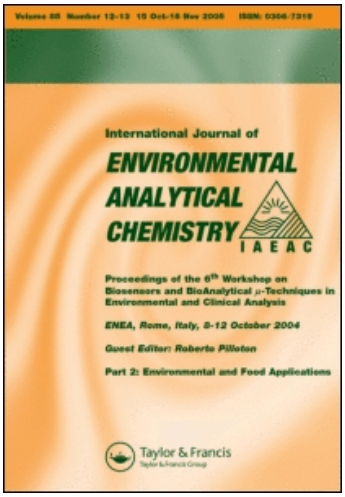

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Enzyme Inhibition and Chromatographic Techniques: Comparative Studies and Application to Pesticide Residue Analysis

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Enzyme Inhibition and Chromatographic Techniques: Comparative Studies and Application to Pesticide Residue Analysis†

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KEY WORDS: TLC, enzyme inhibition, pesticides, organophosphorus, carbamates

The development of enzyme inhibition techniques in relation to pesticide analysis is discussed, along with discussion of (a) principles of thin-layer chromatographic-enzyme inhibition (TLC-EI) technique, (b) general procedures of the technique, (c) the use of enzymes in combination with TLC and colorimetry, and (d) merits and limitations of the techniques. TLC-EI techniques and gas-liquid chromatography are compared based on sensitivity of detection, selectivity, and applicability to pesticide analysis.

The TLC-EI technique is being used and developed further in the Research Laboratories, Health Protection Branch, Ottawa for determination and confirmation of some organophosphorus and carbamate pesticides. Recently, it has been developed to detect methomyl (Lannate(R)) residues in rapeseeds, oils, and meals.

Among several analytical methods, TLC remains an integral and important part of modern pesticide residue analysis. The procedure is simple, sensitive, precise, accurate, versatile, and rapid. Besides, its cost is within the reach of every analytical chemistry laboratory. It is versatile because different modifications can be incorporated into the procedure without changing the basic

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equipment. Different types and thicknesses of gel layer, or various solvents and solvent combinations, can be used to improve the resolution and to characterize the test compounds. Various chemical reactions and visualization techniques can be used to detect the resolved compounds. With the introduction of new and improved equipment, quantitative and automated detection is also achieved.

Cook[1] reported the use of cholinestrase (ChE) to detect pesticides resolved on paper chromatograms. Several investigators[2–6] modified the technique by resolving the pesticides on a TLC plate. The spots corresponding to pesticide locations were then imprinted on a filter paper, which was soaked with a reagent solution and laid over the plate.

In 1965, El-Refai and Hopkins,[7] and Ortloff and Franz[8] simultaneously published an enzymatic technique for detection of pesticides directly on TLC plates. To delineate the locations of the enzyme inhibitors, El-Refai and Hopkins[7] used acetylcholine (ACh) salts and bromophenol-blue indicator; whereas Ortloff and Franz[8] used chromogenic substrates, 1-naphthyl acetate (1-NA) and indoxyl acetate (IA). Subsequently, Mendoza *et al.*[9] evaluated the detection sensitivity of the TLC-EI technique. Besides IA and 1-NA, IA derivatives were also used as enzyme substrates to detect some organophosphorus pesticides and carbaryl. The technique was then developed to screen for organophosphorus pesticides in plant extracts without elaborate clean-up.[10] Different types or sources of enzyme had been used to obtain greater sensitivity and specificity of detection.[9–12] Likewise, chemical derivation using bromine, u.v. light, ammonia, alcohols or other chemicals was used to improve detection and to characterize the compounds.[7,10,13,14] A comprehensive review on TLC-EI techniques was published in the Residue Reviews.[15] A continuation of the review is in preparation. The TLC-EI technique had been used in the analysis of pesticide residues in foods and air,[16] river water,[17,18] well water,[19] and in metabolic studies.[15,20–22] More recently, the technique was successfully used in combination with GLC to confirm and quantitate some carbamates.[23]

MERITS OF THE TLC-EI TECHNIQUE

The TLC-EI technique has advantages that match or surpass those of other methods. (a) It can be used for simultaneous determination of various pesticides. (b) It can be used for simultaneous determination of parent compounds and their metabolites. (c) It detects organophosphorus and carbamate pesticides at picogram to nanogram levels. (d) It can be used for rapid screening and simultaneous analysis of several samples. (e) It is useful in the determination of purity of pesticides used in toxicology and enzymology.

(f) It is a useful tool in metabolic studies. (g) It can be used for samples containing interference too great for GLC. (h) It can be used as confirmatory and semi-quantitative procedures. (i) It can be used to isolate a compound prior to GLC analysis.

LIMITATIONS OF THE TECHNIQUE

The sensitivity of the technique is limited by or varies with the following factors: (a) pesticide chemical properties; (b) type source, or concentration of enzymes; (c) concentration of the enzyme substrate; (d) pH of the spray solution; (e) type of gel; (f) thickness of the gel layer; (g) temperature and humidity of the chromatographic room; (h) degree or quality of resolution; (i) interference due to co-extractives from samples or solvents; (j) treatment (*e.g.*, bromine, ammonia, or u.v. light) used before TLC analysis.

PRINCIPLES OF THE TLC-EI DETECTION

The mechanisms of the enzyme-inhibitor reaction on TLC plates are as follows:

$$1) \quad E+S \rightleftarrows ES \rightarrow E+P$$

$$2) \quad E+I \rightleftarrows EI+S \rightarrow \text{no } P$$

TABLE I

Characteristic colours of spots and backgrounds obtained from some enzyme substrates

Substrate	Colour of	
	Spot	Background
1-Naphthyl acetate	white	mauve
Indophenyl acetate	white	blue-mauve
Indoxyl acetate	white	blue
5-Bromoindoxyl acetate	white	blue
5-Bromo-4-chloroindoxyl acetate	white	turquoise
5-Bromo-6-chloroindoxyl acetate	white	pink
Indoxol or 5-bromoindoxol + Fast Blue RR	white	mauve
Acetylcholine + bromophenol blue	blue	pale yellow

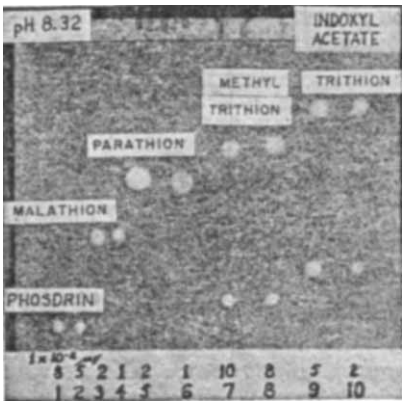

FIGURE 1 A typical chromatogram showing the locations of enzyme inhibitors appearing as white spots against a colour background. The colour obtained after indoxyl acetate hydrolysis is blue. The beef liver extract used was in pH 8.32 tris-HCl buffer. Numbers at the bottom row indicate the spot origin and those at the next row, the amount spotted in 10^{-2} ng.

E = enzyme, S = substrates, I = inhibitor, and P = substrate hydrolytic product. When the enzyme is reacted with an inhibitor, its active sites are blocked and cannot catalyze the substrate hydrolysis. Therefore, no hydrolysis product will be obtained from Reaction 2. The area on the plate where the inhibitor is located will appear as a spot against a uniform background. Table I shows the characteristic colour of spots and backgrounds obtained with different enzyme substrates. Figure 1 shows a typical chromatogram that was sprayed with enzyme and substrate solutions.

COMPARISON BETWEEN TLC AND OTHER CHROMATOGRAPHIC METHODS

In 1968, Hill[24] compared TLC, GLC, paper chromatography (PC) and column chromatography (CC) based on several parameters. Since then, rapid improvement and sophistication in design and capability have been made to these methods. In the advent of new designs in detectors, TLC becomes a more quantitative procedure; CC was modified to what is now known as liquid-liquid chromatography (LLC) to become a quantitative and rapid method. Table II shows an updated comparison of chromatographic techniques.

Table III illustrates general patterns for GLC using six types of detectors and for TLC using enzyme inhibition techniques. It indicates that electron

TABLE II

Comparison of chromatographic methods

Factors	TLC	GLC	PC	CC	LLC
Speed	Fast	Slow-fast	Slow	Slow	Fast
Sensitivity of detection	Fair-high	High	Low	—	High
Resolution	Fair-high	High	Fair-high	High	High
Capacity	High	Low-high	Low	High	Low
Operating temperature	Room	High	Room	Room	Room
Analytical Capability	Semi-quantitative	Quantitative	Semi-quantitative	Preparative	Quantitative
Cost	Low	High	Low	Low	High

TABLE III

General sensitivity patterns for GLC and TLC-EI techniques[a]

Types of compounds	GLC						TLC-EI
	FID	EC	P	S	N	CL	
−P(S)O−	+	+++	++	+	−	−	±
−P(S)S−	+	+++	++	++	−	−	±
−P(O)O−	+	+	++	−	−	−	+++
−P(O)S−	+	+	++	++	−	−	+++
Carbamate	+	+	−	−	++	−	+++
OC	+	+++	−	−	−	+	−

[a] −, not detectable; +, low sensitivity; ++, fairly sensitive; +++, very sensitive.

capture (EC) detection is generally a very sensitive method for the P(S) and organochlorine groups of pesticide. Flame photometric detectors are specific to P or S. However, false P response can be obtained when a large amount of S is present. The flame ionization detector (FID) is not as sensitive as the EC detectors; however, sensitivity and specificity may be improved by using a salt-tipped flame ionization attachment. This attachment greatly and selectively increases the response to P or N compounds. Microcoulometric detectors can be modified to obtain specificity to N-, S-, or Cl-containing chemicals. Similarly, the TLC-EI technique is very sensitive to P(O) and carbamate pesticides. It does not usually detect the P(S) and OC compounds. Additional selectivity may be introduced to this technique by derivatization

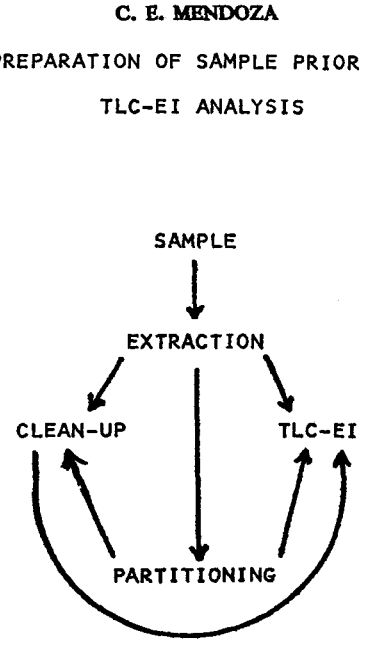

FIGURE 2 Diagrammatic representation of sample preparation prior to TLC-EI analysis.

of pesticides before analysis or by using a particular type of enzyme or enzyme substrate.

SAMPLE PREPARATION

Figure 2 shows a flow diagram illustrating the preparation of a sample prior to TLC-EI analysis. It is evident in the diagram that some samples can be analyzed without clean-up or elaborate partitioning. Others have to be partitioned into another solvent to separate the pesticides from plant extractives or from other types of pesticides. An example of this situation is the partitioning of P(S) compounds into hexane from the aqueous extracts. This procedure leaves behind polar compounds such as P(O) and carbamate compounds and some plant extractives. Intensely coloured carotenoid materials are extractable by hexane. However, these do not interfere with the TLC-EI detection. They are readily destroyed by bromine, which is used to convert P(S) to P(O) or enzyme inhibiting analogues. Other types of samples require a good clean-up procedure to improve the detection limit and quality. Some of the disadvantages associated with the clean-up procedure are as

TABLE IV

Characteristic inhibition of esterases from different livers, by pesticides[27]

Chemicals[a]		Beef	Sheep	Pig	Monkey	Chicken
(1) Carbaryl (1 ng)	NB	O	O	+	O	O
	B	+	+	+	O	+
(2) Dichlorvos (8 ng)	NB	+	+	+	+	+
	B	+	+	+	+	+
(3) Ethion (10 ng)	NB	+	+	+	+	+
	B	+	+	+	+	+
(4) Oxydemeton-methyl (50 ng)	NB	O	O	O	O	O
	B	+	+	+	+	O
(5) Demeton (50 ng)	NB	O	O	+	+	O
	B	+	+	+	+	O
(6) Demeton sulfone thiol isomer (50 ng)	NB	O	O	O	O	O
	B	+	+	+	+	O
(7) Dimethoate (10,000 ng)	NB	O	O	O	O	O
	B	+	+	+	+	+
(8) Dimethoxon (10,000 ng)	NB	+	+	+	+	+
	B	+	+	+	+	+

[a] Amount used follows each name; NB = no bromine, B = with bromine, O = not detectable, + = detectable.

follows: (a) time consuming, (b) loss of residues, (c) a greater chance of encountering more interference from solvents and clean-up columns.

COMPARATIVE STUDIES OF THE TECHNIQUE UNDER DIFFERENT CONDITIONS

Table IV shows the difference in inhibition patterns of beef, sheep, pig, monkey, and chicken liver esterases by seven organophosphorus pesticides and carbaryl. Beef and sheep liver esterases also had identical inhibition properties. Pig and monkey liver esterases had identical patterns except for carbaryl. The monkey liver esterase was not inhibited by carbaryl at the level used, whereas that of pig was sensitive to carbaryl with or without bromine treatment. These two types of esterases were inhibited even by demeton P(S) analogue. It is possible that the enzyme preparations contained components which were capable of converting demeton to a P(O) analogue (*c.f.* parathion conversion to paraoxon by beef liver extracts[12]). Another possibility is that these esterases were naturally sensitive to both demeton P(S) and P(O) analogues. Chicken liver esterase was neither inhibited by oxydemeton-methyl, demeton, or demeton sulfone thiol isomer at the levels used. Ethion

TABLE V

Detection levels (ng) and effects of u.v. on carbamates as determined by a TLC-EI technique using pig liver esterase and 5-bromoindoxyl acetate[2]

Chemicals	Control	Br	U.v.[b]
Carbaryl	0.1 (p)[a]	0.05 (±)	decreased (0.1-10)
Promecarb	0.5 (±)	0.5 (+)	no change (1–10)
C-8353	5.0 (±)	5.0 (±)	decreased (5–100)
Meobal(R)	1.0 (±)	1.0 (±)	no change (1–10)
Butacarb(R)	0.1 (±)	0.1 (±)	no change (1–10)
Methomyl	10.0 (±)	10.0 (±)	decreased (50–1000)
Formetanate (HCl)	10.0 (±)	1.0 (±)	decreased (5–1000)
Mesurol(R)	0.1 (±)	5.0 (±)	decreased (0.1–50)
Aldicarb	25.0 (±)	500.0 (±)	decreased (100–1000)

[a] (+) = spot lasted more than 5 min; (±) = less than 5 min.
[b] Figures in parentheses denote quantity (ng) ranges used in u.v. tests.

inhibited all five types of esterases even without bromine treatment because it readily transformed to a P(O) analogue under ordinary lighting in the chromatographic room (*c.f.* ethion conversion to ethoxon[12]). The inhibition responses due to dichlorvos and dimethoxon were expected because they were both P(O) analogues.

Table V shows the detection limits for nine carbamates, with or without bromine or u.v. exposure, obtained by a TLC-EI technique using pig liver esterase and 5-BIA. The limits ranged from 0.05 ng for carbaryl to 500 ng for aldicarb. The detection sensitivity after bromine treatment was increased for carbaryl and formetanate, decreased for aldicarb and unchanged for the other carbamates. U.v. irradiation had no effect on promecarb, Meobal(R) and Butacarb(R). However, it decreased the detection sensitivity for the other six carbamates.

The use of freeze-dried liver extracts in the TLC analysis of pesticides is shown in Table XI. Although slightly less sensitive than frozen extract, freeze-dried extracts had advantages that should be considered. One litre of frozen extracts could be freeze-dried and reduced to only a few milligrams.

TABLE VI

Comparison between the frozen and freeze-dried enzyme preparations based on detection limits (in nanograms) of carbamate pesticides[12]

Compound	Liver extract			
	Pig		Steer	
	Frozen	Freeze-dried	Frozen	Freeze-dried
Aldicarb	50	100	100	100
Banol	0.5	5	5	10
Carbaryl	0.1	0.5	0.5	5
Carbofuran	1	50	100	100
Carbofuran 3-OH	10	100	100	400
Mesural	0.1	10	10	10
Tranid	100	500	300	100
Zectran	50	50	50	50

The powder required only small containers and readily dissolved in water or buffer solutions. The freeze-dried material could be handled and transported safely and conveniently, unlike the bulky frozen extracts.

Table VII shows an application of the TLC-EI technique for the determination of pesticide residues in peas and carrots. Pig and beef liver esterases were found insensitive to malathion and carbofuran 3-OH, respectively. Parathion was readily detected by beef liver esterase even without bromine treatment because it was converted to P(O) analogue by this enzyme preparation.[12]

Table VIII shows the detection limits for methomyl added to rapeseeds, oils, and meals. The limits were from 0.01 to 0.03 ppm or 40–50 ng. The procedure used for extracting methomyl was described by Mendoza *et al.*[25]

TABLE VII

Detection ratings[a] using pig or beef liver extract-indophenyl acetate sprays for four pesticides added to plant extracts without elaborate clean-up[12]

Type of sample[b]	Parathion	Malathion	Carbaryl	Carbofuran 3-OH
Peas	+(+)	n.d.(+)	+(+)	±(n.d.)
Carrots	+(+)	n.d.(+)	+(+)	±(n.d.)

[a] Based on detections of 20 to 1000 ng per origin spot or 0.1 to 7.25 ppm. + = spots corresponding to pesticides persisted beyond 1 hr; ± = barely visible spot, n.d. = no detectable spots. Symbols in parentheses refer to ratings for beef liver esterase detection.

[b] Test based on three extraction methods: (1) 10% methanol in chloroform, (2) dichloromethane, and (3) acetonitrile, and then partitioned into hexane.

TABLE VIII

Detection limits (in ppm) of methomyl added to rape seeds, meals and oils

	Seeds	Meals	Oils
Limits	0.03	0.01	0.01
Range tested[a]	—	0–20	0–20

[a] Concentrations were 0, 0.01, 0.05, 0.5, 1, 5, and 20 ppm.

TABLE IX

Characteristic mobilities and detection limits of three carbamate pesticides

	Carbaryl	Methomyl (syn)	Methomyl isomer (anti)
hR_f	24.3±1.3	6.5±0.5	18.2±0.2
$hR_{carbaryl}$	100	26.0±1.9	74.2±2.4
TLC limit:			
5-BIA	0.1 ng	10 ng	300 ng
IPA	0.05 ng	10 ng	200 ng
GLC limit:			
EC[a]	0.21 ng	0.21 ng	0.21 ng
MC-N[b]	—	20 ng	—
MC-S[c]	—	2.5 ng	—
EC-N[d]	—	4.3 ng	—
FID-S[d]	—	8 ng	—

[a] Detected as DNPMA, 1 cm peak height, 137 cm × 5.7 mm i.d. column packed with Chromosorb W-HP coated with 4% SE-30 and 6% QF-1, 212°C oven. EC = electron capture detector.
[b] Detected as methomyl, 180 cm × 2 mm i.d. column packed with Gas Chrom Q coated with 4% OV-101 and 6% OV-210, 160°C oven[26] MC–N = microcoulometric–nitrogen detector.
[c] Detected as oxime at 0.02 ppm based on 25-g plant samples, 122 cm × 5.7 mm column packed with Chromosorb W-HP coated with 10% FFAP, 200°C oven.[28] MC-S = microcoulometric sulphur detector.
[d] Detected as oxime, 1 cm peak height, 137 cm × 5.7 mm i.d. column packed with Chromosorb W-HP coated with diethylene glycol succinate, 160°C oven.[29] EC-N—electrolytic conductivity—nitrogen detector. FID-S = flame ionization—sulphur photometric detector.

Fechner *et al.*[16] used the technique to determine 0.5 to 5 mcg of dichlorvos per cubic meter of air.

Table IX illustrates the characteristic mobilities and detection limits of carbaryl, methomyl and methomyl isomer. Methomyl and its isomer are readily resolved by 20% acetone in hexane on plates coated with a 0.5-mm layer of silica gel G-HR. The data also show that the isomer is 3000 to 4000 times less inhibitory to the pig liver enzyme than carbaryl and is 20 to 30 times less inhibitory than methomyl. EC detection was shown for carbaryl, methomyl, and methomyl oxime. Because of the difference in molecular weights, 0.16 ng of methomyl or about 0.21 ng carbaryl is required to obtain about 0.21 ng dinitrophenylmethylamine (DNPMA). Limits obtained by other GLC detectors are shown for methomyl syn isomer only. The detections were based on oxime, or intact methomyl. Williams[26] reported that 20 ng could be measured readily; however, the actual peak height or area was not mentioned. Based on the published graph 20 ng methomyl gave approx. 6% of the full-scale deflection of the recorder pen. Detection based on DNPMA was 12 to 20 times more sensitive than those based on oxime sulfur and nitrogen, respectively.

TABLE X

Per cent recovery of methomyl spotted and enzymatically detected on TLC plates, or "spiked" to gel extracts[a]

	Unsprayed plate		Sprayed plate		
	Control	Spiked	PA	5-BIA	Spiked[b]
Percentage	101	111±1	92±3	91±2	107±3
Number tested	1	3	6	4	5

[a] TLC plates coated with 0.5 mm thick layer of silica gel G-HR and sprayed with pig liver extracts. IPA = indophenyl acetate, BIA = 5-bromoindoxyl acetate.
[b] Pooled values for IPA and 5-BIA.

The data in Table X show the per cent recovery of methomyl spotted and enzymatically detected on TLC plates. Methomyl was scraped off the plate, hydrolyzed, and reacted with 1-fluoro-2, 4-dimitrobenzene (DNFB) to obtain DNPMA. GLC analysis of DNPMA shows 91 to 111% recovery of methomyl from silica-gel plates.

Figure 3 shows typical gas-liquid chromatograms of DNPMA. After the reaction of DNFB and methylamine, the product DNPMA was extracted into benzene and cleaned up through silica-gel G-HR columns. Details of this procedure have been published recently.[31]

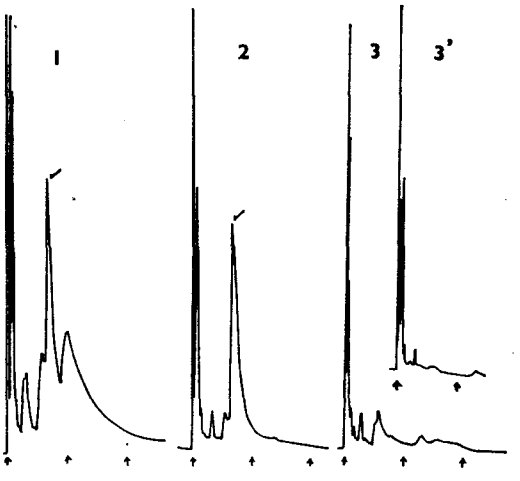

FIGURE 3 Typical GLC-EC chromatograms of DNPMA. The distance between two arrows indicates a 9-min interval. (DNPMA was obtained after reaction of DNFB and methylamine derived from 10 ng methomyl.)

1) DNPMA extract without micro-column clean-up. The peak with a check mark indicates the peak corresponding to DNPMA.

2) DNPMA peak after column clean-up.

3) and 3', Chromatograms of the reaction of blank extracts after micro-column clean up.

TABLE XI

Comparison between TLC-EI detection limits[2] and pI_{50} at 7.5 min reaction time for some carbamates[a,b]

Chemical	TLC limit (ng)	pI_{50} at 7.5 min
Carbaryl	0.05	6.57
Mesurol(R)	0.05	7.16
Promecarb	0.5	7.62
Meobal(R)	0.5	—
Butacarb(R)	0.5	8
C-8353	5	—
Formetanate (HCl)	5	4.64
Methomyl	10	5.09
Aldicarb	25	5.77

[a] $pI_{50} = -\log_{10}(I_{50})$, where I_{50} is the concentration of inhibitor required to give 50% inhibition of enzyme activity at specified conditions.
[b] IPA substrate and pig liver esterases in 0.05M buffer were used at 32°C and pH 8.2.

The sensitivity or ability of some carbamates to prevent pig liver esterase from hydrolyzing IPA sprayed on TLC plates concurred with that obtained colorimetrically (Table XI). Pesticides with low detection limits also gave high pI_{50} values, indicating that they were strong enzyme inhibitors. These results suggest that the method should be useful in toxicological investigations.

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