3rd Generation Stevia Extracts: Neuroscience, Ingredient Technologies and Food Applications

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Sweetener Systems Conference

Neuroscience is the Future of Flavor Technology

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- Taste
- Smell
- Sight
- Sound
- Touch

Neuroscienc e

Sweeteners and Flavors

- Plant-based High potency sweeteners
- Non/low caloric bulk sweeteners
- Natural flavors

Taste Modulation

- Sweetness modulators
- Bitterness modulators
- Enhancement without ingredients

Formulation

- Stacking
- Matrix
- Beverages & Foods

Stevia State of Technology 2018

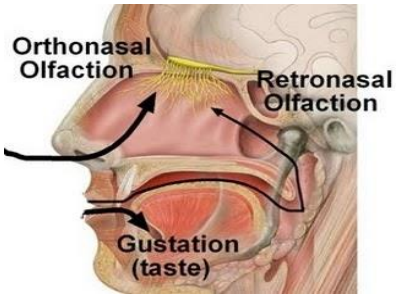
- 2nd generation stevia extracts were all about high purity RA, the higher the purity the better the taste.
- Farm-based 3rd generation stevia extracts are the newer 2-way and 3-way blends of RABCDM for even more sugar like taste but at higher cost. Alternatively, fermentation and bioconversion-based stevia already co-exist with farm-based stevia in 2018.

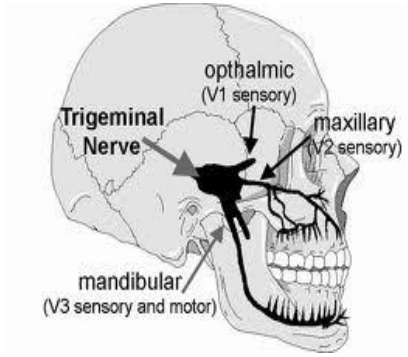
- Enzymatically modified stevia extracts are sweet taste enhancers that can be used as part of the stacking strategy for sugar reduction.
- Stacking is a sugar reduction strategy for building up to the required sweetness intensity and profile while staying below the off flavor thresholds for all the plant-based ingredients used

Agenda

- Sweetness neuroscience
- Stevia as sweetener
- Stevia as flavor
- Stacking

Re-Defining "Flavor" = Taste + Smell + More





- Taste (5+ primary)
- Smell (aroma)
- Somatosensation (Touch):
  - Mechanoreception: Touch, Pressure and Vibration (Prescott, 2015),
  - Thermoception: Temperature,
  - Nociception: Pain (Youseff, 2015), and
  - Up to total 30 senses? (Smith, 2016) can they all be part of somatosensation?
- Vision ("Seeing the flavor". Acree, 2013)
- Sound is the Forgotten Flavor Sense (Spence 2015. Gastropod, 2015)

Three Types of Taste Cells

A taste bud is a cluster of 100 elongated taste cells like an orange segment. Each taste bud cell is taste-specific (One taste, one cell, one brain region. Zuker, 2018). There is integration of gustatory information from different taste cells (Sternini, 2013), that is "sensory processing circuitry" (Bigiani, 2011)

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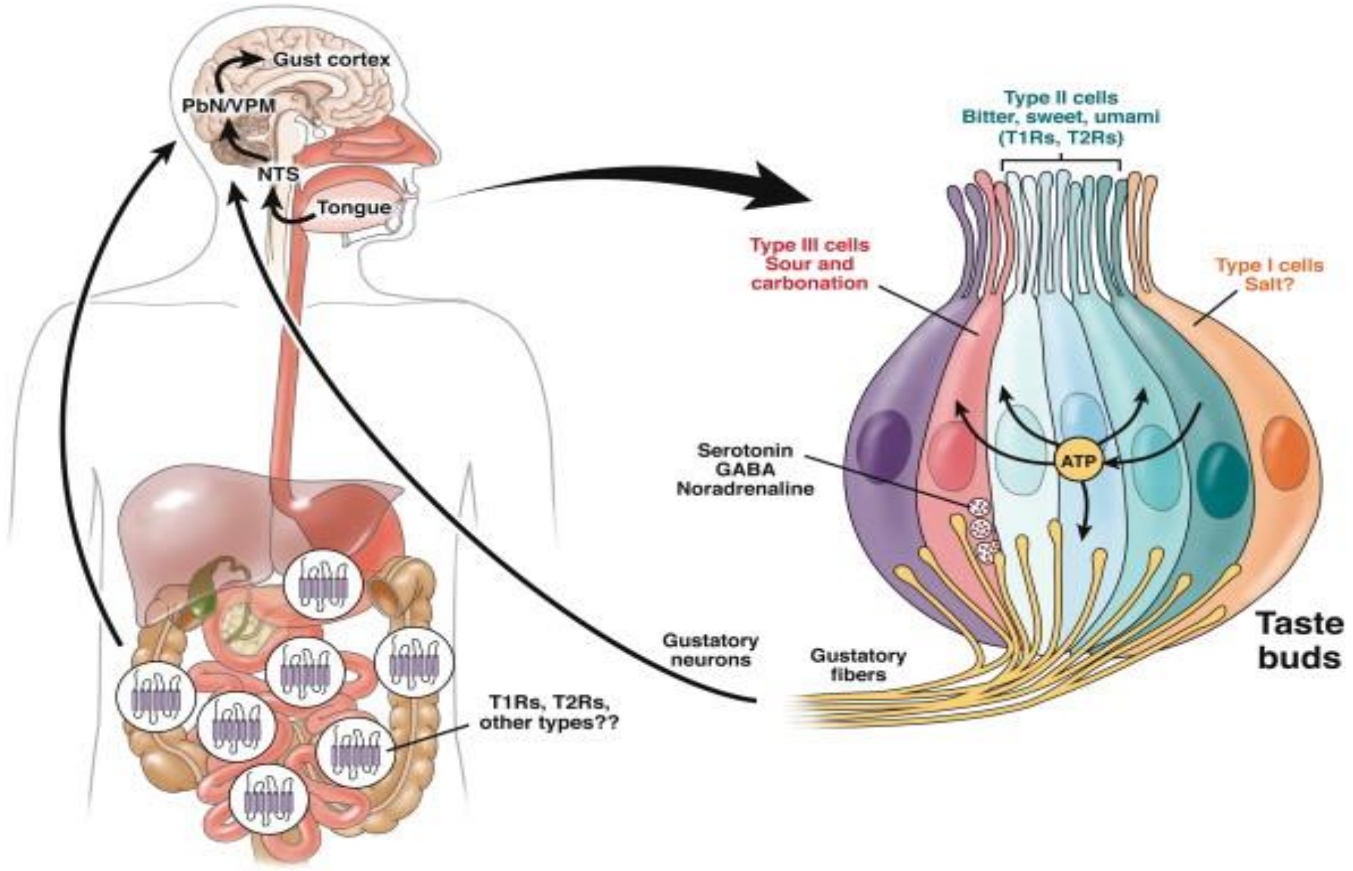

Taste Receptors

Taste receptors had been identified during the rapid advances of taste physiology and neuroscience in the past 15+ years (NIZO, 2011)

- **Bitterness:** 25 Receptors: T2Rs. Family: GPCR. 2000.
- **Sweetness:** 1 Receptor: T1R2/T1R3. Family: GPCR. 2001. And a newly found secondary pathway.
- **Umami:** 1 Receptor: T1R1/T1R3. Family: GPCR. 2002.
- **Sourness:** "Receptor": PKD1L3/PKD2L1. Family: Ion Channel. 2006.
- **Saltiness:** "Receptor": ENaC. Family: Na Channel. 2010. And a newly found secondary pathway in Type III cell.
- **"Fat":** Receptors: CD36, GPR120, FA1. Family: Several GPCR.
- **"Calcium"**: Receptor: CaR. Family: GPCR
- **"Water":** Receptor: Aquaporins. Family: Channel
- **"Starchy"**: Proposed (Lim, 2016)

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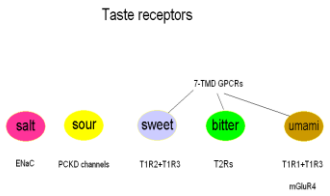

Sweet Taste Receptor

Sweeteners bind to different locations in the taste receptor:
Venus Flytrap Domain, Cysteine-Rich Domain, and 7 Trans Membrane Domain (Masuda, 2012. Lefkowitz and Kobilka, 2012). Binding led to receptor protein conformational change (Nango et al, 2016). Stevia activated both T1R2 and R3 (Mayank, 2015). Sweet receptor cells instructed sweet neurons via SEMA 7A signaling protein (Zucker et al, 2017)

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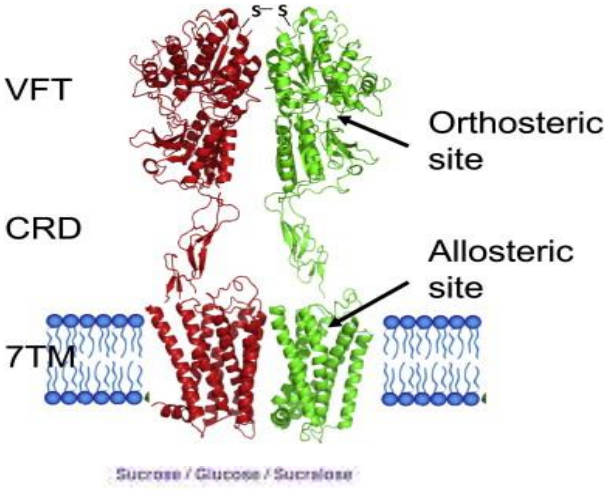
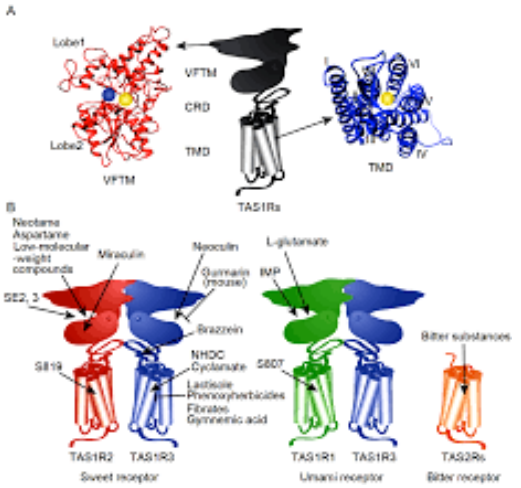
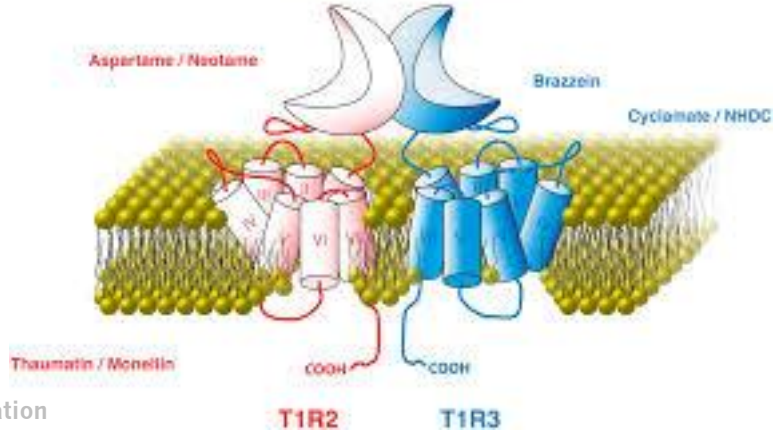
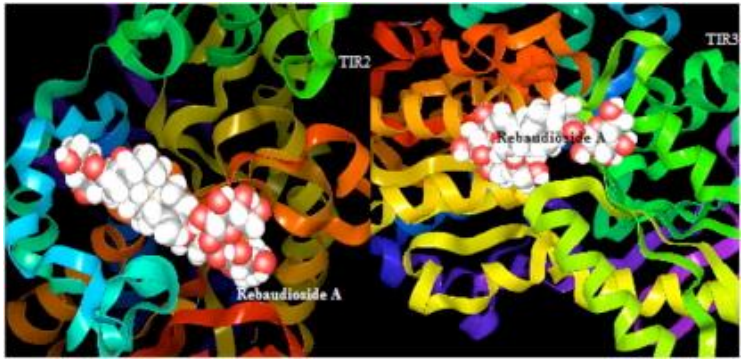


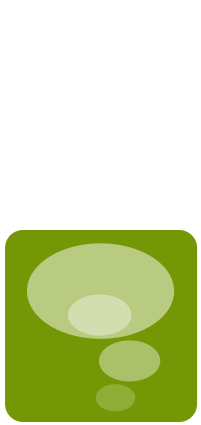






A Secondary Sweet Taste Pathway

Only caloric sucrose and maltose, hydrolyzed into simple sugars by sucrase or maltase next to tongue sweetness receptor, triggered a newly found secondary pathway (Margolskee et al, Monell, 2016)



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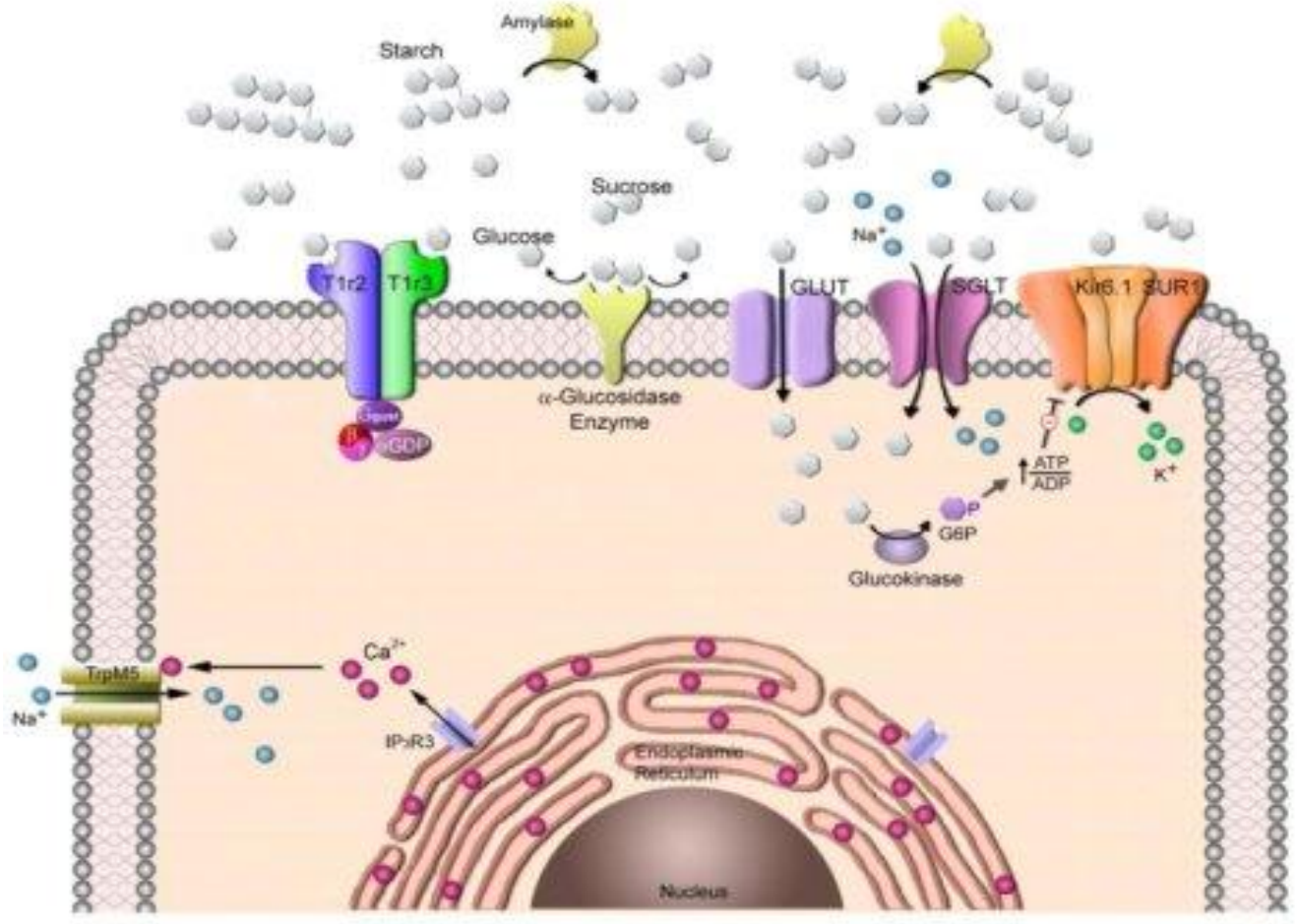

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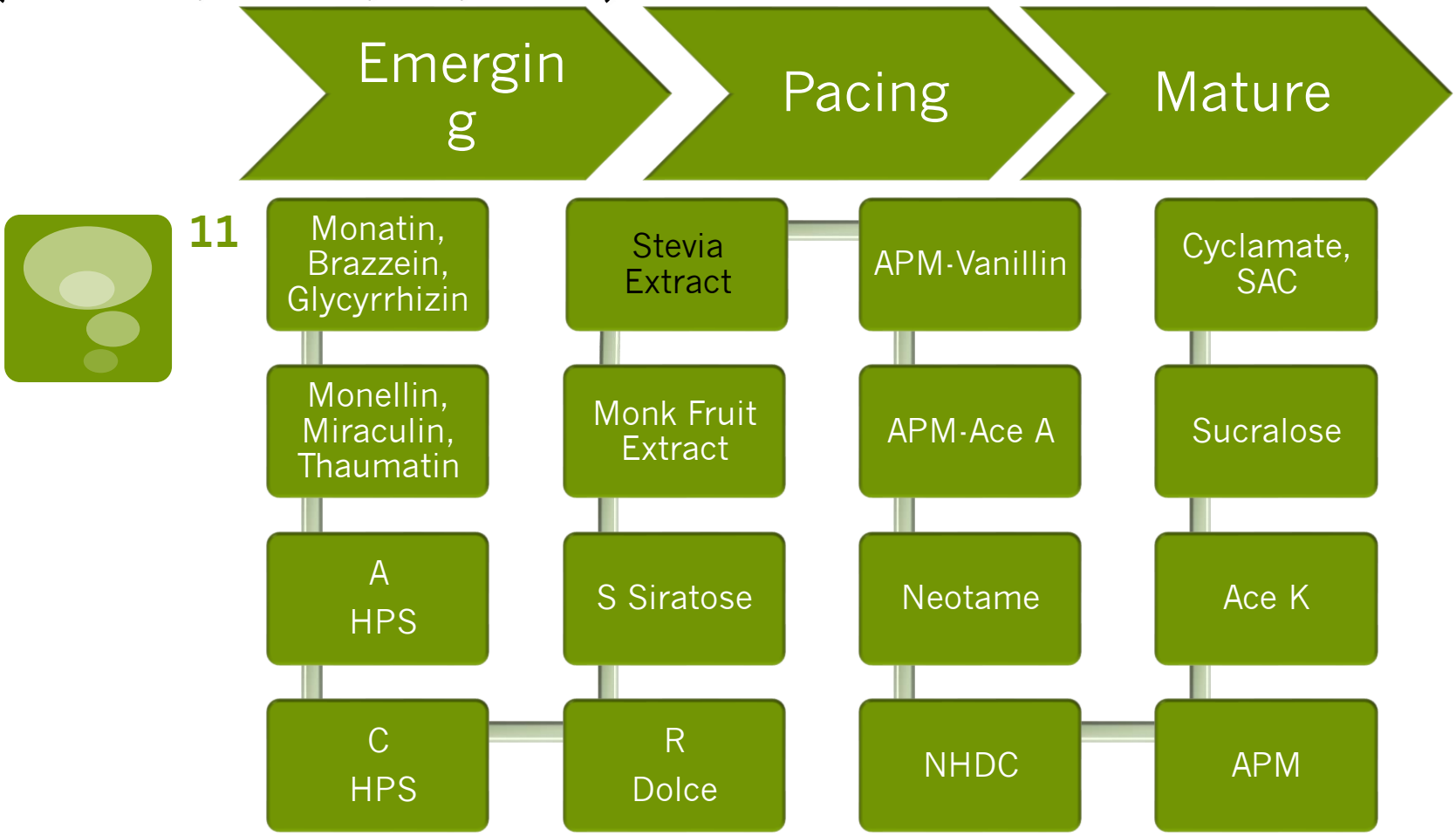
High Potency Sweeteners

Technologies go from “Emerging” (discovered but not yet commercialized) to “Pacing” (first to market sets the pace) and finally to “Mature” (patent expired and technology commoditized) (AD Little, 2000s, AW, 2018)



2nd Generation Stevia Extract is all About Reb A

(Various suppliers' websites, 2018)

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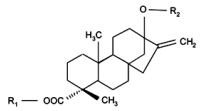
- Found in nature, plant-based
- Non caloric
- GRAS: FDA No Objection Letter 2008
- Approved in >150 countries, limits in EU, Singapore, Thailand, ANZ, Brazil, and Canada.
- Purity: RA 40 to RA100
- ~ 200X as sweet as sugar
- Heat and pH (>3) Stable
- Non-GMO, Kosher & Halal
- 0.02% in beverages = about 5% Sugar Equivalence
- Most commonly labeled as "stevia leaf extract" (USA) and "steviol glycosides" (E960, EU)

Steviol Glycosides: >40 Found.

- >40 SG found (PC, 2016) (Cargill, 2016) (Foods, 2014, 3, 162-175. Prakash et el) (Molecules, 2014, 19(12), 20280-20294, Prakash and Chaturvedula) (JECFA 2017)



Deterpene glycoside	R_1[a]	R_2[a]	Sweening potency (sucrose = 1)
Steviolbioside	H	glc 2—1 glc	100 – 125
Rubusoside	glc	glc	100 – 120
Stevioside	glc	glc 2—1 glc	150 – 300
Rebaudioside A	glc	glc 2—1 glc, glc 3—1 glc	250 – 450
Rebaudioside B	H	glc 2—1 glc, glc 3—1 glc	300 – 350
Rebaudioside C (dulcoside B)	glc	glc 2—1 rhm, glc 4—1 glc	500 – 120
Rebaudioside D	glc 2—1 glc	glc 2—1 glc, glc 3—1 glc	250 – 450
Rebaudioside E	glc 2—1 glc	glc 2—1 glc	150 – 300
Dulcoside A	glc	glc 2—1 rham	50 – 120

[a] glc, β-D-glucopyranosyl; rhamn-L-rhamnopyranosyl.

Figure 1: Structure of some stevia glycosides (Crammer and Ikan, 1987).

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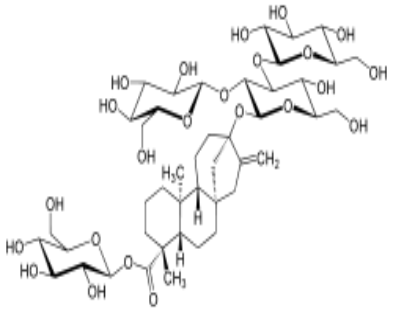
- **All 40+ SG JECFA listed in 2017:**

Sweetener	Reference Number in Text	R-Groups in Backbone Figure Above		Formula	Molecular Weight (g/mol)	Potency *
		R_1	R_2			
Rebaudioside A	1	β-glc-	$(β\text{-glc})_2$-β-glc-	$C_{44}H_{70}O_{23}$	967.01	200
Rebaudioside B	2	H	$(β\text{-glc})_2$-β-glc-	$C_{38}H_{60}O_{18}$	804.88	150
Rebaudioside C	3	β-glc-	(β-glc, α-rha-)-β-glc-	$C_{44}H_{70}O_{22}$	951.01	30
Rebaudioside D	4	β-glc-β-glc-	$(β\text{-glc})_2$-β-glc-	$C_{50}H_{80}O_{28}$	1129.15	221
Rebaudioside E	5	β-glc-β-glc-	β-glc-β-glc-	$C_{44}H_{70}O_{23}$	967.01	174
Rebaudioside F	6	β-glc-	(β-glc, β-xyl)-β-glc-	$C_{43}H_{68}O_{22}$	936.99	200
Rebaudioside M	7	$(β\text{-glc})_2$-β-glc-	$(β\text{-glc})_2$-β-glc-	$C_{56}H_{90}O_{33}$	1291.3	250
Stevioside	8	β-glc-	β-glc-β-glc-	$C_{38}H_{60}O_{18}$	804.88	210
Steviolbioside	9	H	β-glc-β-glc-	$C_{32}H_{50}O_{13}$	642.73	90
Rubusoside	10	β-glc-	β-glc-	$C_{32}H_{50}O_{13}$	642.73	114
Dulcoside A	11	β-glc-	α-rha-β-glc-	$C_{38}H_{60}O_{17}$	788.87	30

glc = glucose; rha = rhamnose; xyl = xylose; * Potency from [1,6,7].

Rebaudioside A (RA): Key Features



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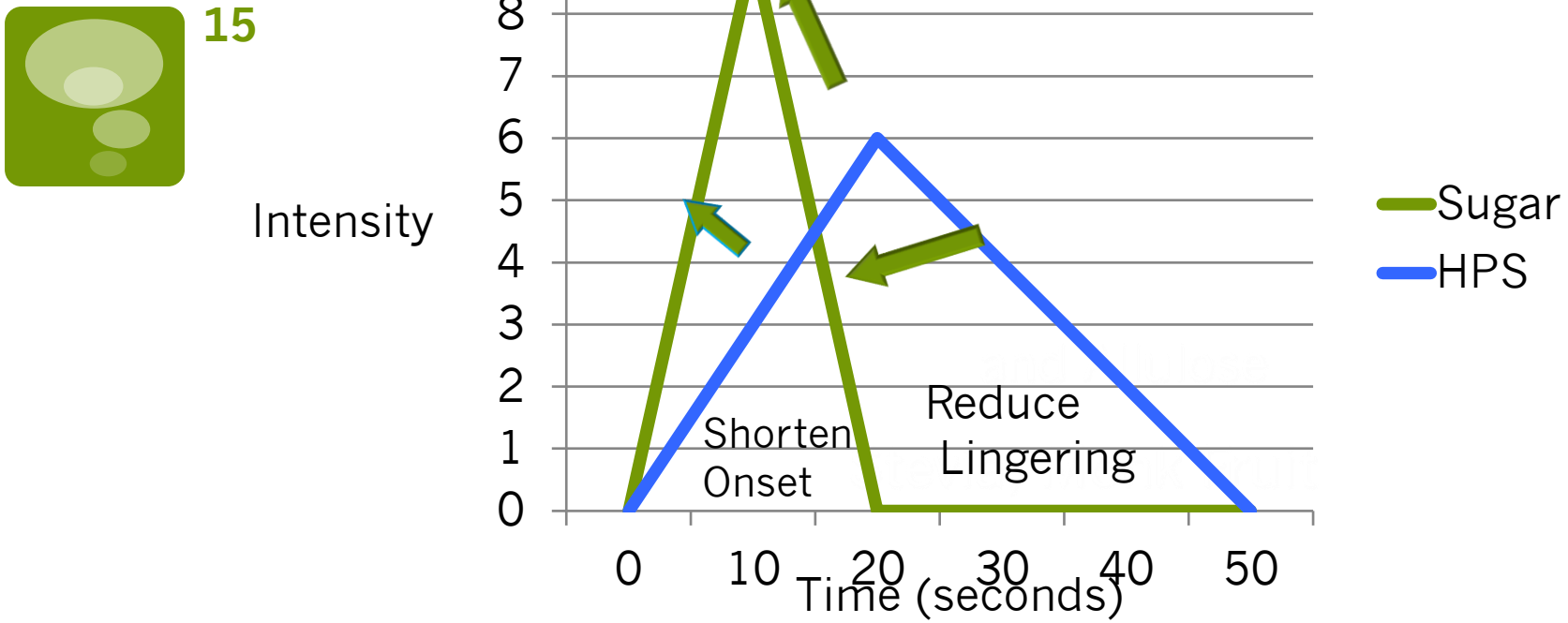
Feature	Details
Chemistry:	"1+3=4 Glucose"
Neuroscience:	Bind to the Venus Fly Trap region of the sweetness receptor at both T1R2 and R3 via H bonding with glucoses (Mayank, 2015)
Taste: • **200X Sugar** • Higher purity and lower the %SE= Higher potency.	• **Higher purity = Cleaner taste** • **SG Triggered 2 out of the 25 bitterness receptors TAS2R 4 and 14 (Meyerhof and Hofmann, 2012)**
Regulatory: • EU: Added approval for energy reduced confections (2017) • Brazil: Now can blend stevia and sugar (2016) • Newest: India (2015)	• EU erased the SG spec minimum "RA+Stev >75%" (EU 2016/1814, 11/2016), meaning **these two SG do not need to be the main components** in 3rd Gen Stevia. • SG analysis by HILIC-MS/MS-SIDA (Well et al, 2013) • **Limits: ~0.024% EU, 0.06% ANVISA**
Suppliers:	• Three leaders N, C, P

The First Perception Problem: Time-Intensity Curve

The sweetness modulation strategy is to shorten the onset, increase the peak, and reduce the lingering of plant-based HPS. (Idealized curves. AW, 2018) (Information including for example DuBois and Prakash, 2012)



The Second Perception Problem: Sweetness & Bitterness Intensity-Concentration Curves

Stevia RA sweetness was nearly linear for the first 200ppm but plateaued at around 7-9% SE, while RA bitterness started above 300ppm and increased dramatically in beverages. (Idealized curves. AW, 2018) (Information including for example Antenucci and Hayes, 2014)

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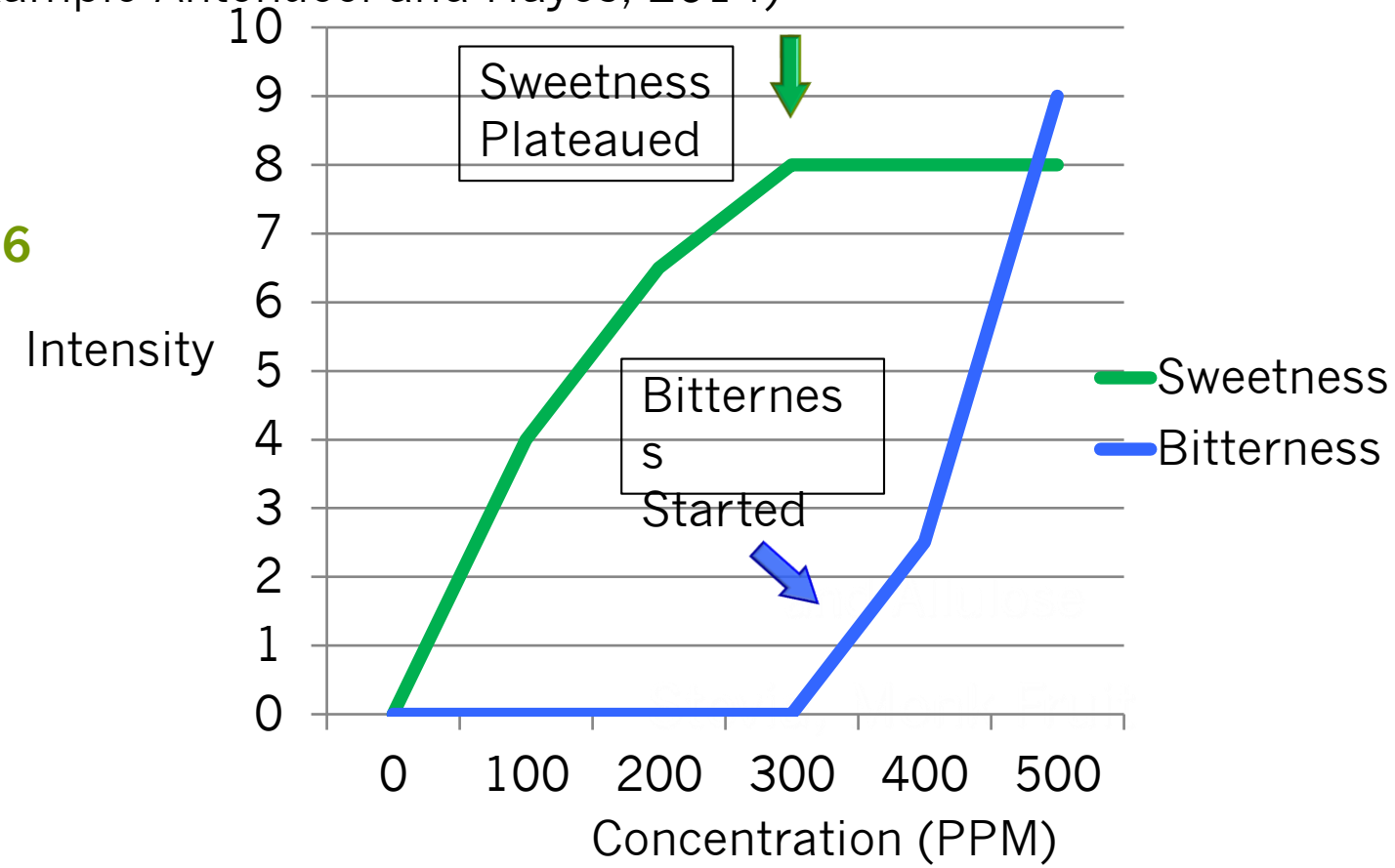

The Third Perception Problem: Quantitative Descriptive Analysis (QDA) Profiles

Stevia extracts at concentrations above 200ppm in beverages typically showed non-sweet undesirable flavor attributes (AW, 2018. Charts from literatures) which may have muted prominent sweetness (Reyes et al, 2017)

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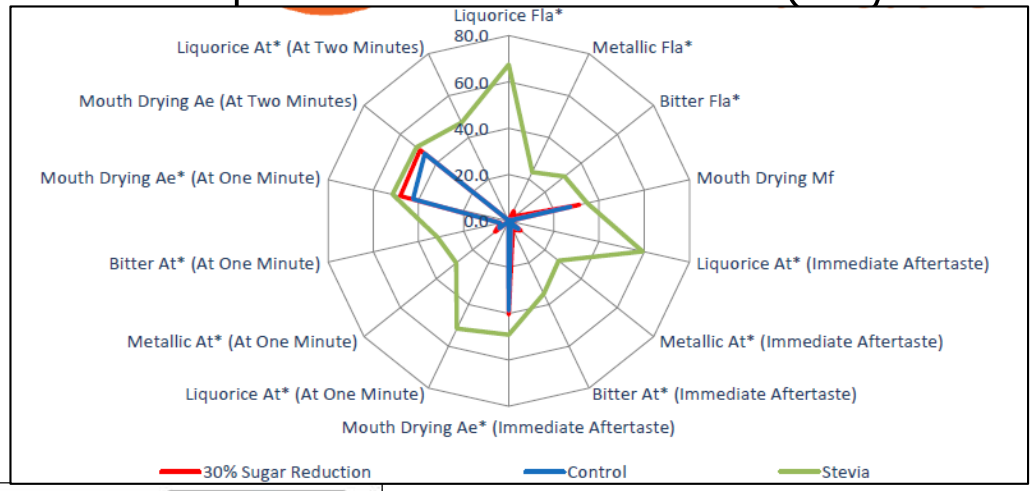
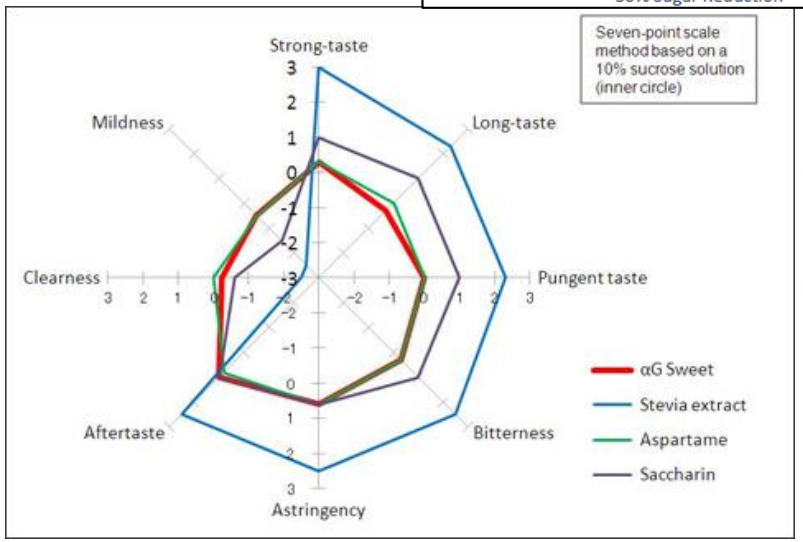
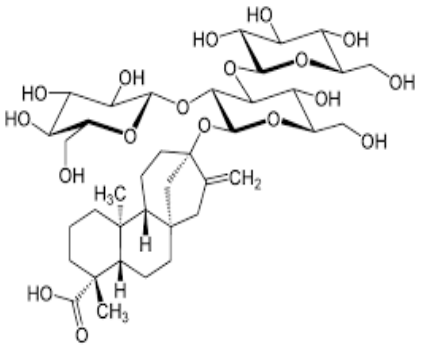




Rebaudioside B (RB): Key Features



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Feature	Details
Chemistry:	**"0+3=3 Glucose" "1 less Glucose than RA"**
Neuroscience:	Possibly same as RA: Bind to the Venus Fly Trap region of the sweetness receptor at both T1R2 and R3 (Mayank, 2015)
Taste: **150X Sugar**	• **Less bitter but also less sweet than RA**: SG Triggered 2 out of the 25 bitterness receptors TAS2R 4 and 14 (Meyerhof and Hofmann, 2012) • **Much less soluble than RA, but RA+RB reportedly synergistic.**
Regulatory:	• EU erased the SG spec minimum "RA+Stev >75%" (EU 2016/1814, 11/2016), meaning these two SG do not need to be the main components in 3rd Gen Stevia. Making RA+RB blends legally possible
Suppliers:	• Two leaders • **2017-2018 patents: Extracting RB, increasing solubility, blending into ABCDM.**

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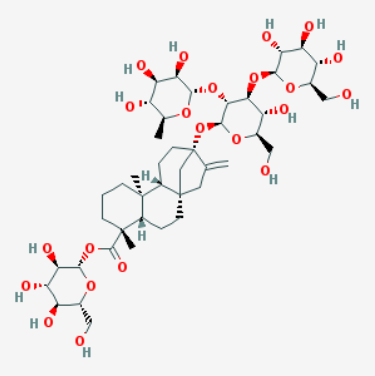
Rebaudioside C (RC): Key Features



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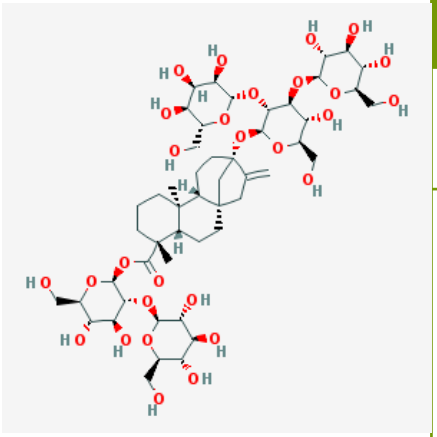
Feature	Details
Chemistry:	**"1+2 Glucose+1 Rhamnose" "Same as RA, except 1 Rhamnose"**
Neuroscience:	Possibly same as RA: Bind to the Venus Fly Trap region of the sweetness receptor at both T1R2 and R3 (Mayank, 2015)
Taste: **30X Sugar**	**Much less sweet than RA**: SG Triggered 2 out of the 25 bitterness receptors TAS2R 4 and 14 (Meyerhof and Hofmann, 2012)
Regulatory:	• **RC80%-95% was FDA GRAS in 2015** • First minor SG proven as a **sugar sweetness enhancer (FEMA 4720)** and labeled as natural flavor • RA+RC blends possible due to EU elimination of SG spec minimum "RA+Stev >75%" (EU 2016/1814, 11/2016)
Suppliers:	• <4 leaders • **2017-2018 patents: High RC cultivars**

FEMA
FLAVOR & EXTRACT MANUFACTURERS ASSOCIATION

EUR-Lex

Rebaudioside D (RD): Key Features



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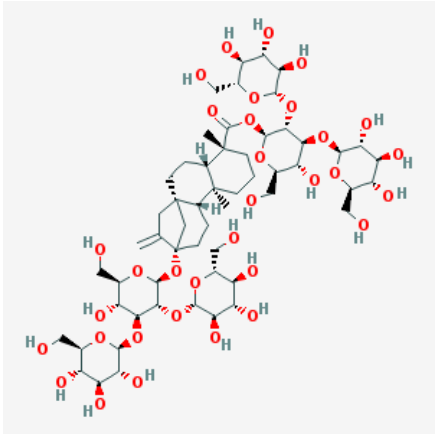
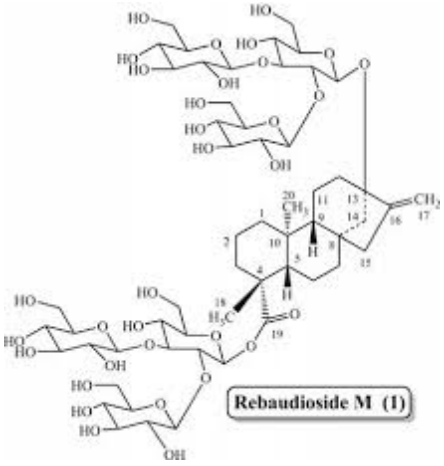
Feature	Details
Chemistry:	**"2+3=5 Glucose" "1 more Glucose than RA"**
Neuroscience:	• RA bound to bitterness receptors TAS2R4 and 14, RD unknown but probably less • RD was less bitter than RA, varied widely in people (Allen et al, Chemosen Perception, 2013) (N Brazil study, 2016)
Taste: **221X Sugar**	• **Much less bitter, slightly sweeter** • **1 of 2 most talked about minor SG for 3rd Gen Stevia, due to taste advantage ("Tastes better", "sugar like TI curve")**
Regulatory:	• RA+RD blends possible due to new EU elimination of SG spec minimum "RA+Stev >75%" (EU 2016/1814, 11/2016) • **FDA GRAS since 2013 (P), and 2014 (G)**
Suppliers:	• Two leaders (farm-based): N, P. (Possibly G, L). Three leaders (farm-based or bioconversion-based): I, C, D • **2017-2018 patents: Increased solubility (polymorphism and purity), and blending into ABCDM.**

Rebaudioside M (RM): Key Features





	Details
Chemistry:	**"3+3=6 Glucose" (Previously as RX) "2 more Glucose than RA"**
Neuroscience:	Possibly same as RA: Bind to the Venus Fly Trap region of the sweetness receptor at both T1R2 and R3 (Mayank, 2015)
Taste: • **250X Sugar** • Coca Cola's publication in Foods 2014, 3(1), 162-175. Prakash et al	• **"Best of all SG" (P and C, 2016) "Clean slightly bitter" (Coca Cola, 2014)** • 1 of 2 most talked about minor SG for 3rd Gen Stevia, due to taste advantage ("tastes better")
Regulatory:	• **FDA GRAS since 2013 (P), 2014 (G, both farm-based), 2016 (C, fermentation-based) and 2017 (I, bioconversion-based)** • RA+RM and RD+RM blends possible due to new EU elimination of SG spec minimum "RA+Stev >75%" (EU 2016/1814, 11/2016) • Recently found and **added to now total 11 EFSA approved SG** (EU 2016/1814, 11/2016) and Canada (Global Stevia Institute, 2016)
Suppliers:	• Two leaders (farm-based): N, P. (Possibly G, L) • Three leaders (fermentation or bioconversion-based): I, C, D, P • **2017-2018 patents: High RM varietals, Increased solubility, blending into ABCDM.**

Application Matrix

Company N Application Matrix, as an example, is a summary of guidelines for selecting the best stevia to use in beverages.

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For sugar free, start with Stevia P.

For 50% sugar reduction, start with Stevia A.

For tabletop sweetener, start with Stevia C.

Replace first 5% sugar with 0.02% or 200ppm stevia, taste and adjust.

Use “Stacking” strategy.

Company N Application Matrix (As of 10/23/2018)

Beverage Applications (SR=sugar reduction, SF= sugar free)



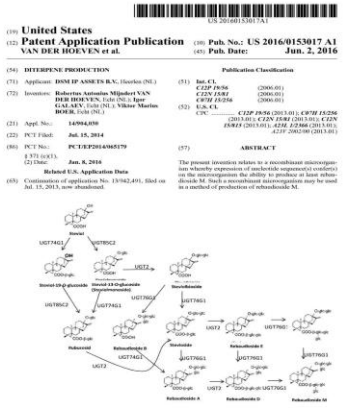
	12% SE: CSD, Juice Drinks			9% SE: Tea, Coffee			5% SE: Water, Table Top		
	33% SR	50% SR	SF	33% SR	50% SR	SF	33% SR	50% SR	SF
1St	A	A	P	A	A	P	A	A	P
2nd	RA97	RA97	RA99	RA97	RA97	RA99	RA97	RA97	C
3rd	RA60	RA60	RA60	RA60	RA60	RA60	RA60	RA60	RA50

Best Taste

Least Cost

Company N 3rd Generation Stevia: Stevia A, P and C.

Stevia Without Farm: Key Features



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(22) PCT Filed: Jul. 15, 2014

(86) PCT No.: PCT/EP2014/065179
§ 371 (c)(1),
(2) Date: Jan. 8, 2016

Related U.S. Application Data

(63) Continuation of application No. 13/942,491, filed on Jul. 15, 2013, now abandoned.

(57) ABSTRACT

The present invention relates to a recombinant microorganism whereby expression of nucleotide sequence(s) confer(s) on the microorganism the ability to produce at least rebaudioside M. Such a recombinant microorganism may be used as a method of production of rebaudioside M.

What was the point?	What does it mean?	What can one do about it?
Chemistry:	• **Fermentation (D, C) or Bioconversion (I, T)** • **Emphasis is on RM**, then RD, some on RB, **RI, RD2, RM2 (last 3= sweetness enhancers).** Also RVWGKA (C, 2018)	• Verify these 3 as enhancers and implement
Neuroscience:	Same detection, same perception	
Taste:	**Same molecule should taste the same**	**Ensure there is no off flavor from impurities**
Regulatory: RD+RM FDA GRAS 2016+2018, labeled as **"Steviol Glycosides" or "Reb D and Reb M"** (C, 2016) or **"Stevia Leaf Reb M"** (I, 2018)	• Canada: "stevia extract" • Each new supplier may need a new GRAS no objection letter • **Non-GMO Project Verified by I**	• Watch and warn • Approved in USA, Canada, and LATAM (4) as of 10/2018
Suppliers:	• 2018: I, C, D, T (R? A?)	

Agenda

- Sweetness neuroscience
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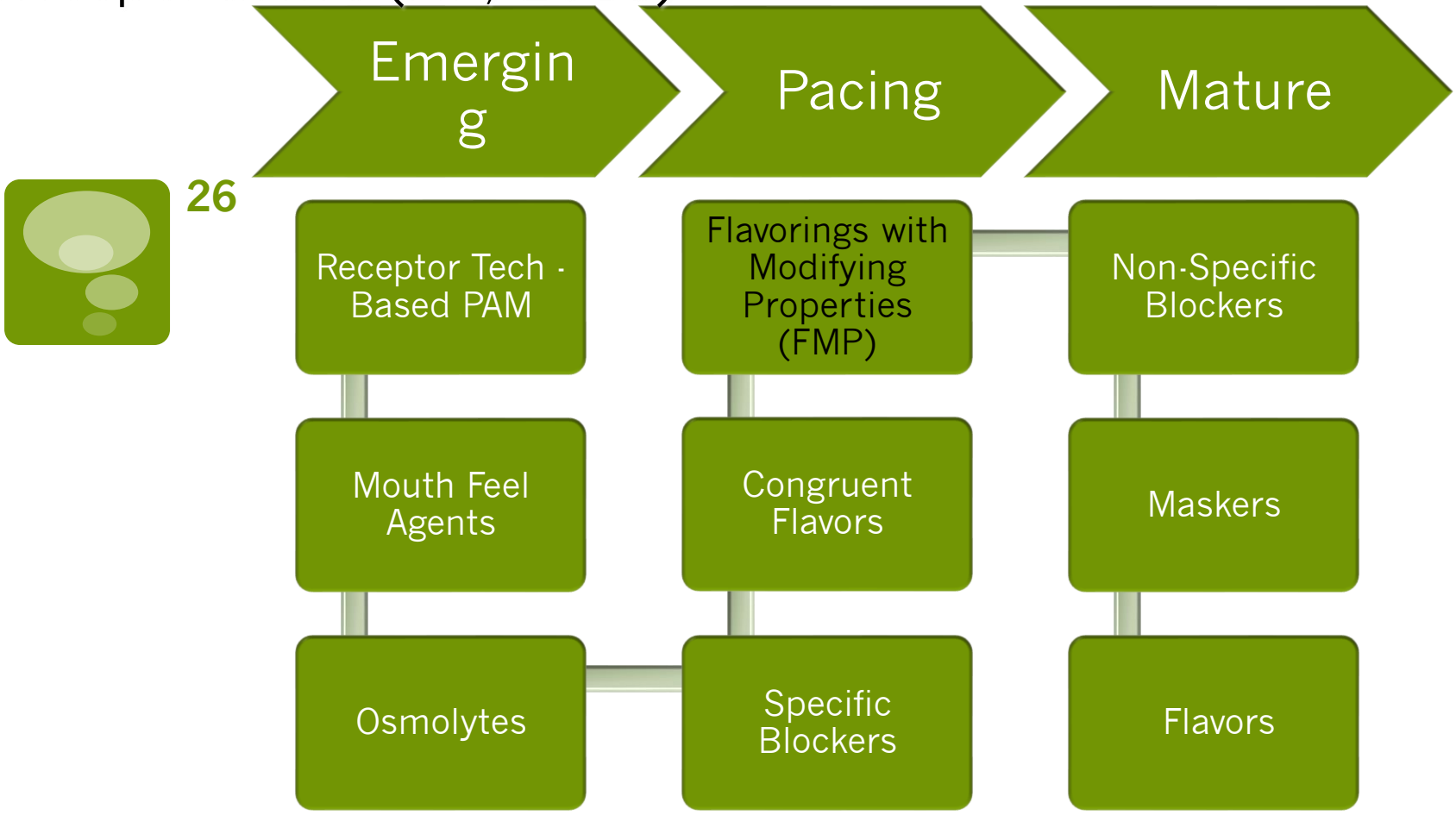
- Stevia as flavor
- Stacking

Taste Modulators Technology Map

This is the technology map for sweetness modulators which are the ingredient technologies based on taste/smell neuroscience and crossmodal correspondence (AW, 2018)



Sweet Taste Modulators: What Do They Do?

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What was the point?	What does it mean?	What can one do about it?
To shorten sweetness onset	• FMP GSG (FEMA 4728), • Erythritol (FEMA 4819) • Allulose (FEMA 4897)	• Understand and implement
To increase sweetness peak	• FMP GSG (FEMA 4728), native stevia • Congruent flavors • Bitterness blockers • PAM (C natural PAM)	• Understand and implement
To reduce sweetness lingering	• Osmolytes • Others with uncertain mechanisms	• Understand and implement
To increase mouth feel	• Hydrocolloids (Gum Arabic, Pectin, Modified Starch) • Others	• Same as above

Stevia as Natural Flavor: GSG (FEMA 28. ISC, 2018)

FEMA

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FEMA GRAS 28	Total SG	GSG	RA	RC	STV	Other SG	Malto dextrin
4728 (GSG, 175ppm)	80-90%	**75-80%**	**1-6%**	NS	2-4%	<3% each	3-20%
4845 (Glucosylated Stevia Extract, 100ppm)	> 80%	NS	<10 %	**<4%**	<5%	<3% Individual	**NS**
4876 (Enzyme Modified Stevia, Stevioside 20%, 120ppm)	**90-95%**	**64-70%**	**10-13%**	NS	**20-22%**	**<1% each**	1-6%

4845: Biggest differences were RC and no maltodextrin.
4876: Biggest difference were higher total SG but lower GSG

Stevia as Natural Flavor: FEMA 4728

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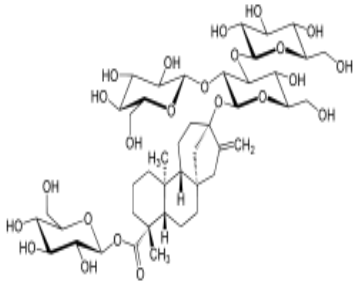
What was the point?	What does it mean?	What can one do about it?
Chemistry: EMSG, also known as Glucosyl Steviol Glycosides **GSG**	• There is a **big market** • **Works as a sweetness enhancer and labeled as natural flavor** • Regulated as FEMA 4728	
Neuroscience:	• FMP	
Taste:	• **Less sweet than 1.5% sucrose at FEMA limit** • Reportedly made sugar, HFCS, polyols, and stevia sweeter	
Regulatory:	• **FEMA: 4728, < 175ppm beverages** • FDA: >175ppm beverages, "artificial" sweetener (?). • Asia = approved in FEMA countries, China and Japan. EU = not approved.	• **Verify and use it as sweetness enhancer and flavor enhancer**
Suppliers:	• Leaders: P, N, A, S. • **Lots of GSG manufacturing patents in 2016-2018**	

Stevia as Natural Flavor: Native Stevia



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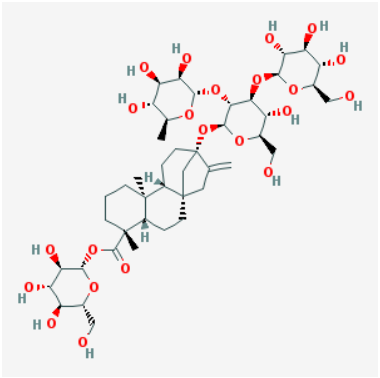


What was the point?	What does it mean?	What can one do about it?
RA22	• FEMA 4805 • Beverages: <70ppm	Use native stevia to create sweet flavor, labeled as natural flavor
RA60	• FEMA 4771 • Beverages: < 30 ppm	
RA80	• FEMA 4772 • Beverages: < 35ppm	
RA99	• FEMA 4601 • Beverages: < 30ppm	
RC90 **RC30** **RC22**	• FEMA 4720 Beverages: <400ppm • FEMA 4796 Beverages < 75ppm • FEMA 4806 Beverages < 100ppm	
Stevioside	• FEMA 4763 • Beverages: <35ppm	
RD95	• FEMA 4921 • Beverages: <32.5ppm	
RM **RM95**	• FEMA 4895 (<20ppm) • FEMA 4922 (<24ppm)	Enhanced sugar and HFCS by up to 3% SE (10039834, 2018, Coca Cola patent)

Agenda

- Sweetness neuroscience
- Stevia as sweetener
- Stevia as flavor
- Stacking

Stacking

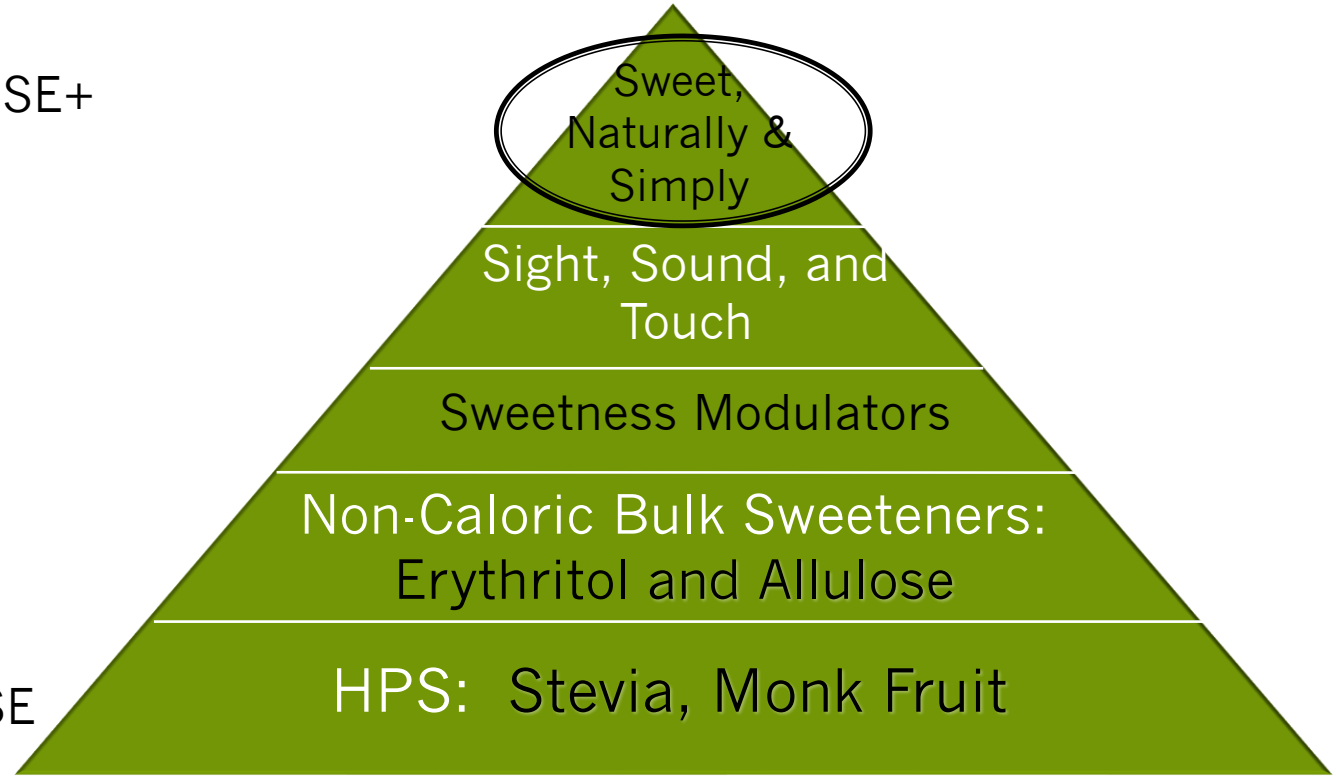
Stacking is a sugar reduction strategy for building up to the required sweetness intensity and profile while staying below the off flavor thresholds for all the plant-based ingredients used (AW, 2018)

Sugar Equivalence (SE)

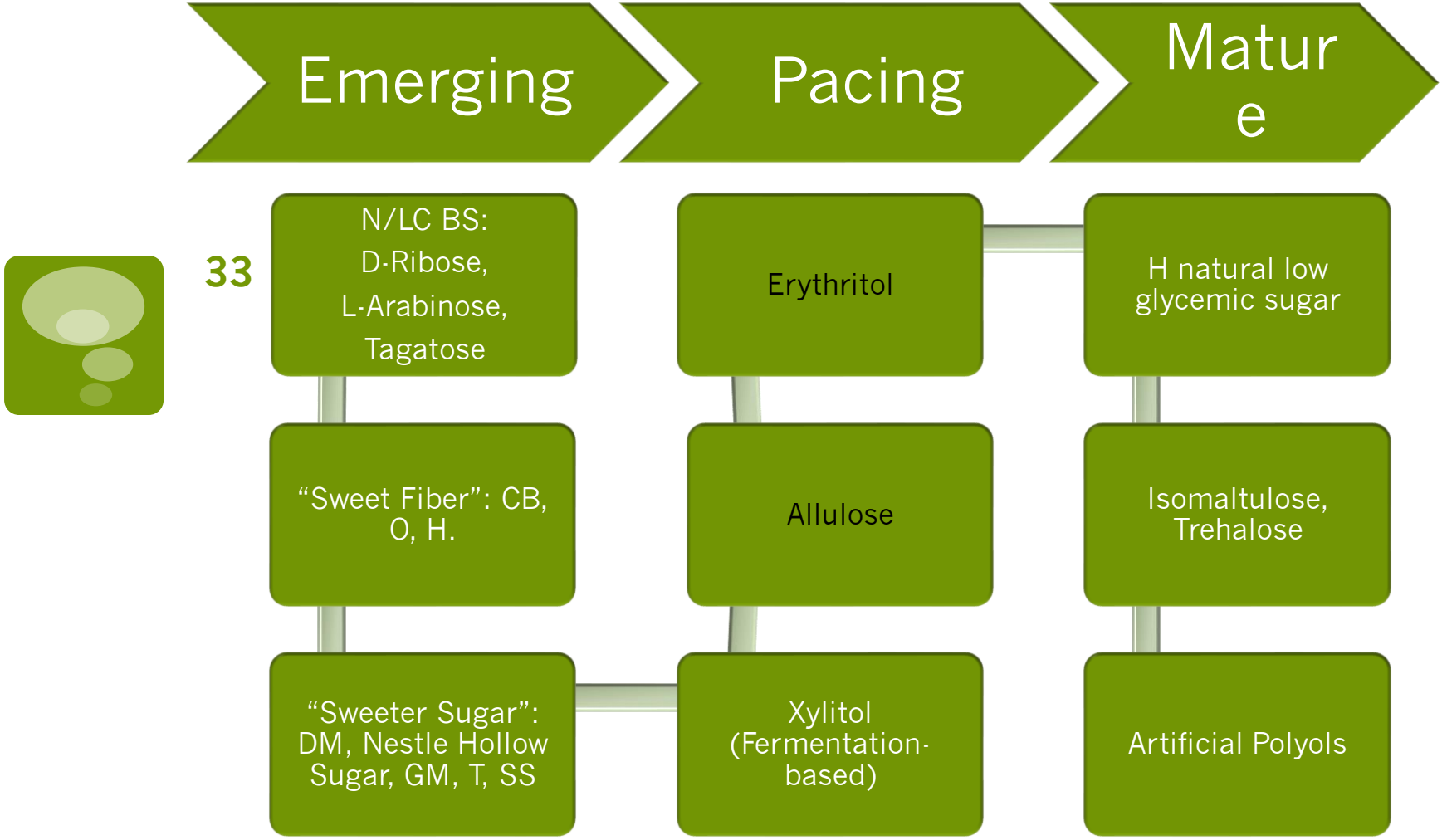
12% SE+

32

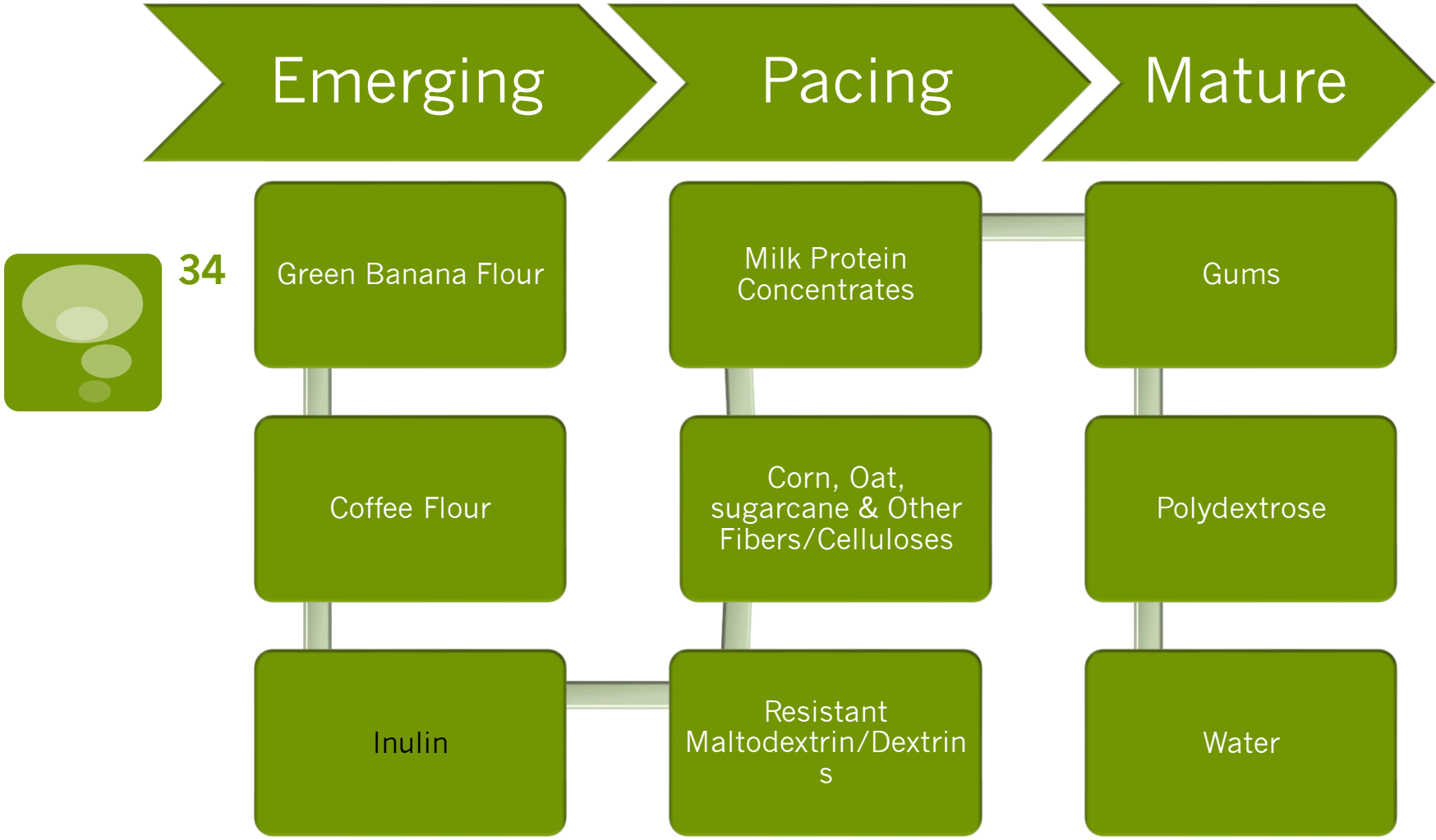
1% SE



Non/low Caloric Bulk Sweeteners
(AW, 2018)



Non/low Caloric Bulking Agents
(AW, 2018)



Stevia State of Technology 2018

- 2nd generation stevia extracts were all about high purity RA, the higher the purity the better the taste.
- Farm-based 3rd generation stevia extracts are the newer 2-way and 3-way blends of RABCDM for even more sugar like taste but at higher cost. Alternatively, fermentation and bioconversion-based stevia already co-exist with farm-based stevia in 2018.

- Enzymatically modified stevia extracts are sweet taste enhancers that can be used as part of the stacking strategy for sugar reduction.
- Stacking is a sugar reduction strategy for building up to the required sweetness intensity and profile while staying below the off flavor thresholds for all the plant-based ingredients used