



p-Synephrine or Phenylephrine: Conclusive Identification of the Primary Biogenic Amine in Bitter Orange

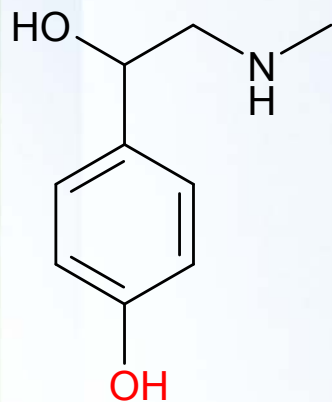
Mark C. Roman
Tampa Bay Analytical Research, Inc.

Literature Confusion!



- **Int. J. Obesity (2002) 26, 1363-1366**
 - “...Bitter Orange standardized for synephrine (phenylephrine);”
- **Mayo Clin. Proc. (2004) 79, 1059-1062**
 - “These formulations typically contain an ephedra-like alkaloid, synephrine (phenylephrine)...”
- **Obesity Reviews (2006) 7, 79-88**
 - “Some authors stated that *C. aurantium* contains m-s, whereas others stated that it contains only p-s.”
- **Natural Products Insider – Posted 11/14/2006**
 - “While some argue whether bitter orange contains both the p- and m-isomers of synephrine...”

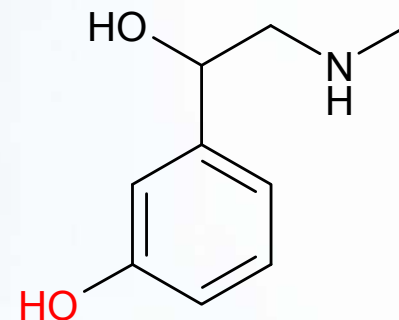
Synephrine vs. Phenylephrine



Synephrine

CAS 94-07-5

Merck Index 13,9104



Phenylephrine

CAS 61-76-7*

Merck Index 13,7370*

*as Hydrochloride



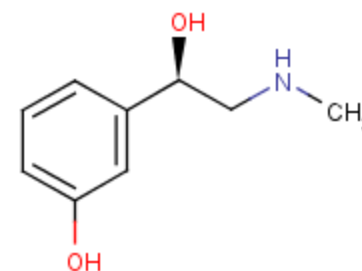
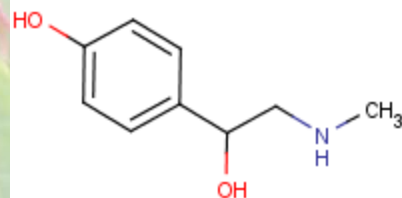
Synephrine vs. Phenylephrine



NIH ChemID Plus Search Results

Search Term: Synephrine

Search Term: Phenylephrine



Oxedrine [BAN]
RN: 94-07-5

Phenylephrine [BAN]
RN: 49-42-7

Synephrine vs. Phenylephrine



- **Synephrine \neq Phenylephrine**



Importance of Distinction



- Phenylephrine is known mydriatic and decongestant.
- Use in pharmaceutical preparations such as Naldecon and Dimetane.
- Phenylephrine has been shown to increase blood pressure.
 - *Br. J. Clin. Pharmacol.* 1991 (32) 705-711.
- Phenylephrine is banned for use by NCAA. WADA monitors phenylephrine but has not yet banned it.
- Synephrine is banned for use by NFL,

Source of Confusion



- J. Clin. Pharmacol. 2001(41) 1059-1063.
 - “Seville (sour) Orange Juice: Synephrine Content and Cardiovascular Effects in Normotensive Adults.”

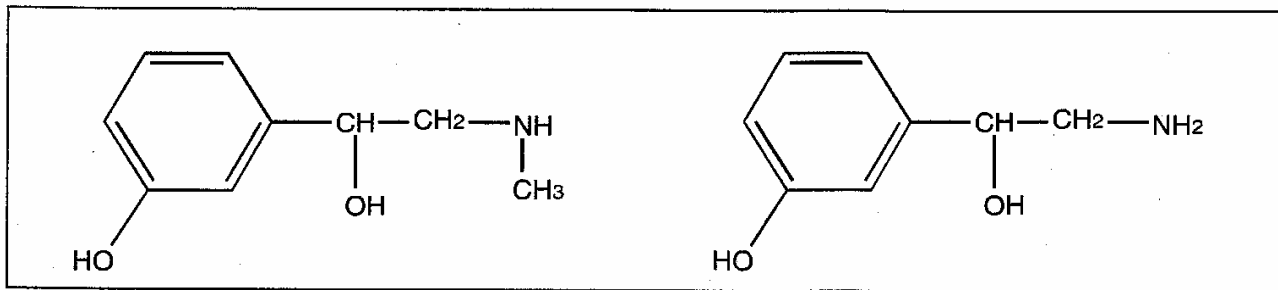


Figure 1. Left: m-syneprine (phenylephrine); right: m-octopamine.



Additional Confusion



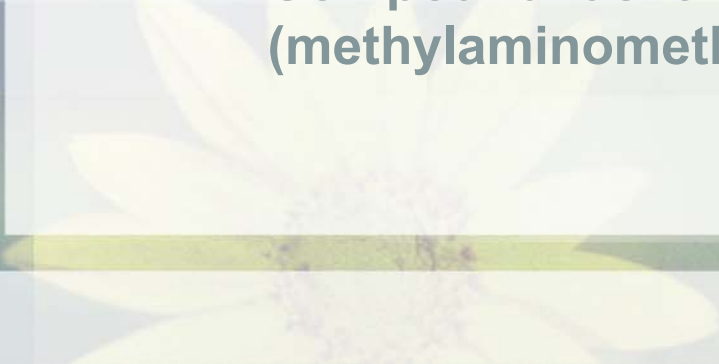
- International Journal of Obesity (2005) 1-4.
 - “Exactly which synephrine alkaloids does *Citrus aurantium* (bitter orange) contain?”
 - Results inconclusive, as authors only tested 1 finished product dietary supplement which specifically claimed to contain *m*-synephrine.



Early Work



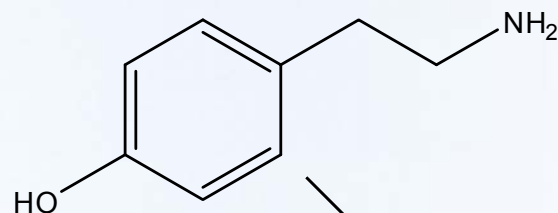
- **J. Biol. Chem. 1964(239) 930-932.**
 - “The Isolation and Identification of *l*-Synephrine in the Leaves and Fruit of Citrus;” Stewart, I. et al.
 - Unknown basic nitrogen compound was detected in tangerine leaf extracts.
 - Compound was extracted, isolated and identified by IR analysis, degradation studies, derivatives, and paper chromatography.
 - Compound identified as *l-p*-hydroxy- α -(methylaminomethyl)benzyl alcohol (synephrine)



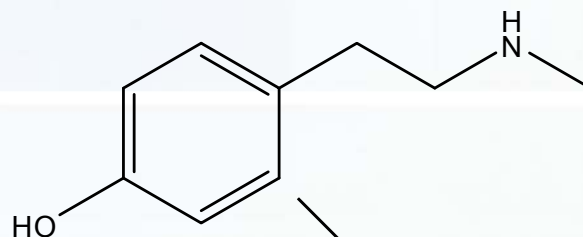
Early Work

•Biosynthetic pathway of synephrine in Citrus species elucidated.

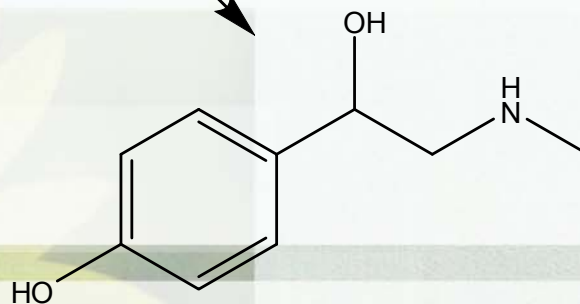
–Phytochemistry 1969 (8) 85-92.



Tyramine



N-Methyltyramine



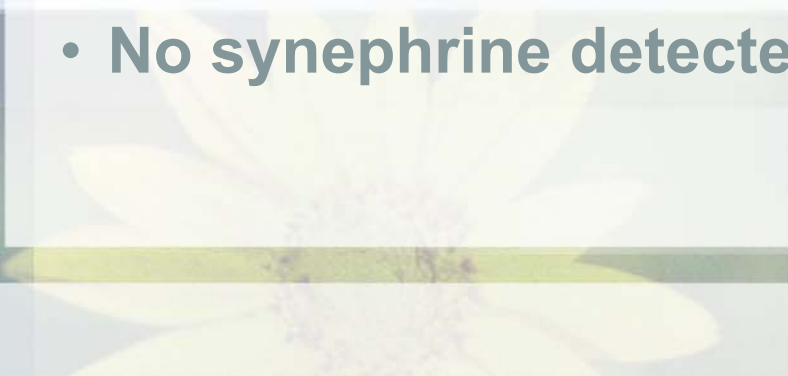
Synephrine



Early Work



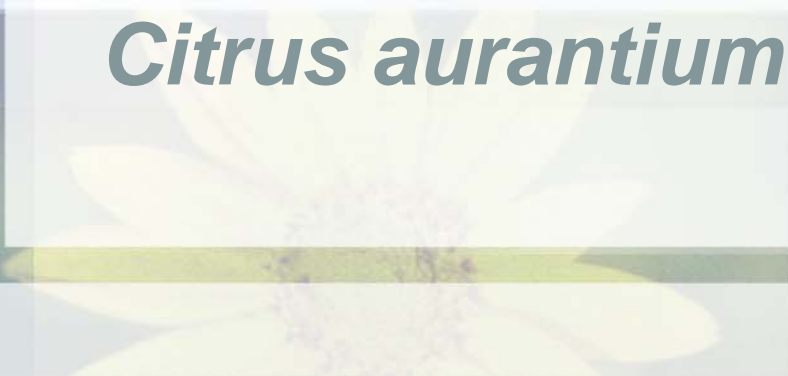
- Lloydia, 1970(33) 244-254.
 - “The Distribution of Tyramine, N-Methyltyramine, Hordenine, Octopamine and Synephrine in Higher Plants.”
 - Synephrine was identified as *p*- isomer.
 - Citrus species examine included *Citrus paradisi* (grapefruit), *Citrus reticulata* Blanco (tangerine), *Citrus sinensis* Osbeck (orange).
 - No synephrine detected in grapefruit.



Summary of Early Work



- *L*-Synephrine was isolated and identified from citrus species.
- Biosynthetic pathway to synephrine in citrus species was elucidated .
- Levels of synephrine in citrus species were determined.
- No work was done specifically on *Citrus aurantium*.





- **Determination of synephrine in *Citrus aurantium*:**

- Hashimoto, K., Yasuda, T., Ohsawa, K. (1992) *J. Chromatogr.* 623, 386-389.
- Kusu, F., Li, X., Takamura, K. (1992) *Chem. Pharm. Bull.* 40, 3284-3286.
- Kusu, F., Matsumoto, K., Takamura, K. (1995) *Chem. Pharm. Bull.* 43, 1158-1161.*
- Kusu, F., Matsumoto, K., Arai, K., Takamura, K. (1996) *Anal. Biochem.* 235, 191-194.*
- He, X., Lian, L., Lin, L., Bernart, M.W. (1997) *J. Chromatogr. A* 791, 127-134.
- Pellati, F., Benvenuti, S., Melegari, M., Firenzuoli, F. (2002) *J. Pharm Biomed. Anal.* 29, 1113-1119.
- Niemann, R.A., Gay, M.L. (2003) *J. Agric. Food Chem.* 51, 5630-5638.
- Avula, B., Upparapalli, S.K., Khan, I.A. (2005) *Chromatographia* 62, 151-157.

*Used phenylephrine as internal standard for quantitation.

Problems with J. Clin. Pharmacol. Paper



- “Synephrine,” “Phenylephrine,” and “m-Synephrine” used interchangeably in paper.
 - Synephrine: 26 times
 - Phenylephrine: 14 times
 - M-Synephrine: 4 times
- Identity of reference standard was not mentioned.
 - No supplier, part number, or characterization data
- Authors did not demonstrate that method could resolve synephrine and phenylephrine.
 - “An additional factor potentially contributing to our results is that we did not separately quantify the respective stereo (+/-)- and positional (*m* and *p*) isomers of synephrine contained in SOJ”



- **Chain of analytical chemistry:**
 - J. Clin. Pharmacol. paper references *Fitoterapia* 1999(70) 586-592 as evidence that CA contains “phenylephrine.”
 - *Fitoterapia* paper only mentions synephrine and references *J. Chromatogr.* 1992(623) 386-389 paper as evidence that CA contains synephrine.
 - *J. Chromatogr.* paper specifically identifies compound as p- isomer of synephrine.

Current Work



- NIH-ODS funded development and validation of method for the determination of 5 biogenic amines in CA and phenylephrine.
 - Synephrine, octopamine, tyramine, N-methyltyramine, hordenine.
- Method was HPLC-UV, and was to be applicable to CA raw materials, extracts, and dietary supplements.

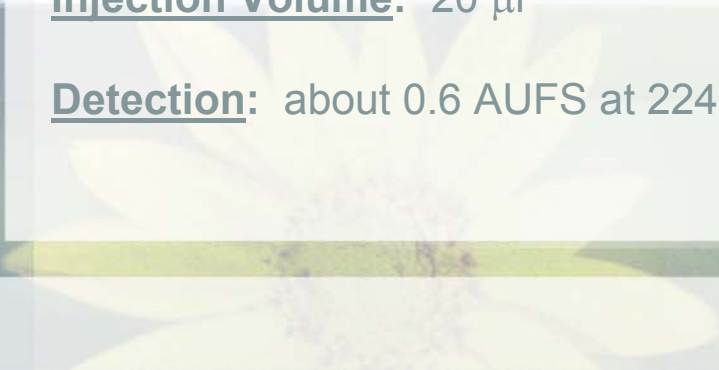
Chromatography



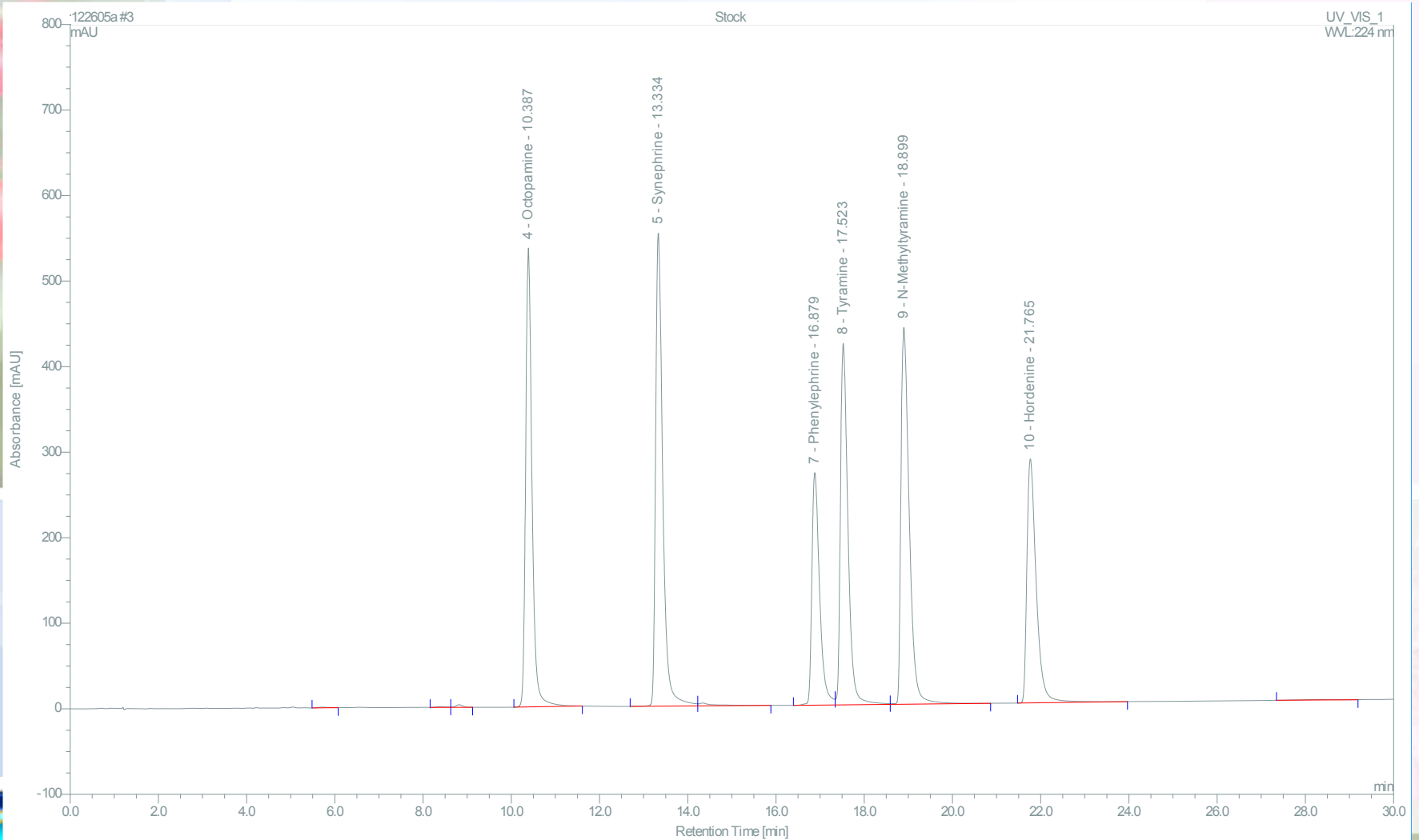
- **Column:** Phenomenex Luna C18(2), 3.0 x 150 mm. 5 μ m particle size
- **Column Temperature:** 35° C
- **Mobile Phase:** A – 10 mM hexansulfonate in 20 mM Borate Buffer, pH 8.2
B – 20:80 (v/v) Acetonitrile:20 mM Borate Buffer, pH 8.2 + 10 mM hexanesulfonate
- **Mobile Phase Gradient:** The mobile phase is changed linearly according to the following program:

<u>Time (min)</u>	<u>%A</u>	<u>%B</u>
0	100	0
30	0	100

- **Flow Rate:** 0.85 mL/minute
- **Injection Volume:** 20 μ l
- **Detection:** about 0.6 AUFS at 224 nm



Chromatography - Standards



Materials in Validation Study



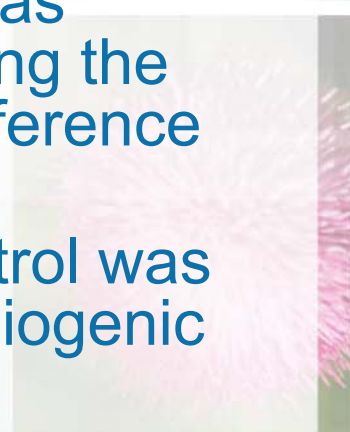
- Whole dried immature fruit of CA
- 30% CA powdered extract
- Powder bitter orange fruit from NIST
- Powdered bitter orange extract from NIST
- 2 Finished product dietary supplements containing CA.



Validation Design



- Repeatability: 5 replicate preparations of each of 6 materials (2 raw materials, 2 extracts, and 2 solid oral dosage forms) were analyzed on each of 4 days (20 total replicates of each).
- Accuracy:
 - Raw Material: Raw materials were exhaustively extracted with Dionex Accelerated Solvent Extractor (ASE 200) to obtain reference values for each biogenic amine. The % recovery was determined as the amount recovered using the method's extraction procedure vs. the reference value obtained.
 - Solid Oral Dosage Forms: Negative control was spiked at 3 levels in triplicate with each biogenic amine, and % recovery was calculated.



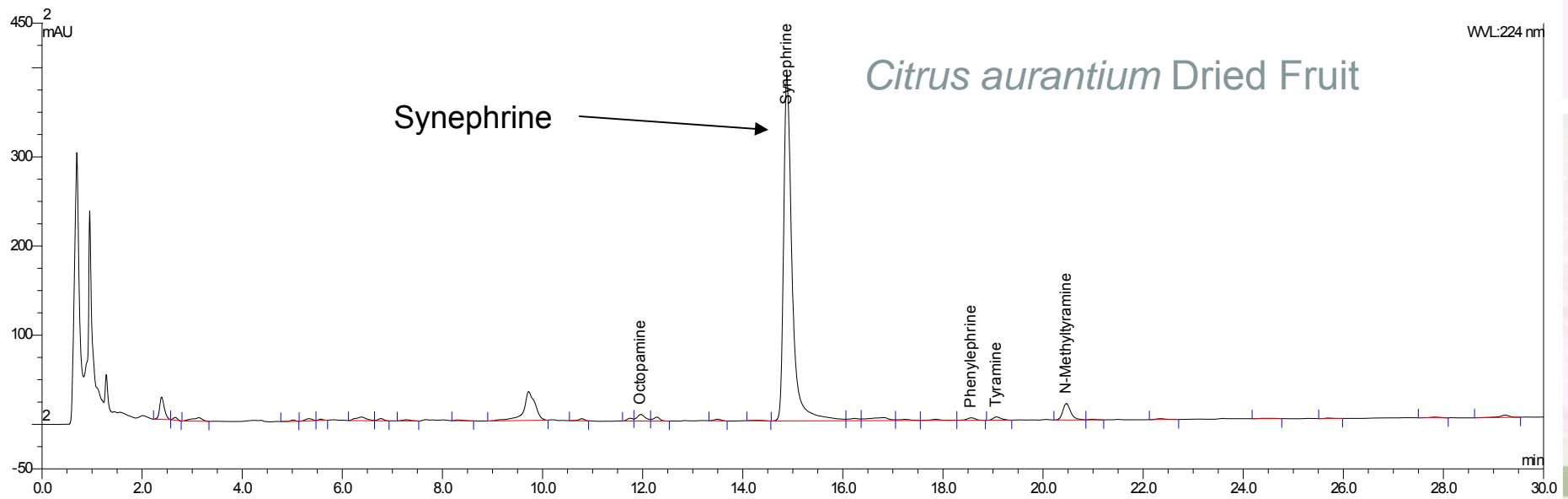
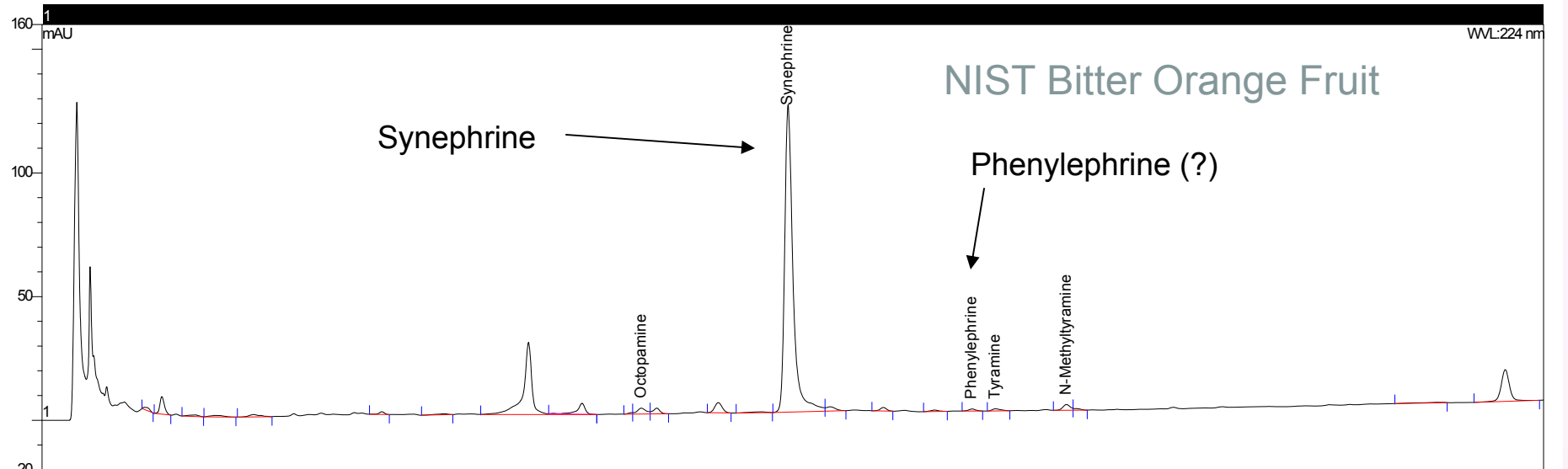
Validaton Design



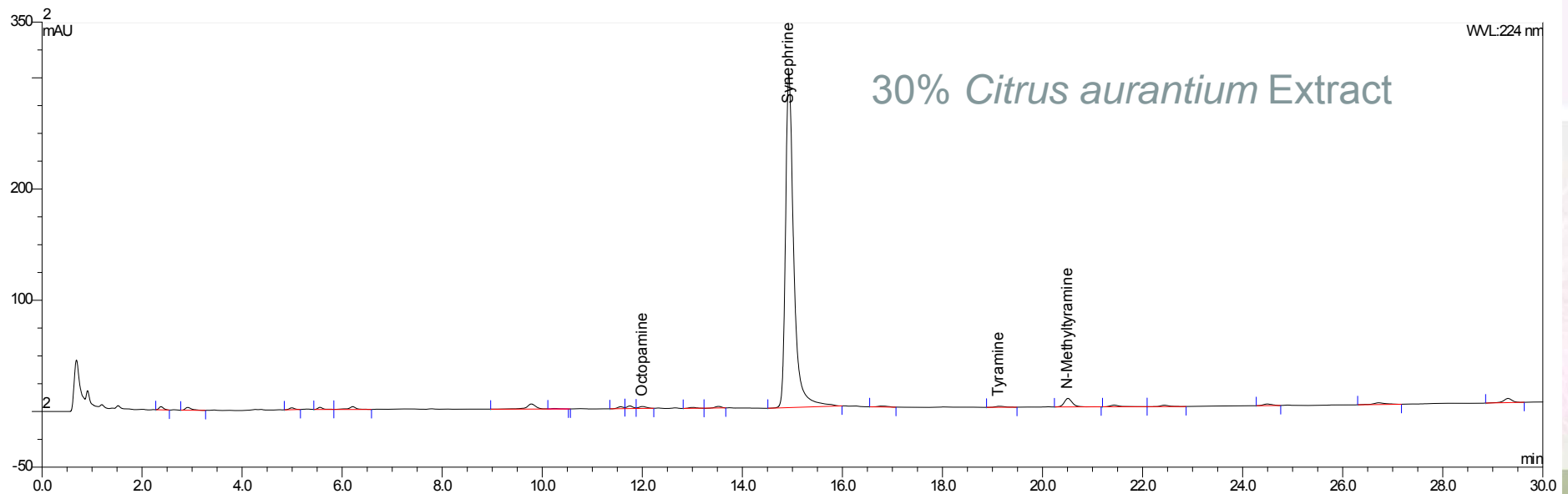
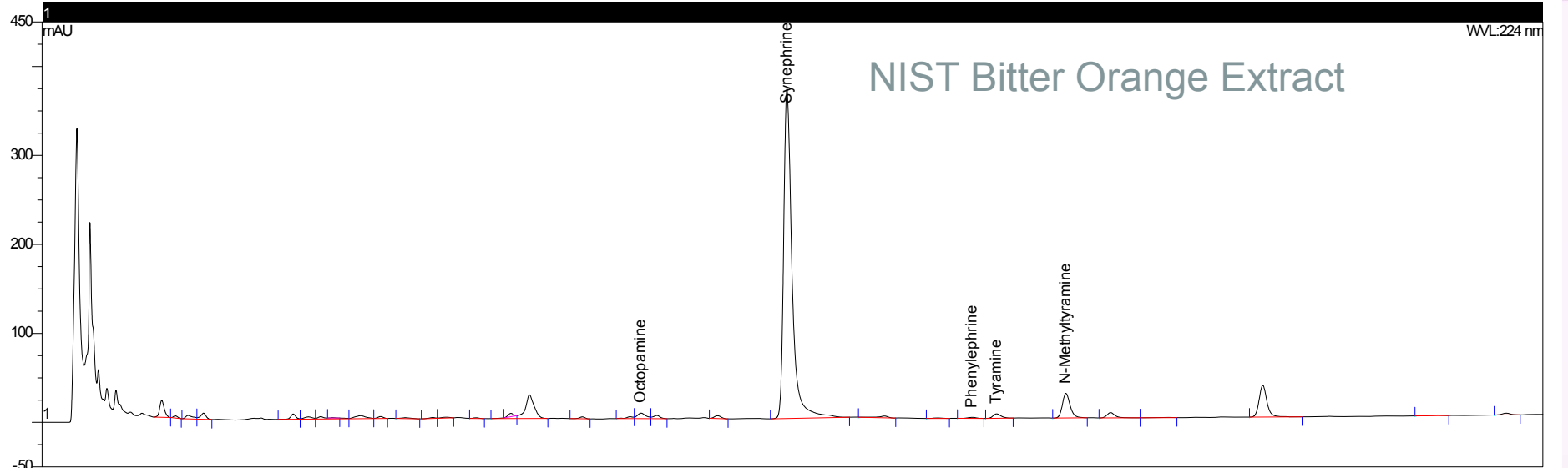
- **Linearity:** A six-point calibration curve covering 2 orders of magnitude in concentration range ($\sim 1 \mu\text{g/mL} - 100 \mu\text{g/mL}$) was generated for each day of analysis. Linear regression was used to calculate the slope and y-intercept of the standard curve for each analyte. The correlation coefficient and % RSD of each standard curve for each day was determined.
- **Selectivity:** Selectivity of the method was confirmed by photodiode array detection (PDA), and by injecting the dietary supplement matrix blank.
- **Ruggedness:** Analyses were performed on 2 different HPLC systems (Dionex Summit and Agilent 1100) by 2 different analysts. In addition, 2 different lots of C18 columns were used, and results obtained using a different brand of C18 column were also compared. Systematic changes in ion-pairing concentration, organic modifier concentration, mobile phase pH, and column temperature were also performed, and their affects on the separation and run time observed



Results of Validation Study



Results of Validation Study



Synephrine Precision



Material	n ¹	Result ²	SD ²	% RSD	PRSD ³	HorRat
A	20	8,290	123	1.48	4.10	0.36
B	20	26,110	439	1.68	3.46	0.49
C	20	66,580	1,585	2.36	3.00	0.79
D	20	292,300	6,898	2.37	2.41	0.99
E	20	11,980	432	3.55	3.88	0.91
F	20	4,686	80.3	1.71	4.47	0.38

A = NIST Bitter Orange Fruit Powder

B = Nutratche *Citrus aurantium* dried fruit

C = NIST Bitter Orange Extract 6% Synephrine

D = Nutratche *Citrus aurantium* Extract 30% Synephrine

E = Product A Capsules

F = Product B Tablets

¹n=Total number of samples tested

²Results in µg/g

³PRSD = Predicted RSD

Validation Results



NIST Bitter Orange Dried Fruit Powder

Compound	Dionex ASE, $\mu\text{g/g}$	Sonication, $\mu\text{g/g}$	% Recovery	% RSD ²
Octopamine	158 ¹	148 ¹	93.6 ¹	9.25
Synephrine	8610	8270	96.0	1.30
Phenylephrine	193 ¹	ND	0	NA
Tyramine	75 ¹	ND	0	NA
N-Methyltyramine	146 ¹	ND	0	NA
Hordenine	22 ¹	ND	0	NA

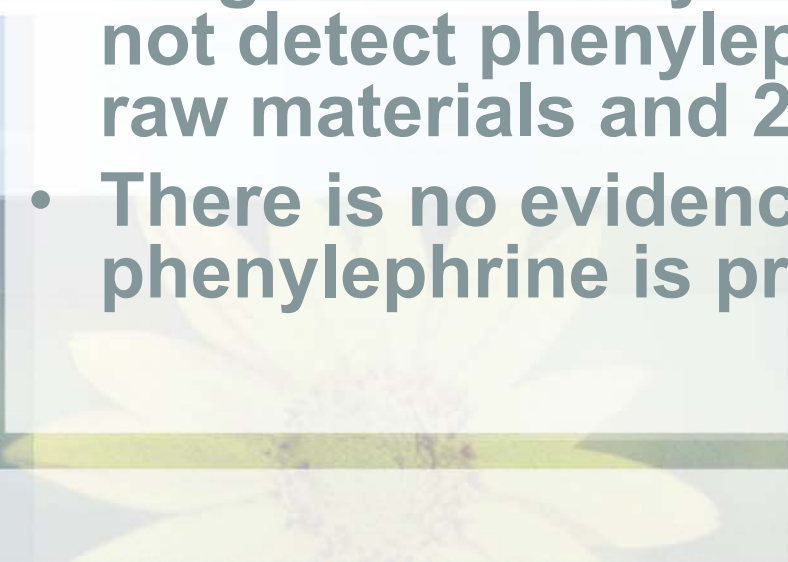
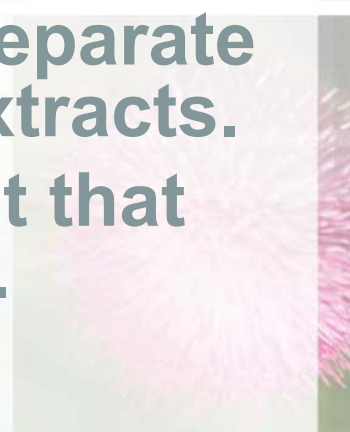
¹Below limit of quantitation of the method

•Phenylephrine was below the reliable limit of detection in every sample (~200 $\mu\text{g/g}$ in raw material)

Conclusions



- Presence of phenylephrine in *Citrus aurantium* is greatly exaggerated.
- Biosynthetic pathway in citrus species to form synephrine has been elucidated.
- Vast bulk of published analytical work on CA identifies the primary biogenic amine as synephrine.
- Single laboratory validation work did not detect phenylephrine in 2 separate raw materials and 2 separate extracts.
- There is no evidence to suggest that phenylephrine is present in CA.



Acknowledgements



Method development and validation project was funded by a contract with the Office of Dietary Supplements, U.S. National Institutes of Health.

