

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





.... Comprehensive multidimensional gas chromatography (CMDGC or GC×GC) is probably <u>the most promising invention in GC</u> 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
- \checkmark 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)

- 1. Seeley, J. V.; Micyus, N. J.; McCurry, J. D.; Seeley, S. K. Am. Lab. 2006, 38, 24–26
- 2. P.Q. Tranchida, F.A Franchina, P. Dugo, L. Mondello. J. Chromatogr A 2014;1359, 271-276
- 3. P.Q. Tranchida, F.A Franchina, P. Dugo, L. Mondello. J. Chromatogr A 2014;1372, 236-244
- 4. R.L. Firor, Application Brief 5989-6078EN, Agilent Technologies, 2007

Differential Flow Modulation with "Forward Fill/Flush" dynamics





Adapted from Agilent 5989-9889EN

Differential Flow Modulation with "Forward Fill/Flush" dynamics





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

- 1. Q. Gu et al. J. Chromatogr. A 1217 (2010) 4448–4453
- 2. G. Semard et al. J. Chromatogr. A 1218 (2011) 3146–3152
- 3. J. Krupcík et al. J. Chromatogr. A 1280 (2013) 104-111
- 4. P. Manzano et al. J. Sep. Sci. 2014, 37, 675–683

Differential Flow Modulation with "Reverse Fill/Flush" dynamics

Loading



* 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

Injection



* Rough representation of internal channel





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]

- 1. J.F. Griffith et al. J. Chromatogr. A 1226 (2012) 116-123
- 2. C. Duhamel et al. J. Chromatogr. A 1387 (2015) 95–103

Differential Flow Modulation with "Reverse Fill Flush" dynamics



trans-2-hexenyl acetate - variable amount



Essential Oils, Extracts and (Volatiles) fractions

Essential oils¹ (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. [1] AFNOR NF T 75-006 Feb 1998 [2] European Pharmacopoeia 8th Edn. 2008

Clevenger circulatory distillation apparatus reported in the *European Pharmacopoeia*

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).



Volatiles 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



Journal of Chromatoaranhy A 203 (1995) 3-15

Sample dimensionality: a predictor of order-disorder in component peak distribution in multidimensional separation J. Calvin Giddings Field-Flow Fractionation Research Center, Department of Chemistry, University of Utuh, Salt Lake City, UT 84112, USA *"... there is some intrinsic property of analytical samples (other than the number <u>m</u> <i>of components) that determines their amenability to multidimensional techniques.*

... the key property is related to sample variability...and is defined as sample dimensionality <u>s</u>"

"The parameter s is the number of independent variables that must be specified to identify the components of the sample"



Characterize sample composition (<u>detailed profiling</u>) 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

System optimization column settings



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).







System optimization column settings



I. "Recommended Configuration"



<u>30m×0.25mm×0.25µm</u> He carrier @ **0.35mL/min** ²D - Medium polarity OV1/ <u>5.0m×0.25mm×0.25μm</u> He carrier @ <u>25mL/min</u>

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)

6-Methyl coumarin	Damascenone
α-(Z)- santalol	δ-Damascone
α - Terpineol	Eugenol
α-Damascone (Z)	Eugenyl Acetate
α-Pinene	(E,E)-Farnesol
Amyl Cinnamal	(E,Z)-Farnesol
Anethole	Geranyl Acetate
Benzaldehyde	Hexadecanolactone
Benzyl Alcohol	Hexil Cinnamal (E)
Benzyl Benzoate	Isoeugenol (E)
Benzyl Salicylate	Isoeugenyl Acetate
β-(Z)-santalol	Limonene
β-Caryophillene	Linalool
β-Damascone (Z)	Linalyl Acetate
β-Pinene	Menthol
Camphor	Methyl Salycilate
Carvone	Salicylaldehyde
Cinnamal	Sclareol
Cinnamyc Alcohol	Terpinolene
Citronellol	Vanillin
Coumarin	



I. "Recommended Configuration"



He carrier @ 0.35mL/min

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 <u>Analysis time 75' (last eluted sclareol)</u> Few critical pairs



System optimization column settings

I. "Recommended Configuration"





✓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 <u>Modulation period: 2.5 s</u> Injection: 0.11 s <u>Analysis time 35'</u> (last eluted *sclareol*) **Few critical pairs**





DSTF

I. "Recommended Configuration"





III. Alternative Configuration ApMp2 $\underbrace{FT \ RFF}_{FP \ e^{-union}} \underbrace{FID \ (49\%)}_{MS \ (51\%)}$ $\underbrace{Po \ Apolar \ OV1}_{10m \times 0.10m \times 0.40\mu m}_{ME \ carrier \ @ \ Add mL/min}$

Added features:

- \checkmark 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



Oven programming 50°C(1') to 280°C(10') @ 3°C/min <u>Modulation period: 4 s</u> Injection: 0.11 s <u>Analysis time 60'</u> (last eluted *sclareol*) **Fully-resolved pattern**



System optimization column settings



I. "Recommended Configuration"



II. Alternative Configuration ApMp1



III. Alternative Configuration ApMp2



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

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

Added features:

✓ higher polarity ²D

Expectations

- ✓ improved "orthogonality"
- ✓ improved ²D peak-widths
- ✓ reduced analysis time (faster rates)



IV. Alternative Configuration ApP3



Oven programming 70°C(1') to 280°C(10') @ 5°C/min <u>Modulation period: 4 s</u> Injection: 0.11 s <u>Analysis time 40 min (last eluted sclareol)</u> Fully-resolved pattern



System optimization column settings



System optimization column settings







DELTA DAMASCONE

TA-DAMASCONE

System optimization column settings

GERANYL ACETAT

ALPHA-DAMASCONE (Z)





System optimization *performance evaluation*





Performance parameters <u>Re-injection pulse width (σ_{i}^{2}) [1]</u> <u>Net separation measure $(S_{GC\times GC})$ [2]</u> Modulation Ratio (M_{R}) [3] <u>Separation space used [4]</u>



Lavender EO Alt. Conf. PMp4 (PEG-OV1701) - Oven $50^{\circ}C(1')$ to $260^{\circ}C(10') @ 5^{\circ}C/min$ Modulation period: 4 s - Injection: 0.11 s - Analysis time 44 min

- 1. M. Klee et al. (2015) J. Chromatogr. A 1383, 151-159
- 2. L. M. Blumberg (2003) J. Chromatogr. A 985, 29 38
- 3. W. Khummueng et al. (2006) Anal. Chem. 78, 4578 4587
- 4. D. Ryan et al. (2005) J. Chromatogr. A. 1071, 47 53

System optimization performance evaluation

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

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





Re-injection pulse width (σ_i^2)

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

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



Modulation period: 4 s - Injection: 0.11 s - Analysis time 60 min

System optimization *performance evaluation*

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





 1 D σ (s) first and last eluted peak



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





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

1. W. Khummueng et al. (2006) Anal. Chem. 78, 4578 – 4587



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 280°C(10') @ 3°C/min Modulation period: 4 s Injection: 0.11 s Analysis time 50 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



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

200-300 peaks

Mentha x piperita L. (pepperita L. (pepperita L. (spearmint)

Lavandula angustifolia Mill. (lavender)



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

Real-world samples





Haiti type vetiver EO Conf. 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

Chemical signatures



2D peaks-different chemical entities

583 or *Brazil* 540 for *Java* 553 for *Haiti* 733 for *Bourbon*



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



Bourbon (ref) vs. Haiti (anal) Colorized fuzzy ratio



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





AN/2

Lavandula angustifolia Mill. (lavender)





Lavender spp. 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



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)





WARNING

Regulated substances

according with Quality Standards for Product Conformity Assessment **MS confirmatory methods are mandatory**

(Commission Decision EC 657/2002)



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



∣▼

LAVANDULOL

WARNING

100-

-

Peak spectrum for Blob 25 - Peak Value: 149646.0000

Real-world samples



FID (49%)

MS (51%)

Conclusions

Matthew Klee1, Leonid Blumberg2

Le.04

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

11th GC×GC Symposium Riva del Garda, Italy



••••

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 <u>reliable and satisfactory results in</u> profiling and fingerprinting medium-to-high complexity EOs



The system has <u>acceptable operational costs</u> Relative <u>ease of use and simple maintenance</u>

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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