Quantitative Analysis of Labdanoids in Tobacco Leaves by Gas Chromatography

KASHIMA Takasumi, ISHIDA Naoyuki,
GOHARA Yoshito and YOKOI Michinori

JAPAN TOBACCO INC.

Outline

- Background & Objective
- Identification of labdanoids in cured Oriental tobacco leaves
- Quantification for identified labdanoids
- Comparison of the quantified labdanoids in various tobacco leaves

Background

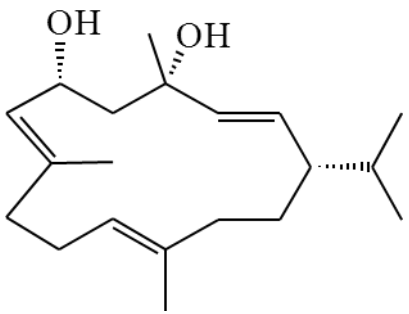
Tobacco leaf

Leaf surface resin

- Sugar ester
- Hydrocarbon
- Fatty alcohol
- Fatty acid
- Diterpenoid

Low polarity

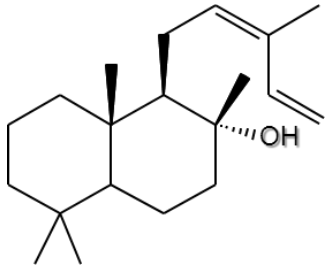
Cembranoids



Cembra-2,7,11-triene-4,6-diol

- Major diterpenoids present in *Nicotiana species*
- More than 100 compounds are known

Labdanoids



cis-Abienol

- Major diterpenoids in Oriental (ORI)
- More than 50 compounds are known
- Giving taste and aroma specific to ORI
- Considered to be generated from *cis*-abienol during curing process[1)]

1) Wahlberg I., *et al*, *Acta Chem. Scand.*, B32, 203-215, 1978

Objective

Comprehensive quantification of labdanoids in cured tobacco leaves has not been accomplished.

- **To identify major labdanoids in cured Oriental tobacco leaves**
- **To quantify labdanoids and compare their amounts among various cured tobacco leaves**

Experimental procedure for identification

Cured Oriental tobacco leaves

Cured tobacco leaves

Pulverization

Pulverized tobacco leaves

Extraction by *n*-hexane

Shaken for 30 min at 200 rpm

Filtration

Evaporator

Conc. *in vacuo*

Silica gel column chromatography

Silica gel C.C.

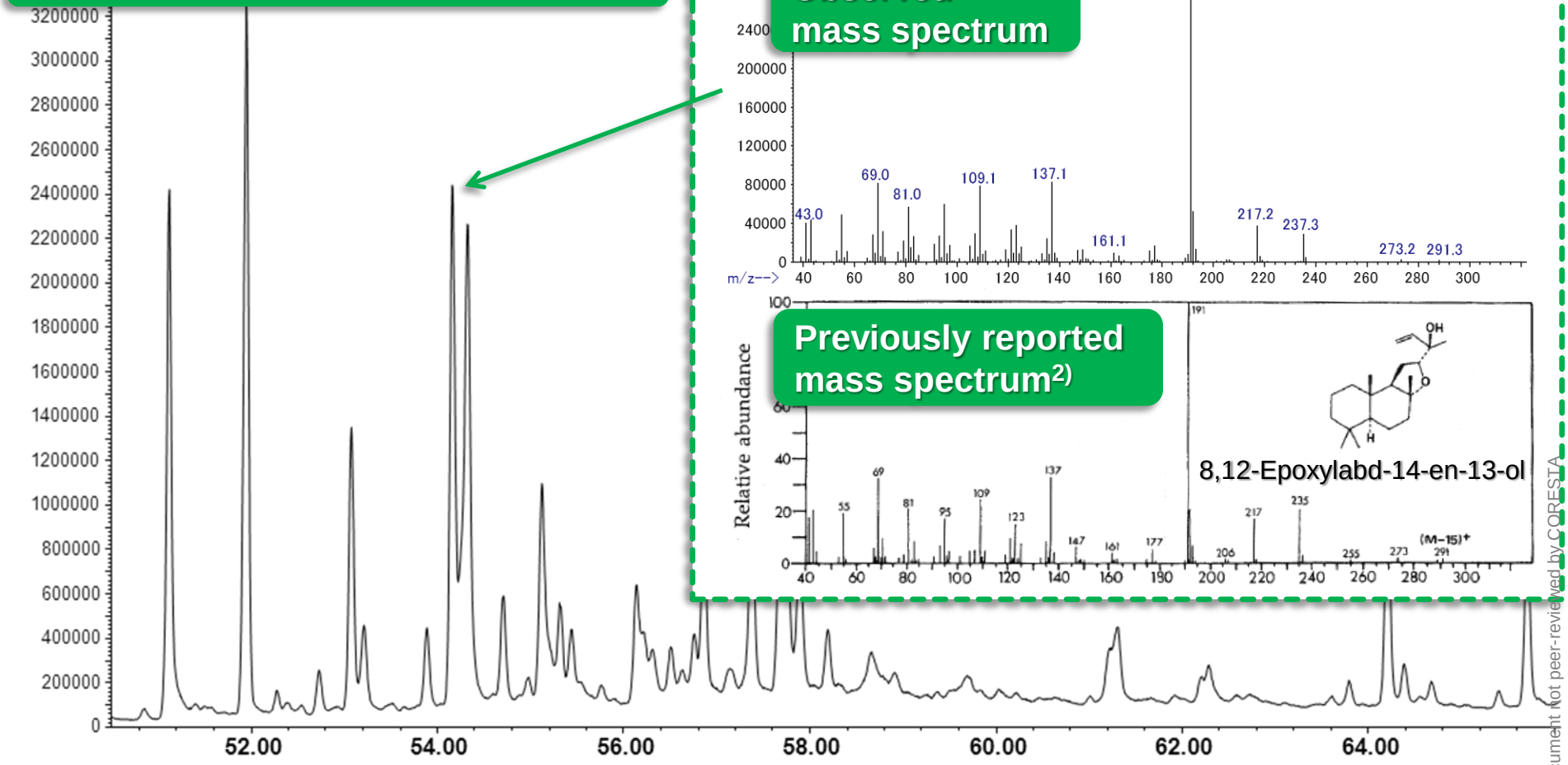
Stepwise elution with *n*-hexane/EtOAc

GC/MS analysis

Agilent 7890A GC and 5975C MSD
(Agilent Tech., CA, USA)

Identification of labdanoids by mass spectrum

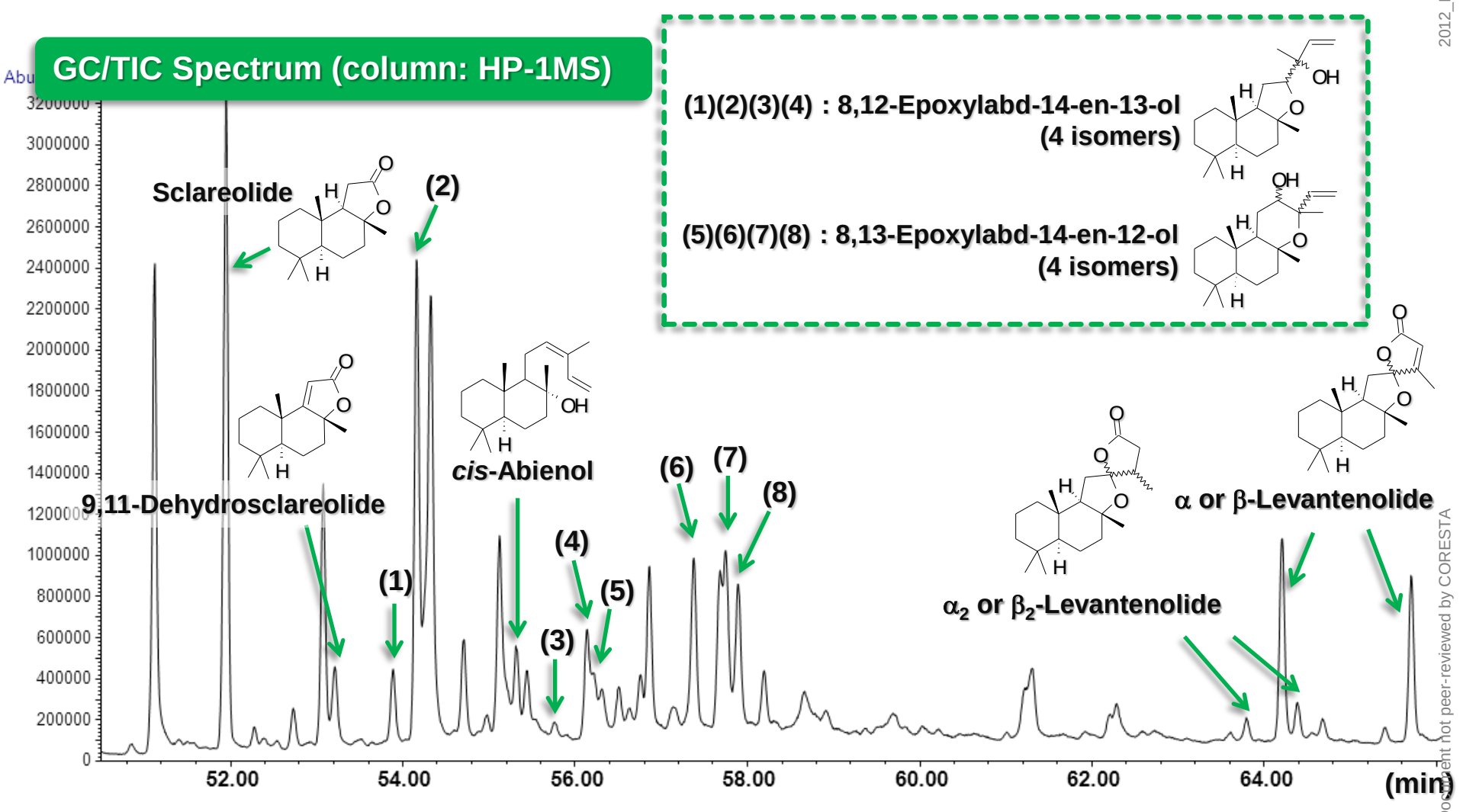
GC/TIC Spectrum (column: HP-1MS)



- **Obtained spectrum was compared to the previous report.**

2) Enzell C. R., *et al*, *Mass Spectrom. Rev.*, 3, 395-438, 1984

Identified major labdanoids



- **15 labdanoids were identified on the chromatogram.**

Modification of method for quantification

Some techniques were applied to method for quantification.

- **Authentic standard**
  → Sclareolide was chosen as an authentic standard compound to quantify each individual labdanoid.

- **GC Detector**
  → A flame ionization detector (FID) was selected owing to its organic-component-versatility enabling quantification by using only sclareolide.

- **Internal standard**
  → *n*-Hepadecanol was added in advance to extraction solvent.

- **Sample preparation**
  → *n*-Hexane extract from tobacco sample was charged on solid phase extraction cartridge (SPE) without concentration.

- **GC column**
  → Appropriate Column (DB-35MS) was selected to ensure the separation of labdanoids.

Quantification method

Sample preparation

Pulverized tobacco (1.0 g)

▼ Extracted with 10 mL of *n*-hexane containing ISTD (*n*-heptadecanol)

▼ Filtration

Filtrate (2.5 mL)

▼ Charged on SPE (silica gel, 500 mg)

▼ Washed with *n*-hexane (4 mL) and, then eluted with EtOAc:Hex = 40:60 (4 mL)

Eluate

▼

GC-FID & GC-MSD

Instrument condition

Instrument

Agilent 7890A/5975C

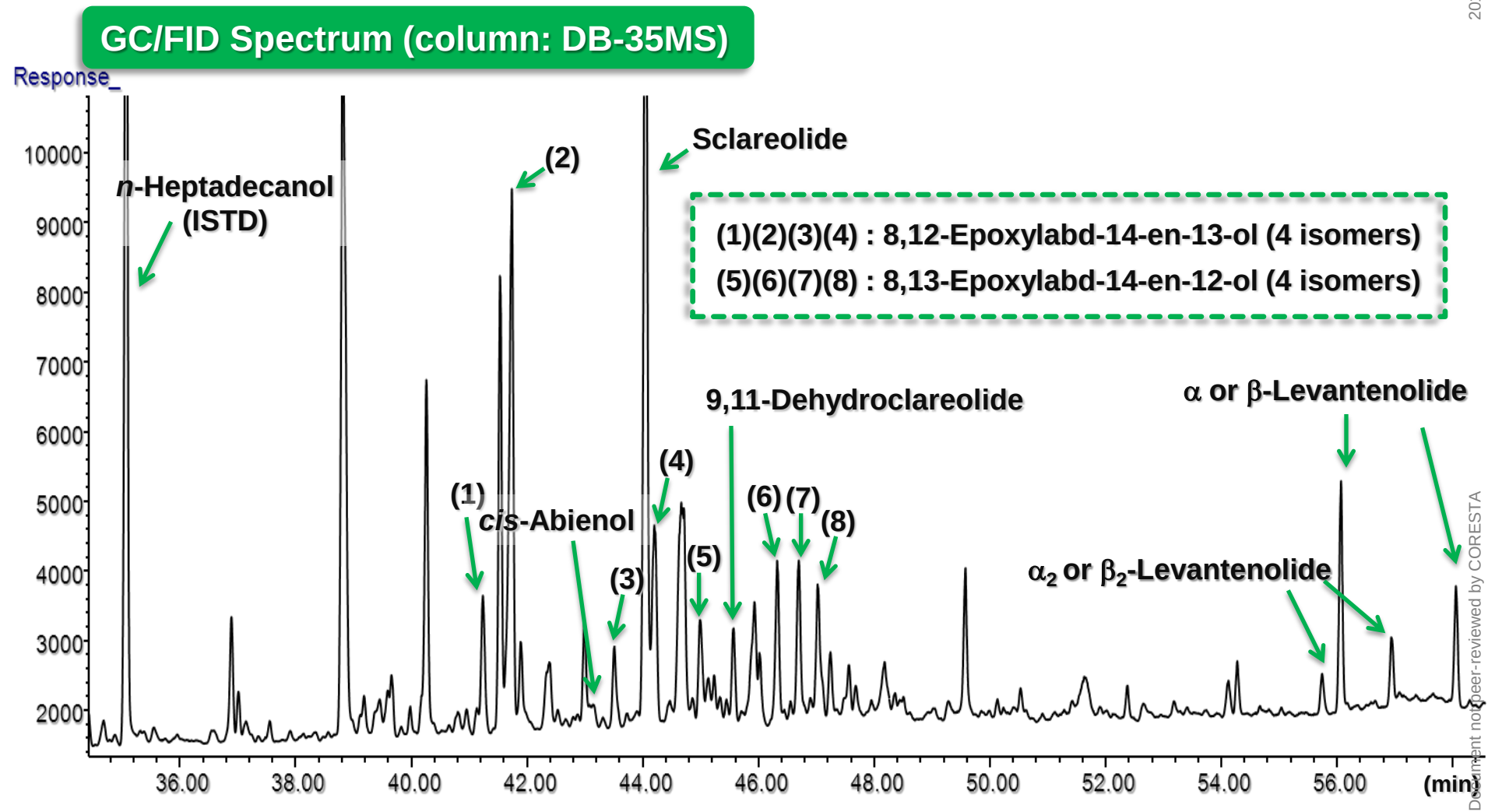
GC condition

Column : DB-35MS, 30 m, 0.25 mm i.d., 0.25 mm f.t.
Oven : 100˚C (2min) →(3˚C/min)→300˚C (10min)
Injection : Pulsed splitless, 30 psi, 2μL
Carrier gas : He (1.3 mL/min, constant flow)

Detector condition

FID	Temperature : 330˚C
	Make up : N_2 (45 mL/min flow)
	H_2 flow : 40 mL/min
	Air flow : 450 mL/min
MSD	Mode : EI (70eV) scan mode
	Transfer line temp. : 280˚C
	Ion source temp. : 230˚C
	Quad temp. : 150˚C
	Scan range : 35-550 m/z

Chromatogram for quantification



- **Labdanoids were well-separated with the modified method.**
  (Peak identification was carried out by GC/MS under the same GC condition.)

Validation

Validation study was conducted by using sclareolide.

➢ Linearity

- ♦ Desirable correlation coefficient (R^2 = 0.9996) was obtained. (0.5 to 32.0 μg/mL)

➢ RSD, LOD and LOQ

Repetition	Sclareolide μg/mL
1	1.14
2	1.11
3	1.08
4	1.05
5	1.08
6	1.10
7	1.08
8	1.07
9	1.10
10	1.07
Mean	1.088
SD	0.024
RSD%	**2.18**
LOD※	**0.071**
LOQ※※	**0.237**

※ LOD : SD × 3
※※ LOQ : SD × 10

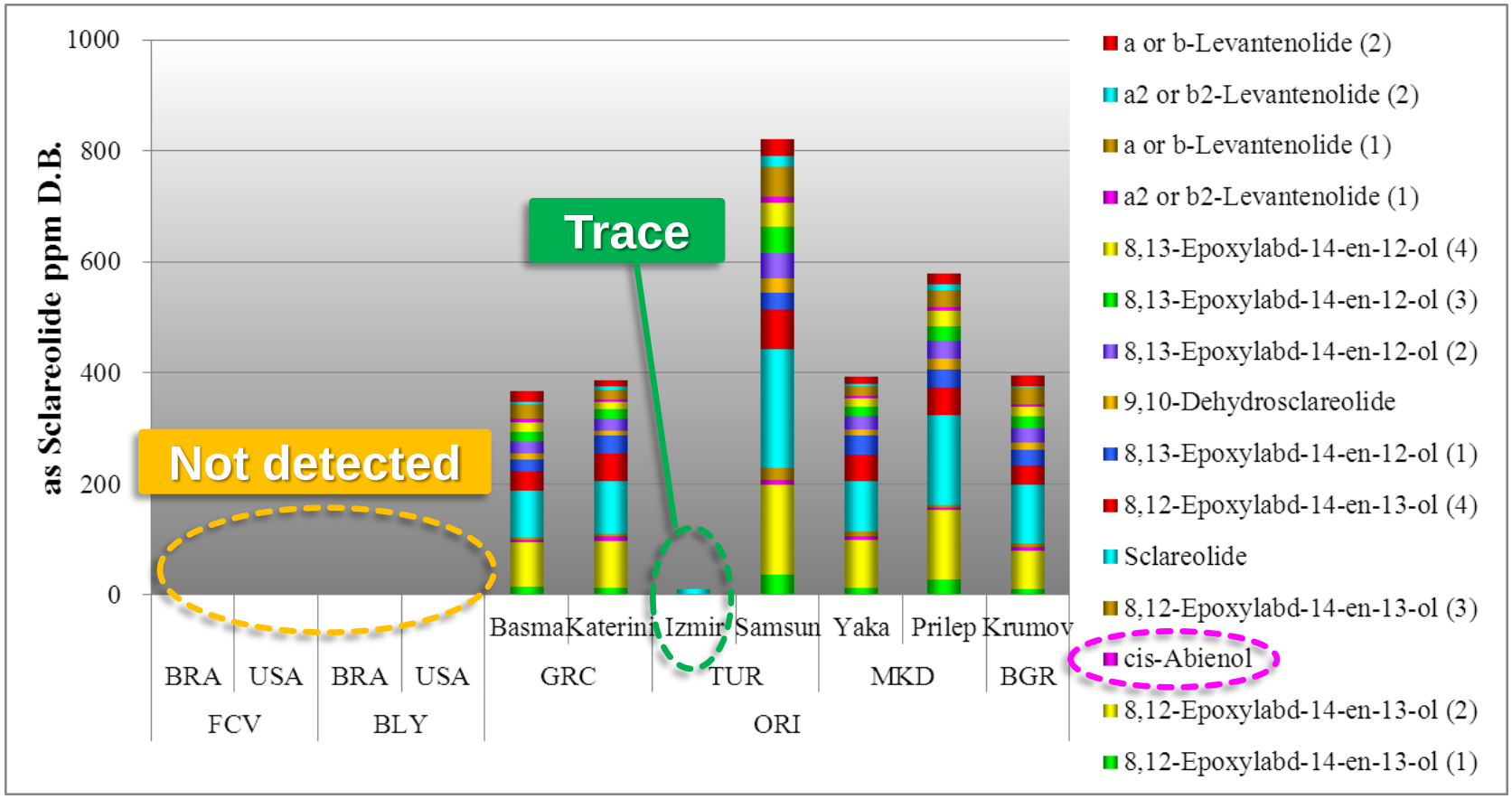
- ♦ Measurements were replicated 10 times.

➢ Recovery

Repetition	Sclareolide μg/mL			
1	3.59	4.77	7.63	20.46
2	3.47	4.55	7.56	20.62
3	3.76	4.64	7.86	20.17
4	3.64	4.64	7.80	20.70
5	3.44	4.68	7.70	20.58
6	3.66	4.68	7.69	20.35
7	3.54	4.43	7.64	20.36
8	3.73	4.85	7.92	20.22
9	3.66	4.73	7.91	20.84
10	3.43	4.71	7.73	20.39
Mean	3.59	4.67	7.74	20.47
Spiked Conc.	0.00	1.02	4.07	16.30
Recovery%	-	**105.57**	**101.88**	**103.55**

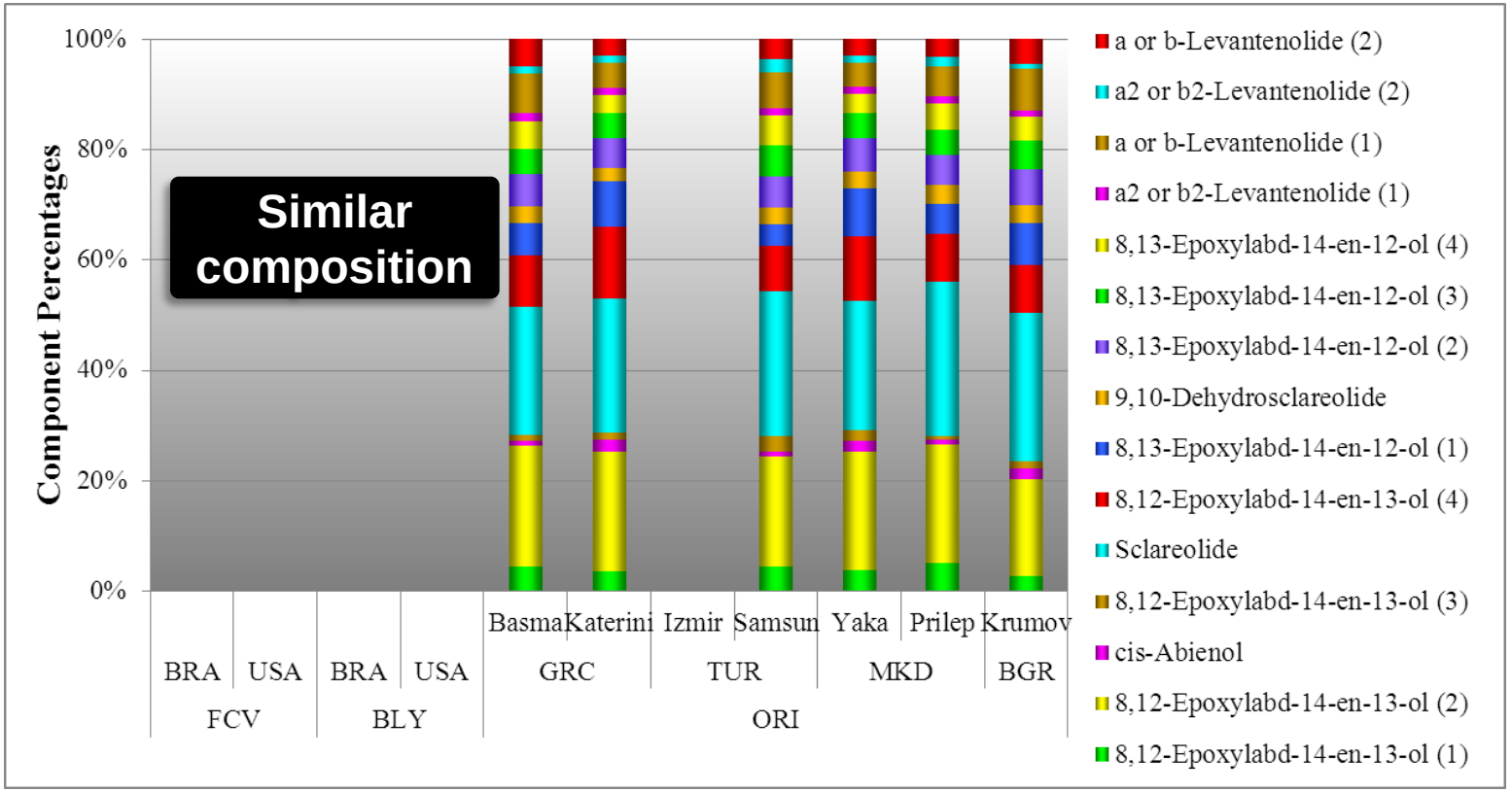
- ♦ Tobacco samples were spiked with sclareolide at the time of extraction.

Quantification for various tobacco leaves



- Not detected in flue-cured Virginia (FCV) and Burley (BLY)
- Trace of labdanoids in Izmir
- Low amount of *cis*-abienol in cured Oriental
- Total amount of labdanoids varied among cultivars

Quantification for various tobacco leaves



- **Oriental leaves rich in labdanoids showed similar composition.**
- **Labdanoids might be generated from oxidation of *cis*-abienol during curing process.[1)]**

Even though cultivars and growing districts differ, the mechanisms of dominant *cis*-abienol oxidation must pass through common pathway.

Summary

➢ Identification

***cis*-Abienol**

Sclareolide and its derivative

Epoxylabdan

Levantenolide

15 compounds were identified in cured Oriental leaves.

➢ Quantification and Comparison

Total amount :

Samsun > Prilep > Basma, Katerini, Yaka, Krumov >> Izmir
(No labdanoid in FCV, BLY)

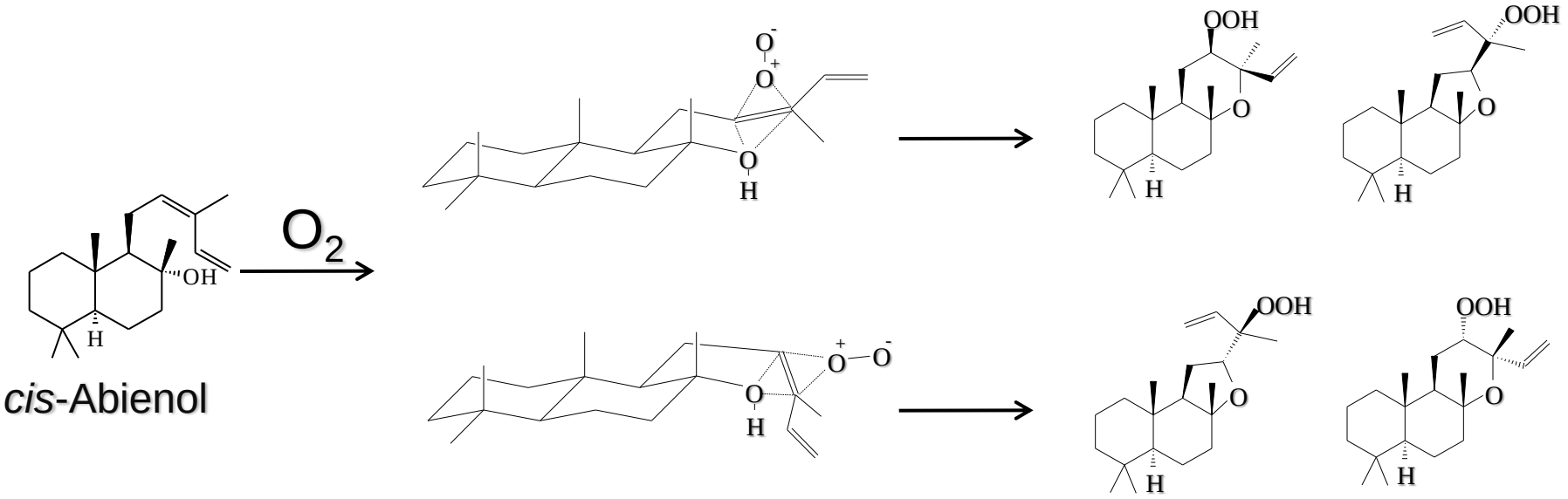
Composition :

Similar pattern among different cultivars and growing districts

Appendix

Oxidative degradation of *cis*-abienol

- **Degradation mechanisms of *cis*-abienol during curing process were explained by biomimetic sensitized photo-oxygenation.[1,7)]**
  - ✓ Sensitized photo-oxygenation of *cis*-abienol gives several types of epoxylabdans through peroxide transition state.



cis-Abienol

1) Wahlberg I., *et al*, *Acta Chem. Scand.*, B32, 203-215, 1978

3) Wahlberg I., *et al*, *Acta Chem. Scand.*, B33, 437-442, 1979

Biosynthesis of *cis*-abienol

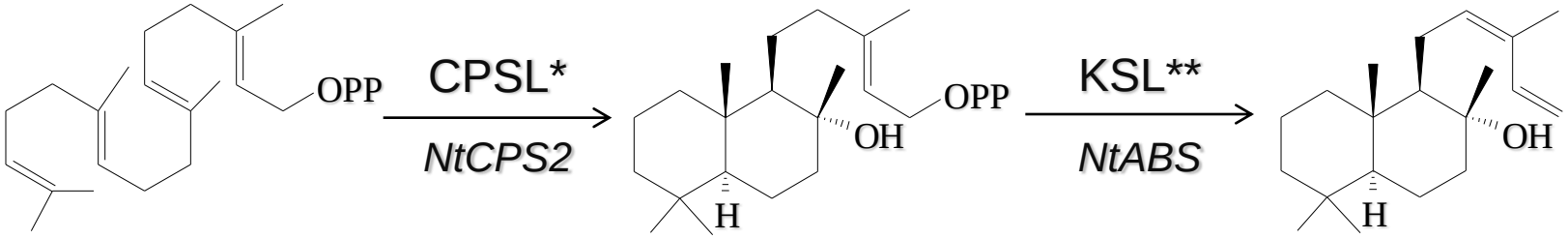
- **Biosynthesis of *cis*-abienol was found to be genetically controlled by a single locus, *Abl*.[4)]**

  N. sylvestris : has neither *Abl* locus nor *cis*-abienol accumulation

  N. tomentosiformis : has *Abl* locus and *cis*-abienol accumulation

4) Vontimitta V., *et al*, *J. Agric. Food Chem.*, 58, 294-300, 2010

- **Two genes for the biosynthesis of *cis*-abienol in *N. tabacum* were characterized.[8)]**



GGPP (Geranylgeranyl pyrophosphate)

8-OH-CPP (8-Hydroxycopalyl diphosphete)

cis-Abienol

* Copalyl diphosphate synthase – like enzyme

** Kaurene synthase – like enzyme

5) Sallaud C., *et al*, *Plant J.*, in press, 2012