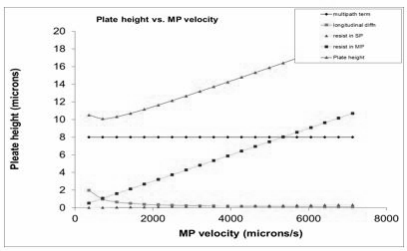
Basic Theory

Liquid Chromatography	MP → Liquid ▸ Actively participate in equilibrium process
	SP → Quasi/porous solid ▸ Most common do reverse-phase chromatography SP ▸ Film thickness → Very small (monolayer)
	D_m 10D_s ▸ B/U 0
	d_f 0 ▸ $C_s U$ 0
	Always carried-out in packed columns
Advantage	More versatile than GC
	Adaptable to needs
Disadvantage	Much less efficient than GC
	Diffusion coefficient of analyte is orders of magnitude smaller than in GC ▸ Bounced into other molecules →Diffusion rate is small
	In liquid phase (not gas)
Improve efficiency	Use small particle → Narrow range velocity ▸ Dependent on particle diameter (d_p) ▸ Smaller particle size = smaller plate height at any given velocity
	Compensate for travel distance of analyte to reach surface of SP ▸ Minimize the space between the particles that the analyte have to diffuse across
	Analytical LC → < 5μm
	HPLC → >3μm
	UHPLC → < 3μm

Theory Equations

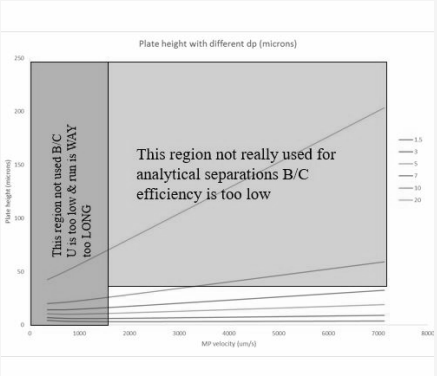


$$A = 2\lambda d_p \qquad \frac{B}{U} = \frac{2\gamma D_m}{U} \approx 0$$

$$C_s U = \frac{f_s(k')d_f^2}{D_s} U \approx 0$$

$$C_m U = \frac{f_m(k')d_p^2}{D_m} U$$

LC Velocity Range



Column

Packed Column	Packed full of particles
	Usually composed of stainless steel, etc.
	Length ▸ 2-20cm(analytical) ▸ Large column → Use for preparative scale ▸ Small column → Packaged inside a capillary (75-100um diameter) → Couple efficiently to MS

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Column (cont)

Efficiency (N)

- 3000-20000
- Dramatically lower than GC
- Rule of thumb (GUESTIMATE ONLY): N~ 3500 *L(cm) / d_p(um)

Sample Capacity

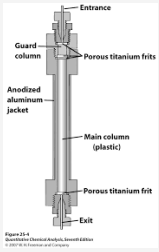
- Depending on size of column and packing
- 5mg/g {[fa-arrow-right]} C18/silica
- 10mg → "vanilla" column

Thickness of SP

- ~ 1-2nm

Resistance to mass transfer in MP and multipath terms dominate

HPLC Column



HPLC System

MP reservoirs	Stores MP in inert glass bottles - Platic coated Pyrex bottle (common) → $300/pc
	Degas solvent - Add element for filtering/degas - Minimize amount of oxygen dissolved into MP → Oxygen reactive in high pressure (increase oxidation of analyte) - Small bubbles can form → Result intensive undesirable peaks
	Connected to a computer(pump) - Control mixing value to produces desired MP mixture

HPLC System (cont)

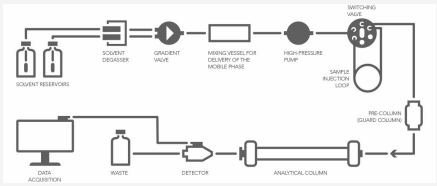
Analytical Column	Wide variety - Diameter → ~0.5cm (general) - Length → 10-20cm (general)
Injector	Manual - Syringe with sample - Inject needle into port and release - Liquid flow into loop (at atmospheric pressure) - When rotate lever to 60 degree → Rearrange injector (set of valves) - Switches loop into flow path = swept down into analytical column
	Operates at very high pressure - If inject sample into septum → Shatter syringe
	Autosampler - Prepare in vials → Tightly seal for sample to not evaporate - Program computer - Runs separation overnight - ~100 samples
Detector	Record data and integrate peak area - Quantitation
High-Pressure Pump	Direct MP through system
	Use high pressure - Analytical column is filled with fine particles
Dynamic Mixer	Needed to blend the different fluids (MP)
	Provides the correct percentages of fluids dynamically as the separation goes

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HPLC System (cont)

Guard Column	Avoid column killers ▸ Species that strongly store in SP → Never eluted ▸ Contaminated column ▸ Can change separation → Destroy separation in terms of its analytical quality
	Very small column ▸ Contains same type of SP as analytical column
	Contamination is trapped inside ▸ Periodically replace cartridges → Preserves analytical column
	Optional
Narrow Bore Tubing	Tubes that connects components
	Has to be rated for HPLC ▸ Can handle high pressure ▸ Has to be narrow bore → Don't want MP to mixing + dilute sample peaks
	Use short length as much as possible
	If fitting not installed correctly ▸ Can result in dead volume → Analyte that gets trapped in dead volume = gets broaden
Thermostat Oven	Constant temperature ▸ 30-45 degrees ▸ For a given MP → Equilibrium is constant
Fraction Collector	Robots that periodically move tubes of the eluted species from detector
	Collect in vials ▸ Sophisticated → Deposit 1 peak per vial ▸ Less sophisticated → Periodically move from one vial to the next

HPLC System Diagram



Stationary Phase

Control Retention	Control retention → Control distribution constant(K)
	Control by: ▸ Adjust type of MP and SP ▸ Adjust "strength" of MP and/or SP ▸ Add additives to MP → Interact specifically with analyte, SP, MP ▸ MP velocity → Does not alter retention (K or K')
Stationary Phase	Most use silica support particles ▸ Not great in high pH → Use alumina(high pH resistance) ▸ Low pH → SP can come off of support (hydrolyzed) → Use polymera support
	Almost always a "pure" SP ▸ Not mixed
	Use chemistry reaction to anchor SP to wall/surface
	Almost always a "monolayer" SP ▸ $d_f \sim 0$
	Wide range of polarities ▸ Use chemical reactions to adjust the SP
	Can use chiral SP ▸ Separate enantiomers ▸ Reasonable environment conditions

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Stationary Phase (cont)	
Silane Reaction	Use to anchor/bond silicones to silica surfaces ▸ In packing materials (particles) ▸ FS capillaries
	Use to deactivate silanols
	Silanol ▸ Very reactive ▸ Highly polar ▸ Expose on surface of silica
	Deactivate silanol ▸ Use chloro silane ▸ Ex: C18 ▸ Result in silanization of surface
	Residual silanol ▸ SP is usually of a different polarity (non-polar) ▸ Results in tailing of analytical peak
	After reacting surface with SP ▸ Use a short chain alkyl ▸ Take care of residual silanol
	If silanol peaks present ▸ Column is old ▸ Molecules of SP are desorbs or removed from surface
Silanol Interactions	"Standard" silica → SP support particles ▸ Has silanols on surface → Si-OH ▸ ~50% of Si-OH are reacted to Si-O-Si-C18

Stationary Phase (cont)	
	Residual Si-OH ▸ When close to a metal in the silica ▸ Are "acidic" and deprotonate easily leaving a $Si\text{-}O^-$ on the surface
	Act as ion-exchange sites for basic analyte ▸ Reverse-Phase MP is not suited to ion-exchange separation ▸ Very poor peaks are obtained for basic analyte → Tailing peak
	Alternative options ▸ Use a high purity silica column → Less acidic silanol ▸ Purchase a deactivated silica column
	Affects only basic compounds ▸ Neutral and acidic compounds does not show tailing
Particle and Surface Area	Terms that dominate: ▸ Overall plate height ▸ Overall plate number ▸ d_p → VD eq.: A and C_mU
	Spherical Particle ▸ Surface area/Volume scales with $1/d_p$ ▸ ↑ A/V = ↓ Retention ▸ More SP packed = ↑ Retention (K') = ↑ Resolving Power (R')
	Size ▸ Nearly all SP are um scale silica particles ▸ Impacts VD equations

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Stationary Phase (cont)

	Porous Particles ▸ ↑ Surface area per particle ▸ ↑Amount of SP inside column ▸ ↑ Retention and sample capacity = Better R' ▸ Smaller the pore→ the larger the surface area/g of support
	Diffusional Trap of small pores ▸ Loss of analyte → Tailing peaks ▸ Large MW analyte go into small pores → Never gets eluted
	Separation molecules ▸ Small molecules 80Å ▸ Large proteins 120-300Å
Normal Phase (NP)	Developed initially ▸ Used raw silica as SP → Polar silanol
	Separation based ▸ Polar-polar interactions with silanol ▸ Non-polar elute earlier ▸ Polar analyte elute later
	In general ▸ MP is opposite polarity to SP ▸ Works well for polar species only
Reverse Phase (RP)	Use non-polar SP → Silane reaction ▸ Use polar MP (Water based)
	Separation based ▸ Nonpolar-Nonpolar interactions ▸ Reverse separation of normal phase ▸ Polar species elute first ▸ Non-polar species elute later

Stationary Phase (cont)

More popular

▸ Organic solvents used for MP → Expensive/dangerous

▸ Most analytes are made our of biological origin → Soluble in water-based MP

Controlling Retention

SP Polarities	Retention depends on ▸ Mass of SP ▸ Type of SP
	Mass ▸ Control by chain length ▸ Density of SP on silica→ % of silanol reacted ▸ Surface area →Porisity
	Selectivity depends on ▸ Type of SP ▸ Chain length ▸ Linker type/length
Chain Length	↑Chain length and/or % organic (carbon) load of SP = ↑ Retention (K')
	Example ▸ C4 → C8 chain ▸ Double chain length → Double volume of SP → Double retention
	If within the same type of SP ▸ Ex: Alkyl chains ▸ No significant changes in selectivity ▸ Only shrinking/expanding the chromatogram about t m ▸ Shrink c-gram = reduce carbon ▸ Stretch c-gram = Increase carbon load

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Controlling Retention (cont)

	If analyte is not retained ▸ Increase % carbon of SP/chain length
	If analyte is excessively retained ▸ Reduce % carbon
	Significant changes in selectivity → Resolving power ▸ By changing the type of SP ▸ Overall retention should remain roughly constant ▸ Keeping the % carbon constant
Effect of MP Strength	MP plays an active role in retention ▸ Distribution constant
	Common solvents can be sorted according to their polarity
	Polarity of MP ▸ Main factors of controlling K → K'
	When changing MP strenght ▸ Can calculate the retention under new MP
	Equation (Only for RP): ▸ K'new/K'old= 10((P'new-P'old)/2)
	Equation (Polarity) P'MP= Weighted Polarity = %A*PIA + %B* PIB ▸ PI= Polarity
	Equation (Only for NP): ▸ K'old/K'new= 10((P'old-P'new)/2)
Example	1. Look at K' of first and last peak ▸ K'old = (last peak - first peak)/first peak →K' = (2.8-1.8)/1.8 = 0.5 ▸ K'new → Want it at 10
	2. Replace terms in equation ▸ K'new/K'old = 10/0.5 = 20

Controlling Retention (cont)

	3. Old MP polarity ▸ If old MP is 20% water/80% Acetonitrile ▸ P'old= (0.2)(10.2)+(0.8)(5.8) = 6.68
	4. New MP polarity ▸ P'new = 2log(K'new/K'old) + P'old ▸ P'new= 2log(20) +6.68 = 9.28
	5. Solve new MP components ▸ P'new = (x)(10.2) + (1-x)(5.8) = 9.98 ▸ 79.1% water and 20.9% ACN
	Rule of 3 ▸ Tool to check/estimate results (not used in calculations) ▸ Change of 20% water ~ 3x change in K'
MP Gradient	Dynamically adjust MP
	Some sample contain wide range of analytes ▸ Low or high retention ▸ no single MP that will elute them all in a satisfactory range of k’
	MP gradient ▸ MP strength is initially "weak"→Analyte well retained ▸ Those with low retention→Elute at reasonable K' ▸ Strengthen MP over the course of separation ▸ Strongly retained species can be eluted→At a reasonable K" and R'
	MP≠constant → Changing strength ▸ K and K' ≠ constant ▸ Can no longer be predicted

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Controlling Retention (cont)

MP Selectivity

Alter selectivity (α) by changing type of solvent

Resolving power

- R= (α-1)(K'/(1+K'))($\sqrt{N}$/4)
- Sensitive to selectivity → Critical pairs in peaks

Selectivity

- Depends on nature of MP
- Change selectivity = change type of MP

Try and keep

- P'old~P'new
- Overall retention is roughly the same
- Selectivity of peaks change

Selectivity changes cannot be predicted

Chiral Separation

Basic Theory

Important to bioanalyses and pharmaceutical separation

Separation of chiral species

- Enantio selective

Chiral SP or chiral additives to MP → Separation of enantiomers

Possible to separate structural isomers → Strength of interaction changes as a function of isomer

Ion-Exchange

Ionic Species

Small "hard" ions

- Inorganic ions
- Cannot use ion pairing
- Ions can interact with appropriate SP → Ionic SP

Ion-Exchange (cont)

Basic Theory

Ion-exchange

- Equilibrium-based separation
- Discreet soption and displacement process
- Carry throughout column

Column

- Does not use silica particles
- Use polymer resin
- Attach with a strong anion or cation

Example

Using a strong anion SP

- Cation exchange column

1. SP sulfonic acid (IEX resin) → Anion surface particle (SO_3^-)
   - Wash column with acid solution {{fa-arrow-right} Cations (H^+)
   - Sulfonic acid is protonated (SO_3H)

2. Inject sample with cation analytes → Metal ion (M^{2+})
   - Metal ions interact with SP
   - Metal ions displace some of H^+ from resin

3. Unbind analyte from SP
   - Introduce a higher concentration of protons behind analytes → MP gradient
   -
   - H^+ displace weakly bound analytes the move onto strongly bound analyte (cation) → Exchange process
   - Cation analytes is displace off of surface and solubilize in MP

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Ion-Exchange (cont)

	4. Analytes move down the column in strength of MP ▸ Each type of analyte elute as a peak ▸ MP ahead of each analyte is too weak → Bound ▸ MP behind each analyte is too strong → Fully displace
Equilibrium Constant	If it behaves like an equilibrium → There is an equilibrium constant ▸ Expect to behave like an LC → Produce peaks
	Ion-exchange equilibrium constant would behave like a distribution constant ▸ Obtain similar result of chromatogram peaks ▸ MP controls retention
	Equation ▸ K_{iex}=[exchange&analyte]$_s$/[analyte]$_m$ = C_s/C_m

Optimization

Process of Separation	1. Carry out initial separation ▸ Choose a strong MP ▸ Ensure everything is eluted and fast separation
	2. Adjust MP strength ▸ Retention of last peak is within the right region ▸ Depending on the complexity of sample ▸ Simple sample → K' 10 ▸ Complex sample → K' 20 ▸ Do calculations for an estimate adjusting needed

Optimization (cont)

	3. Examine if peaks are within the acceptable region ▸ Examine if all analytes are well resolved
	4. Consider if a gradient is required ▸ Presence of large area of empty baseline
	5. If needed ▸ Switch MP type to alter selectivity ▸ Gradient to reach acceptable retention and resolution
	6. Consider using additives in MP ▸ Help alter selectivity
	If MP type/mix strength does not achieve required separation ▸ Change SP type ▸ May consider type of separation
Summary of MP Effects	Very powerful tool → Versatile ▸ Control retention and selectivity
	Directly affects distribution constant
	↑ MP strength = ↓ K'
	MP "strength" is polarity ▸ Effect are opposite in RP vs NP ▸ RP → Non-polar solvent (organic) = Stronger solvent ▸ NP → Polar solvent = Stronger solvent
	Ramped MP → Gradient ▸ Helps dynamically adjust K'
	Useful to make separation less intuitive → R↓ = K' ↓ ▸ Gradient runs → R ↑ = K' ↓

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LC Detectors

Ideal Detectors	High sensitivity ▸ Steep slope
	High stability ▸ Minimal drift ▸ Minimal noise on baseline
	Very low DL
	Long LDR
	Can accept MP over wide range ▸ Need reference to null out MP gradients
	Fast response ▸ Independent of MP
	Easy to use, maintain and repair
	Inexpensive
	Selective/universal ▸ Can be either depending on properties
	Non destructive ▸ Can collect fractions
1λ: UV- Vis Detector	Volume ▸ ~1-10 uL (very small) ➔If V is too large, the signal becomes constant and we see a square shaped peak
	Pathlengths ▸ ~5-10mm ▸ Longer = better Bigger absorbance for same concentration (beer--lambert law)
	Window material ▸ Quartz

LC Detectors (cont)

	D_2 lamps ▸ Good broad UV source ▸ 185-400nm ▸ Spectrometer to isolate narrow band of wavelength ▸ More simple than FAA ➔UV does not have to compensate for a flame
	2 sensors ▸ Sample diode (I) ➔Intensity coming through the sample ▸ Reference diode ($I_\circ$) ➔ Intensity from the light source ▸ Equation ➔$A= -\log(I/I_\circ)= -\log(T)$
	Chromatogram ▸ Abs vs time ▸ Use peak area for quantitation
Many λ: Photodiode array (PDA) Detector	Chromatogram ▸ Collect many chromatograms across many wavelength (a spectrum)
	Sensitivity ▸ Can choose/use chromatogram that provides the greatest sensitivity for each analyte ▸ Find wavelength where analyte has least interference from neighbouring peaks

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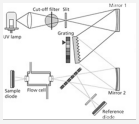
LC Detectors (cont)

	Application ▸ Useful to verify which peaks is which when MP is changed
Refractive Index (RI) Detector	Uses refractive index of analyte compared to MP ▸ Snell's law ➔ The rays will bend if there is a mismatch in refractive indices of the outside and the inside ▸ When refractive indices match ➔ Rays not refracted
	Chromatogram ▸ If RI match (only MP) ➔ Full intensity reaches sensor ▸ If RI does not match (analyte eluting) ➔ Reduced intensity reaches sensor ▸ Plot signal vs time
	Properties ▸ Universal ▸ Sensitivity ➔ ~3 orders of magnitude less sensitive than UV ▸ Absorbance ➔ Optically silent ▸ Reference flow ➔ Limited gradient capability

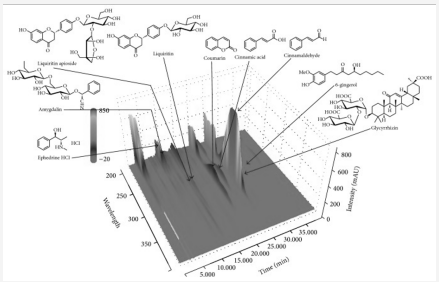
LC Detectors (cont)

Evaporative Light Scatter (ELS) Detector	How it works ▸ Uses nebulizer to produce aerosol ▸ MP evaporates ➔ Leaves behind analyte fine crystals ▸ Scattering of light (usually laser) ➔ Only when crystals are present
	Analyte ▸ Needs to produce crystals ➔ Very low volatility ▸ Can work for non-absorbing analytes ▸ Response is nearly uniform for all analytes
	Buffers (MP) ▸ Must be volatile ➔ Restricts choices ➔ Can't use inorganic buffers: Leads to buffer salts
	Better than RI detector ▸ Higher sensitivity ▸ Longer LDR

UV-Vis Detector Diagram

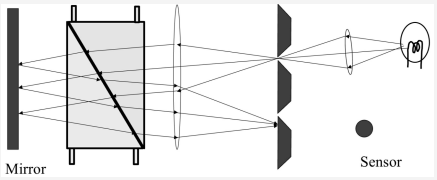


Photodiode Array Chromatogram



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Refractive Index Detector Diagram



LC-MS

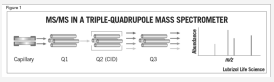
Properties	Electrospray ▸ Sample goes through nebulizer ▸ High voltage is applied → Produces charged droplets
	Fine metal capillary tube ▸ ~0.5-1mm ▸ Connected to the outlet ▸ Charged with high voltage
	Signal ▸ MP is pumped ▸ Charged droplets are attracted to MS interface ▸ Droplets dry down in flight → charge density ↑ until charge repulsion causes coulombic explosion
Single Quadrupole MS	Mass spectrum ▸ Simple ▸ MP evaporates away → Leaves $[M+H]^+$ ions → no fragments
	Problem ▸ Difficult for definitive ID ▸ Potential m/z overlap
Triple Quadrupole MS	Allows the production of fragments ▸ Contains Q1, Q2(CID) and Q3
	Q1 → Parent ions are selected Q2(CID) → Collision induced dissociation → Selected ions collide with Ar/He/N2 (Creates fragments) Q3 → Fragment ions are filtered/scanned → Detected to produce mass spectrum

LC-MS (cont)

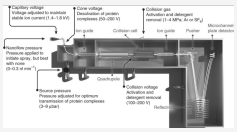
	Mass spectrum ▸ Scanning mode → Produce full spectrum → For method development ▸ Multiple reaction monitoring (MRM) → Only selected fragments are measured → For quantitation
	Better than single quadrupole ▸ Lower DL → Less interferences ▸ Longer LDR ▸ Allows positive ID of analyte ▸ Better selectivity with MRM
	Problem ▸ Q3 scans across m/z range pretty slowly (1-30 spectra/s) ▸ Lowering resolution allows faster scanning → Can't get a full detailed spectrum
QTOF MS	Quadrupole time of flight MS
	Advantage ▸ Can scan 10000 spectra/s → Many are averaged together to improve quality (better than QQQ) ▸ Allows more analytes to be measured simultaneously ▸ Higher mass accuracies and resolution → Permits greater ID power ▸ LDR>5 orders of magnitude
	Problem ▸ Not as precise as QQQ ▸ Expensive

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Triple Quadrupole Detector Diagram



QTOF MS Detector Diagram



Summary and Applications

Advantage	MP plays a critical role in controlling separation ▸ Retention → "Strength" ▸ Selectivity → "Type"
	Wide range of MP available ▸ Diverse set of separation conditions ▸ Within the same SP and column ▸ Allow to quickly try different separation conditions ▸ Allow to quickly arrive to a newer optimization separation
	No requirements of volatile analyte ▸ Needs to be soluble in MP
	Wider range of SP available ▸ Can choose type ▸ Can change particle size ▸ Can choose the amount of SP/unit of column
	Easy to collect purified analyte
Disadvantage	Much lower N compared to GC-FSOT ▸ Degrades R and ↑ Overlapping peaks ▸ Many LC have low N → 1000-5000
Detector Comparison	Selective or universal
	DL
	LDR

Summary and Applications (cont)

	Cost ▸ Purchase ▸ Maintenance
	Sample capacity
	Immune from MP gradients?
	Amendable to using IS?
Key Factors if LC is useful	1. Analytes soluble in liquid MP
	2. Concentration of analytes are high enough ▸ Can load larger volumes/concentraton on columns ▸ Combine with sensitive detectors
	3. Does sample require a high R' separation ▸ GC favored over LC
	4. Need to recover analyte ▸ LC > GC
	5. Slower than GC
Applications	Anti-dopping and forensics
	Pharmaceutical ▸ Process control ▸ Quality control ▸ R&D ▸ Metabolic ▸ Proteomic
	Food and Beverages ▸ Vitamins ▸ Pesticides ▸ Contaminants
	Environmental ▸ Pesticides ▸ Industrial materials

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Summary and Applications (cont)

R&D

- Organic synthesis
- Catalysis

Industrial

- Feedstock

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