample cell had a 5-mm ruby bead check valve at the bottom, and approximately 6.6 g of 1 mm glass beads were added to ensure a laminar flow of artificial saliva. Loose MST products, weighing around 1 g, were directly added to each flow-through cell. Additionally, approximately 6.6 g of 3 mm glass beads were used to maintain the position and prevent floating of the tobacco product in the flow-through cell. The dissolution testing followed FDA guidance, with 12 replicates of one product and dissolution profiles taken

at intervals of up to 15 min. Each replicate was dissolved into 9 fractions. Fractions 1–5 were collected for 4 minutes each, resulting in a final volume of 16 mL per fraction. Fractions 6–9 were collected for 10 min each, resulting in a final volume of 40 mL per fraction. The total dissolution time was 60 min. After collecting all 9 fractions from each sample replicate, 0.1 mL of each dissolution fraction was added to an autosampler vial. Subsequently, 0.1 mL of ethyl benzoate (1 mg/mL) was added as an internal standard, followed by 0.8 mL of artificial saliva. The nicotine and flavor markers concentrations in $\mu\text{g/mL}$ were quantitated in all fractions collected from the 12 replicates using the previously described UPLC-PDA method (24). The concentration of nicotine and flavor markers based on sample weight ($\mu\text{g/g}$) was determined using the instrument calculating nicotine and flavor markers concentrations ($\mu\text{g/mL}$), the weight of the sample analyzed, and the volume of the dissolution fraction. The cumulative concentrations of nicotine and flavor markers ($\mu\text{g/g}$) for each tested product were calculated by summing the averaged nicotine and flavor markers released at each fraction time point from all 12 replicates. This sum represented the total amount of nicotine and flavor markers released up to each time point. The percentage relative to the total nicotine and flavor markers released at each time point was then calculated and plotted to provide the total release profile. The relative percentage to the total nicotine and flavor markers released was calculated by dividing the amount of nicotine and flavor markers released up to each time point for each fraction by the cumulative amount released in 60 min.

RESULTS AND DISCUSSION

We have enhanced our previously published analytical method by including three flavor markers, in addition to nicotine. The method remained unchanged except for the addition of these flavor markers. We validated the calibration curve, accuracy, precision, specificity, and system suitability for the determination of these flavor markers in MST products.

Figure 1 shows the LC chromatograms of nicotine, methyl salicylate, ethyl salicylate, and glycyrrhizic acid obtained in the presence of the three loose MST product extracts in fraction 1. Retention times of 5.05, 5.90, 7.73, and 8.43 min were observed for glycyrrhizic acid, nicotine, methyl salicylate, and ethyl salicylate, respectively. These retention times reflect expectations based on the chemical structures (i.e., polarity) of these compounds; methyl and ethyl salicylates feature a phenyl group along with a methyl or ethyl group contributing to their longer retention time compared to nicotine and glycyrrhizic acid (i.e., highest in polarity). The nicotine and flavor markers calibration curves were analyzed on three separate days to evaluate the calibration model, assessing the slope, intercept, coefficient of determination (R^2), and percent relative concentration residual (%RCR). The calibration curves were confirmed to be linear within the concentration range typically found in MST product dissolution fractions, indicating its suitability for analysis. The coefficient of determination (R^2) for all calibration curves over three days was ≥ 0.998 , and the

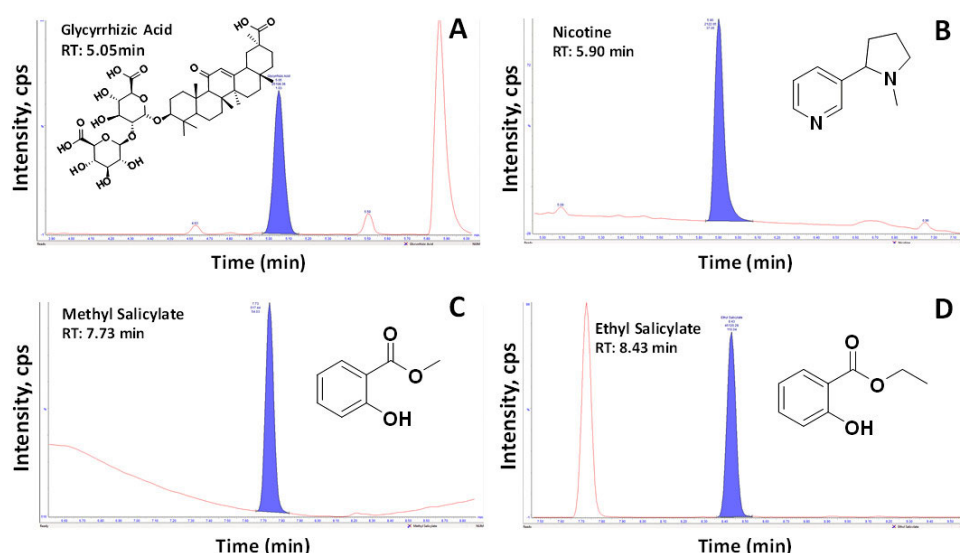


Figure 1. UPLC-PDA chromatograms of (A) glycyrrhizic acid, (B) nicotine, (C) methyl salicylate, and (D) ethyl salicylate in fraction 1 from products -C, -A, and -B, respectively. The inserts show the chemical structures of nicotine and the three flavor markers.

percent relative concentration residual (% RCR) for nicotine standards was less than 5%. To measure the accuracy of the analytical method, recovery was calculated from three fortification levels (3, 10, and 30 $\mu\text{g/mL}$) in triplicate, using pooled fractions of artificial saliva and loose MST products. To calculate the recovery, we analyzed three replicates of each fortified pooled fractions sample, along with unfortified pooled fractions samples for each product. The calculated nicotine and flavor markers recovery values for all fortification levels and matrix types ranged from 83.8% to 108%. Instrument precision, based on 10 injections of lowest standard and sample extracts, was determined to be < 2 percent relative standard deviation (% RSD). Inter-day precision was assessed by analyzing six replicates of each MST product within a single day and was determined to be $< 2\%$ RSD. Intermediate method precision, determined by analyzing six replicate samples over three days ($n = 18$ replicates), was found to be $< 10\%$ RSD. The specificity of the method was demonstrated by the absence of matrix interference in all matrices and artificial saliva, indicating its ability to accurately quantify nicotine and flavor markers in the presence of sample matrix components (Figure 1). Prior to each analytical batch, we evaluated system suitability by ensuring that the lowest standard injected five times produced a signal-to-noise ratio greater than 10 and a peak response % RSD less than 10%.

Figure 2 shows the cumulative release profiles (Figure 2A) and percent (%) of total release (Figure 2B) of nicotine from the three studied loose MST products. We have followed the FDA guidance for industry by determining the dissolution profile of 12 replicates from each studied product (25). The amount of total nicotine released within 60 min from these products varies slightly, which is expected given that these are natural products that contain tobacco leaves which have inherent variability of nicotine depending on crop year and source (Figure 2A). However,

equivalent % of total release profiles were obtained for nicotine as indicated by the overlapping release profiles (Figure 2B). To further confirm this observation, we analyzed the nicotine release profiles by calculating the difference factor (f_1) and similarity factor (f_2) by adopting a methodology referenced in the Guidance for Industry from FDA's Center for Drug Evaluation and Research (CDER) (24–26). f_1 and f_2 values were found to be 6.6 and 64.5 when comparing product-A to product-B and 3.5 and 77.6 when comparing product-A to product-C. The f_1 and f_2 values demonstrate equivalency of the nicotine release from these products with calculated f_1 lower than 15 and f_2 higher than 50. This observation was expected and confirms our previous published findings for loose MST products (24).

The quantity of flavor formulation added to MST products varies depending on the manufacturer and the product design. Each flavor formulation consists of multiple flavor compounds added at varying concentrations. In this study, we have identified a specific flavor marker for each MST product. Consequently, each product contains the flavor marker at a different concentration. It is important to note that the amount of flavor marker dissolved after 60 minutes of dissolution may not correspond to the initially added amount to the formulation. The amount of dissolved flavor markers varied in each brand with the highest amount (11.7 mg/g) for methyl salicylate in product-A and the lowest amount (1.5 mg/g) of glycyrrhizic acid in product-C (Figure 3A). The amount of ethyl salicylate dissolved over 60 min in product-B was found to be 4.0 mg/g (Figure 3A). These amounts also depend on the variable amount used for the flavor markers in each formulation. The % dissolution release profiles in Figure 3B indicate that the dissolution release rate for the flavor markers from each of the studied loose MST products strongly depends on the physico-chemical properties of the compound, namely polarity and solubility in aqueous media such as saliva.

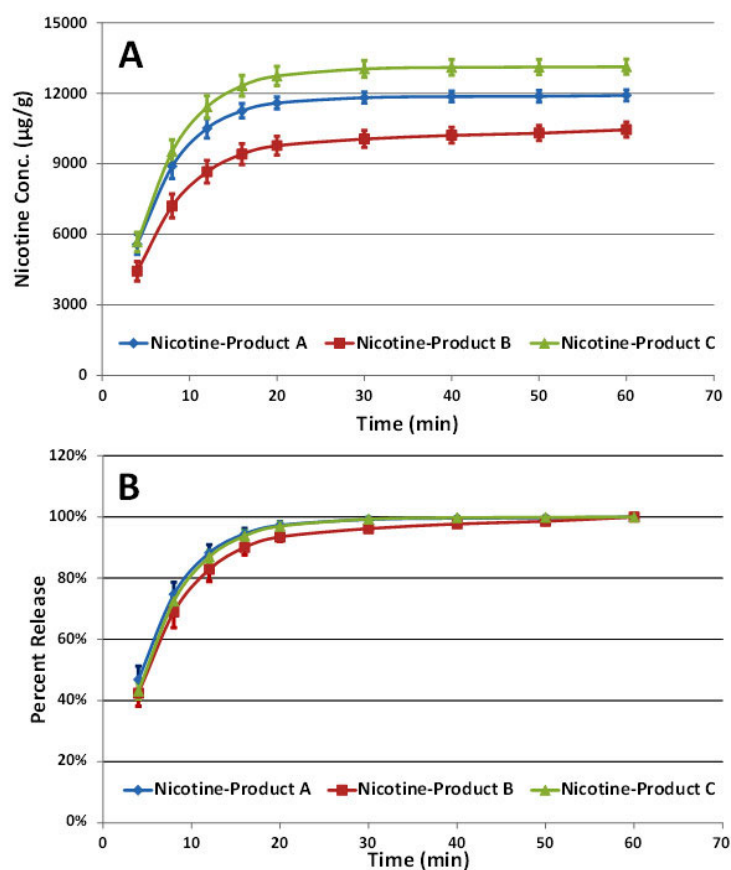


Figure 2. (A) Cumulative release and (B) percent of total dissolution release profiles (n = 12) of nicotine collected from products -A, -B, and -C. (Error Bars ± 1 SD).

In the profile region between zero and 20 min, a rapid dissolution was observed for glycyrrhizic acid and nicotine with % of total release of 95% whereas only 74% and 46% for methyl salicylate and ethyl salicylate were released from product-A and product-B, respectively. Glycyrrhizic acid and nicotine exhibited the fastest release profiles (highest polarity), while ethyl salicylate displayed the slowest release rate (lowest polarity). These findings indicate that the physicochemical properties of compounds play a very important role in their dissolution from loose MST products under the same experimental conditions.

CONCLUSION

We have demonstrated that the dissolution release profiles of nicotine are equivalent across all three tested tobacco products. In contrast, the release profiles of the studied flavor markers exhibited distinct differences, primarily influenced by their chemical properties, particularly polarity. The insights gained from this investigation can inform manufacturers and researchers on the release profiles of nicotine and flavor markers in smokeless tobacco products. Furthermore, regulatory bodies can utilize this knowledge to establish guidelines and standards for the development and evaluation of smokeless tobacco products, promoting consumer safety and informed decision-making.

AUTHOR INFORMATION

The authors declare no competing financial interest.

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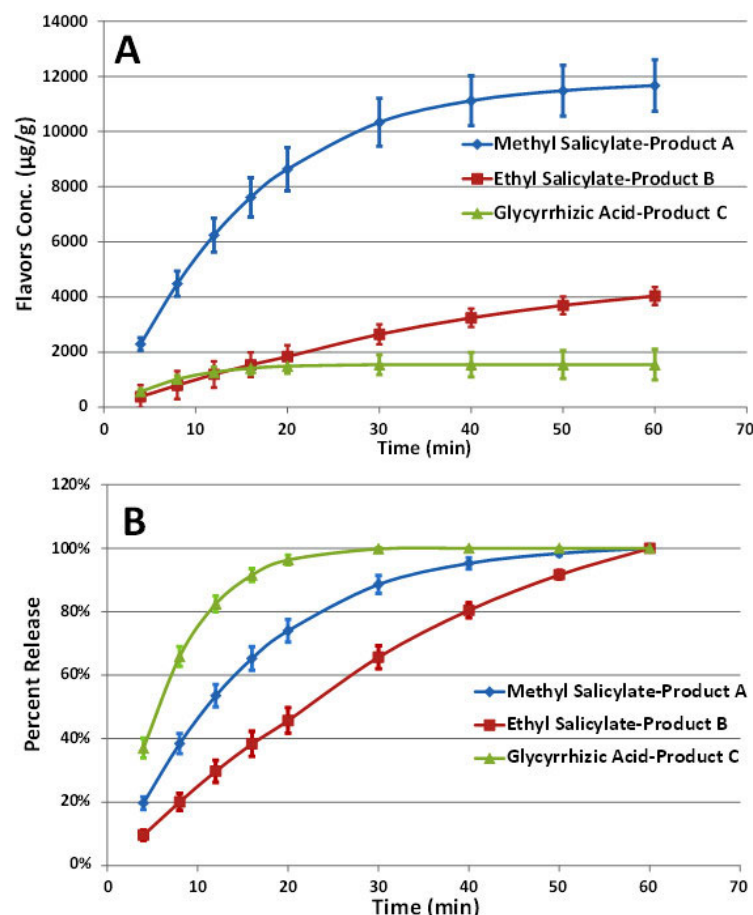


Figure 3. (A) Cumulative release and (B) percent of total dissolution release profiles (n = 12) of flavor markers collected from product-A, -B, and -C. (Error Bars ± 1 SD).

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