Potential of the Reversed-Inject Differential Flow Modulator for Comprehensive Two-dimensional Gas Chromatography in the Quantitative Profiling of Complex Natural Samples

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Foreword

Basics of Differential Flow Modulation with Reverse Inject dynamics
✓ system configuration
✓ principles of operation
✓ challenges

Complex Vegetal Samples
✓ compositional characteristics
✓ sample dimensionality
✓ investigation strategies: profiling and fingerprinting

System optimization: column settings and performance parameters
✓ Peak capacity, selectivity exploitation and information dimensions
✓ Model Mixture of volatiles of interest in the F&F field

Real-world samples
✓ full quantitative assessment by GC×2GC-FID/MS - Mint and Lavender EOs
✓ fingerprinting and classification by chemical signature - Vetiver EOs

Concluding remarks
A CRITICAL ASSESSMENT OF THE CURRENT STATUS AND POTENTIAL FUTURE OF GC×GC

Matthew Klee¹, Leonid Blumberg²

.... Comprehensive multidimensional gas chromatography (CMDGC or GC×GC) is probably the most promising invention in GC since discovery of capillary columns more than half a century ago.

The approach has the potential to provide considerably more sample information in the same timeframe as single dimension GC analyses.

But...
Differential Flow Modulation with “Forward Fill/Flush” dynamics

Simplified design J. Seeley et al. [1]
- low operational costs
- robust hardware

Fully-flexible configurations [2,3]
- adjustable sample loop (length & diameter)
- extended re-injection periods
- column configuration extremely flexible
- compatibility with MS detection

Commercial device - Agilent 2006 [4]
- Capillary Flow Technology (CFT) microfluidic plates
- Forward Fill/Flush (FFF) dynamics [1]
- Sample loop fixed volume
- Operative limitations (columns diameter and volumetric flows)

Differential Flow Modulation with “Forward Fill/Flush” dynamics

Adapted from Agilent 5989-9889EN
Differential Flow Modulation with “Forward Fill/Flush” dynamics

Injection

Modulation Valve

He from Aux EPC

Loop Collection channel

1D column

S/SL inj

2D column

Detector

Successful applications

✓ fatty acids methyl esters [1]
✓ hydrocarbons in light cycle oils [2]
✓ gasoline and kerosene [3]
✓ volatiles roasted almonds [4]

Adapted from Agilent 5989-9889EN

Differential Flow Modulation with “Reverse Fill/Flush” dynamics

* Rough representation of internal channel

Length and diameter of the restrictor capillary are chosen according to pressure/flow conditions of columns to provide flow equivalent to the output of the first dimension.
Differential Flow Modulation with “Reverse Fill/Flush” dynamics

Advantages of the RFF dynamics

- higher efficiency of band re-injection
- improved 2D peak-widths
- improved 2D peak symmetry
- “adjustable” collection channel volume (bleed capillary restriction)
- better handling of the overloading phenomenon [1,2]

Differential Flow Modulation with “Reverse Fill Flush” dynamics

“Streaking” effect due to collection channel overloading

Non-Gaussian 2D profile “Apparent” overloading of the 2D column

trans-2-hexenyl acetate - variable amount

2D peaks of improved symmetry

Magnified resolution in the 2D

Complex Vegetal Samples
compositional characteristics

Essential Oils, Extracts and (Volatile) fractions

**Essential oils**¹ (EO): product obtained by hydro-, steam- or dry-distillation or by a suitable mechanical process without heating (for *Citrus* fruits) of a plant or of some parts of it.


Distillates and/or extracts selectively concentrate volatiles:
✓ **Simultaneous Distillation-Extraction** (SDE);
✓ Normal pressure or vacuum (hydro-)distillation;
✓ **Solvent Assisted Flavour Evaporation** (SAFE);
✓ **Ultrasound or microwave-assisted hydrodistillation**
✓ **Ultrasound or microwave-assisted extraction** (USE, MAE);
✓ **Selective and/or pressurised (or accelerated) solvent extraction** (ASE);
✓ **Supercritical fluid extraction** (SFE).

**Volatile fraction** can be also extracted in the “vapour” phase through headspace (HS) sampling approaches:
**Static Headspace** (S-HS) extraction, **Dynamic Headspace** (D-HS) and **High Concentration Capacity HS** techniques (SPME, HSSE, MME, MESI etc.).
Samples of vegetable origin (EOs, extracts, volatile fractions):
secondary metabolites with common/similar skeleton
common biosynthetic pathways
very variable abundance (from % to µg/Kg)
differing polarity (hydrocarbons, oxygenated derivatives, aromatics etc.)

Samples are characterized by 100-1000 components
Challenge for mono-dimensional separation platforms

“... there is some intrinsic property of analytical samples (other than the number m of components) that determines their amenability to multidimensional techniques.
... the key property is related to sample variability...and is defined as sample dimensionality s”
“The parameter s is the number of independent variables that must be specified to identify the components of the sample”
Complex Vegetal Samples
Investigation strategies

Characterize sample composition (detailed profiling)
Quantification of informative analytes
   (bio)-markers
   toxic compounds
   regulated substances (e.g. volatile suspected allergens)
   potent odorants (e.g. key-aroma compounds)
Detect adulterations - origin assessment
Classification based on chemical signatures (fingerprinting)

GC×GC with thermal modulators
   effective (sensitivity gain and peak capacity)
   reliable (identification/quantitation)

But...
Quality Control Laboratories needs
   Low operational costs
   Simple design and maintenance
Agilent 7890B GC equipped with 7650A autosampler and 5977A MSD operating in EI mode at 70 eV - FID detector
Scan speed 20,000 amu/s Etune option

Reverse-inject differential flow modulator
Prototype consisting of a CFT microfluidic plate
Aux PCM He
Three-way solenoid valve

Capillary columns, unions and non-purged tees were from Agilent
Bleeding capillary was calibrated to counterbalance the 1D column effluent during the filling stage.
To verify the absence of bleeding the capillary was connected to the FID and signal collected during the analytical run.

Raw data was acquired by Enhance MassHunter (Agilent Technologies)

2D data was processed by GC Image® GC×GC Edition Software, Release 2.5 (GC Image, Lincoln NE, USA).
I. “Recommended Configuration”

1. **1D - Apolar SE52**
   - 30m×0.25mm×0.25µm
   - He carrier @ 0.35mL/min

2. **2D - Medium polarity OV1701**
   - 5.0m×0.25mm×0.25µm
   - He carrier @ 25mL/min

Model mixture of volatiles
- Mono, sesqui and diterpenoids
- Synthetic odor-active compounds
- Functionalities: hydrocarbons, alcohols, carbonyls, esters and aromatics
- LRI interval (apolar) 900-2350

Medium complexity Essential Oils
- Mint spp. and Lavender spp. (200-250 peaks)

High complexity Essential Oils
- Vetiver (*Chrysopogon zizanioides* L.) (500-600 peaks)

### Table of Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound</th>
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<tbody>
<tr>
<td>6-Methyl coumarin</td>
<td>Damascenone</td>
</tr>
<tr>
<td>α-(Z)- santalol</td>
<td>δ-Damascone</td>
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<tr>
<td>α - Terpineol</td>
<td>Eugenol</td>
</tr>
<tr>
<td>α-Damascone (Z)</td>
<td>Eugenyl Acetate</td>
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<tr>
<td>α - Pinene</td>
<td>(E,E)-Farnesol</td>
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<tr>
<td>Amyl Cinnamal</td>
<td>(E,Z)-Farnesol</td>
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<td>Anethole</td>
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<td>Benzaldehyde</td>
<td>Hexadecanolate</td>
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<td>Benzyl Benzoate</td>
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<td>Terpinolene</td>
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<td>Citronellol</td>
<td>Vanillin</td>
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<tr>
<td>Coumarin</td>
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</table>
I. “Recommended Configuration”

1D - Apolar SE52
30m×0.25mm×0.25µm
He carrier @ 0.35mL/min

2D - Medium polarity OV1701
5.0m×0.25mm×0.25µm
He carrier @ 25mL/min

Oven programming
80°C(2’) to 280°C(10’) @ 3°C/min
Modulation period: 2.5 s
Injection: 0.11 s
Analysis time 75’ (last eluted sclareol)
Few critical pairs
I. “Recommended Configuration”

1D - Apolar SE52
30m×0.25mm×0.25µm
He carrier @ 0.35 mL/min

2D - Medium polarity OV1701
5.0m×0.25mm×0.25µm
He carrier @ 25 mL/min

II. Alternative Configuration ApMp1

1D - Apolar SE52
10m×0.10mm×0.10µm
He carrier @ 0.40 mL/min

2D - Medium polarity OV1701
two parallel 1.0m×0.10mm×0.10µm
He carrier @ 6 mL/min

Added features:
✓ 1D narrow-bore column
✓ two 2D columns (doubled loading capacity - halved flow resistance)
✓ 2D flows compatible to MS
System optimization

column settings

Oven programming
50°C(1’) to 280°C(10’) @ 5°C/min
Modulation period: 2.5 s
Injection: 0.11 s
Analysis time 35’ (last eluted *sclareol*)
Few critical pairs
I. “Recommended Configuration”

1D - Apolar SE52
30m×0.25mm×0.25µm
He carrier @ 0.35 mL/min

2D - Medium polarity OV1701
5.0m×0.25mm×0.25µm
He carrier @ 25 mL/min

II. Alternative Configuration ApMp1

1D - Apolar SE52
10m×0.10mm×0.10µm
He carrier @ 0.40 mL/min

2D - Medium polarity OV1701
two parallel 1.0m×0.10mm×0.10µm
He carrier @ 6 mL/min

Added features:
✓ thicker film in the 1D
✓ longer 2D columns

Expectations:
✓ higher overall sensitivity
✓ lower carries flows in the 2D
✓ possibility to increase MP

III. Alternative Configuration ApMp2

1D - Apolar OV1
10m×0.10mm×0.40µm
He carrier @ 0.40 mL/min

2D - Medium polarity OV1701
two parallel 1.5m×0.10mm×0.10µm
He carrier @ 4 mL/min
III. Alternative Configuration ApMp2

\[ ^{1}\text{D} - \text{Apolar OV1} \]
10m×0.10mm×0.40µm
He carrier @ 0.40 mL/min

\[ ^{2}\text{D} - \text{Medium polarity OV1701} \]
two parallel 1.5m×0.10mm×0.10µm
He carrier @ 4 mL/min

Oven programming
50°C(1’) to 280°C(10’) @ 3°C/min
Modulation period: 4 s
Injection: 0.11 s
Analysis time 60’ (last eluted sclareol)
Fully-resolved pattern
System optimization
Column settings
I. “Recommended Configuration”

1D - Apolar SE52
30m×0.25mm×0.25µm
He carrier @ 0.35 mL/min

2D - Medium polarity OV1701
5.0m×0.25mm×0.25µm
He carrier @ 25 mL/min

II. Alternative Configuration ApMp1

1D - Apolar SE52
10m×0.10mm×0.10µm
He carrier @ 0.40 mL/min

2D - Medium polarity OV1701
two parallel 1.0m×0.10mm×0.10µm
He carrier @ 6 mL/min

Added features:
✓ higher polarity 2D

Expectations
✓ improved “orthogonality”
✓ improved 2D peak-widths
✓ reduced analysis time (faster rates)

III. Alternative Configuration ApMp2

1D - Apolar OV1
10m×0.10mm×0.40µm
He carrier @ 0.40 mL/min

2D - Medium polarity OV1701
two parallel 1.5m×0.10mm×0.10µm
He carrier @ 4 mL/min

CFT RFF
FID (75%)
MS (25%)

Tee-union

IV. Alternative Configuration ApP3

1D - Apolar OV1
10m×0.10mm×0.40µm
He carrier @ 0.40 mL/min

2D - Polar PEG
two parallel 1.5m×0.10mm×0.10µm
He carrier @ 4 mL/min

CFT RFF
FID (49%)
MS (51%)

Tee-union
**System optimization column settings**

### IV. Alternative Configuration ApP3

1\(^{D}\) - Apolar OV1
- 10m×0.10mm×0.40µm
- He carrier @ **0.40 mL/min**

2\(^{D}\) - Polar PEG
- two parallel 1.5m×0.10mm×0.10µm
- He carrier @ **4 mL/min**

**Oven programming**
- 70°C(1’) to 280°C(10’) @ 5°C/min
- Modulation period: 4 s
- Injection: 0.11 s
- Analysis time 40 min (last eluted *sclareol*)

**Fully-resolved pattern**
System optimization

**Column settings**

- Eucalyptol
- β-Damascenone
- β-Damascone (Z)
- Geranyl acetate
- β-Caryophyllene
- Amyl Cinnamal
- α-(Z)-santalol
- β-(Z)-santalol
- (E,Z)-Farnesol
- (E,E)-Farnesol
- Isoeugenol
- β-Damascone (Z)
- δ-Damascone
- α-Damascone (Z)
- α-(Z)-santalol
- β-(Z)-santalol
- (E,Z)-Farnesol
- (E,E)-Farnesol
V. Alternative Configuration PMp4

1D - Polar PEG
10m×0.10mm×0.10µm
He carrier @ 0.40 mL/min

2D - Medium Polarity OV1701
two parallel 1.5m×0.10mm×0.10µm
He carrier @ 4 mL/min

CFT RFF
Tee-union
FID (49%)
MS (51%)

Added features:
✓ 1D volatility/polarity driven separation

Expectations
✓ improved 1D peak-widths and symmetry
✓ shorter analysis time (faster rates)
System optimization

**column settings**

Oven programming
50°C(1’) to 260°C(10’) @ 5°C/min
Modulation period: 4 s
Injection: 0.11 s
Analysis time 44 min (last eluted benzyl salicilate)

**Fully-resolved pattern**
**System optimization**

**Performance evaluation**

**Performance parameters**

- Re-injection pulse width ($\sigma^2_i$) [1]
- Net separation measure ($S_{GC\times GC}$) [2]
- Modulation Ratio ($M_R$) [3]
- Separation space used [4]

**Re-injection pulse width ($\sigma^2_i$)**

- Lavender EO Alt. Conf. PMp4 (PEG-OV1701) - Oven 50°C(1’) to 260°C(10’) @ 5°C/min
- Modulation period: 4 s - Injection: 0.11 s - Analysis time 44 min

System optimization
performance evaluation

Re-injection pulse width ($\sigma^2_i$)

Very effective re-injection bands
genometry of the CFT plate
re-injection dynamics (RFF)

Values are in agreement with those
reported by Duhamel et al. [1]

Net separation measure ($S_{GC\times GC}$)

$$S = \Delta t \delta_{av}$$

$$S_{GC\times GC} = S_1 \times S_2$$

$S_1$: first and last eluting 2D-peak
$S_2$: 2D hold-up time and MP

Model Mixture Alt. Conf. ApMp3 (OV1-OV1701) - Oven 50°C(1’) to 260°C(10’) @ 3°C/min
Modulation period: 4 s - Injection: 0.11 s - Analysis time 60 min

System optimization performance evaluation

Net separation measure ($S_{GC\times GC}$)

Peak variance

$^{2D} \sigma$

$^{1D} \sigma$

$^{1D} \sigma$ (s) first and last eluted peak

$^{2D} \sigma$ (s) first and last eluted peak

$S_{GC\times GC}$

Recom. Config. SE52-OV1701 OV1-OV1701 OV1-PEG PEG-OV1701

8711 13512 27466 16955 35724

$S_{GC\times GC}$

$^{1D} \sigma$ (s) first and last eluted peak

Recom. Config. SE52-OV1701 OV1-OV1701 OV1-PEG PEG-OV1701

3.93 5.08 4.19 3.96 4.24 3.36 2.41 3.91

$^{2D} \sigma$ (s) first and last eluted peak

Recom. Config. SE52-OV1701 OV1-OV1701 OV1-PEG PEG-OV1701

8711 13512 27466 16955 35724

$S_{GC\times GC}$

$^{1D} \sigma$ (s) first and last eluted peak

Recom. Config. SE52-OV1701 OV1-OV1701 OV1-PEG PEG-OV1701

3.93 5.08 4.19 3.96 4.24 3.36 2.41 3.91

$^{2D} \sigma$ (s) first and last eluted peak

Recom. Config. SE52-OV1701 OV1-OV1701 OV1-PEG PEG-OV1701

0.11 0.10 0.11 0.10 0.10

0.05 0.06 0.07 0.07 0.08

Net separation measure ($S_{GC\times GC}$)
**System optimization performance evaluation**

**Separation space used** [1]

- degree of correlation between dimensions
- nature of the stationary phases
- changes of selectivity operated by temperature programming


**Separation space used** [1]

2D area (s*s) occupied by solute separation (between the first and the last eluted analytes in both dimensions) and the 2D available area above the hold-up time

**Area ratio (pixels)**

pixel-based area ratio
boundary area (pixels) around the elution pattern (blue boundary in **Figure**) and the available retention time area

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Model Mixture of volatiles  Conf. ApMp2 (OV1-OV1701) - Oven 50°C(1’) to 280°C(10’) @ 3°C/min Modulation period: 4 s Injection: 0.11 s Analysis time 50 min

- α-pinene
- vanillin
- sclareol

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<tr>
<th>Model</th>
<th>SE52-OV1701</th>
<th>OV1-OV1701</th>
<th>OV1-PEG</th>
<th>PEG-OV1701</th>
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</thead>
<tbody>
<tr>
<td>Recom. Config.</td>
<td>0.66</td>
<td>0.95</td>
<td>0.74</td>
<td>0.98</td>
</tr>
<tr>
<td>Area Ratio (pixel values)</td>
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<td>0.43</td>
<td>0.33</td>
<td>0.63</td>
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<tr>
<td>Separation space used</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.41</td>
</tr>
</tbody>
</table>
Real-world samples

**IV. Apolar - Polar OV1-PEG**

1D - Apolar OV1
10m×0.10mm×0.40µm
He carrier @ 0.40 mL/min

2D - Polar PEG
two parallel 1.5m×0.10mm×0.10µm
He carrier @ 4 mL/min

Lowest degree of correlation
2D peaks spreading maximized

500-800 peaks

*Chrysopogon zizanioides* L. (vetiver) EOs
Different “types” *Haiti, Java, Brazil & Bourbon*

**V. Polar - Medium Polarity PEG-OV1701**

1D - Polar PEG
10m×0.10mm×0.40µm
He carrier @ 0.40 mL/min

2D - Medium Polarity OV1701
two parallel 1.5m×0.10mm×0.10µm
He carrier @ 4 mL/min

Highest peak-capacity ($S_{GC×GC}$)
Very high efficiency for polar analytes

200-300 peaks

*Mentha x piperita* L. (peppermint)
*Mentha spicata* L. (spearmint)

*Lavandula angustifolia* Mill. (lavender)
Real-world samples

*Chrysopogon zizanioides* L. (vetiver) EOs
Different “types” Haiti, Java, Brazil & Bourbon

**Haiti type vetiver EO**
Mod. ApP3 (OV1-PEG) - Oven 120°C(2') to 280°C(10') @ 2.5°C/min
Modulation period: 5s Injection: 0.11 s Analysis time 45 min

**FID channel - 550 peaks**
Vol. Threshold 30000 SNR>25

**Chemical signatures**
2D peaks-different chemical entities
583 or Brazil
540 for Java
553 for Haiti
733 for Bourbon
Real-world samples

*Chrysopogon zizanioides* L. (vetiver) EOs
Comparison Haiti vs. Bourbon type

Templates of un-targeted peaks
EO “type” chemical signature

**Fingerprinting approaches**
*Visual features*
*Peak-region features*

**Image comparison**
Pseudocolor comparisons
*Colorized fuzzy ratio*
Red-green regions reveal compositional differences
Quality Control & Authenticity assessment

Area Percentage (Area %) intervals
Ratios between markers

- limonene
- 1,8-cineole
- menthone
- menthofuran
- isomenthone
- menthyl acetate
- isopulegol
- menthol
- pulegone

European Pharmacopoeia [VIII ed. 2014]
United States Pharmacopeia
ISO References
Quality Control of lavender EOs

Area Percentage (Area %) intervals

Ratios between markers

- linalool
- linalyl acetate
- lavandulyl acetate
- 4-terpineol
- lavandulol
- 1,8-cineole
- camphor
- borneol

European Pharmacopoeia [VIII ed. 2014 ]
ISO References

Suspected allergens (restrictions)
Regulated substances according with Quality Standards for Product Conformity Assessment

**MS confirmatory methods are mandatory** (Commission Decision EC 657/2002)

- Identify / confirm ID by EI-MS spectrum
- Quantify by FID (external calibration and Response Factors) and by MS

The system operating with parallel separation/detection enables to:
- Identify / confirm ID by EI-MS spectrum
- Quantify by FID (external calibration and Response Factors) and by MS

### Alignment of FID-TIC MS signals
raw data chromatograms
Target analyte: camphor

### MS data (Signal m/z 95)
- Pk-pk S/N  Corrected signal/Pk-pk noise 258
- FID Signal
  - Pk-pk S/N = Corrected signal/Pk-pk noise 304
Regulated substances according with Quality Standards for Product Conformity Assessment

**MS confirmatory methods are mandatory**
(Commission Decision EC 657/2002)
Differential flow modulated GC×GC with reverse fill/flush dynamics is a promising approach to popularize MD methods in F&F.

The system has shown to provide reliable and satisfactory results in profiling and fingerprinting medium-to-high complexity EOs.

The system has acceptable operational costs. Relative ease of use and simple maintenance.

But...

Issue to overcome data elaboration and interpretation require a change of mind compared to conventional 1D-GC. Chromatographers (old and young) are very conservative.
Thank you for your attention

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Prof. Dr. Patrizia Rubiolo

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