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Potential of the reversed-inject differential flow modulator for comprehensive two-dimensional gas chromatography in the quantitative profiling and fingerprinting of essential oils of different complexity

Chiara Cordero[a,*], Patrizia Rubiolo[a], Luigi Cobelli[b], Gianluca Stani[b], Armando Miliazza[c], Matthew Giardina[d], Roger Firor[d], Carlo Bicchi[a]

[a] Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Turin, Italy
[b] SRA Intruments SpA, Cernusco sul Naviglio, Milan, Italy
[c] Agilent Technologies Italia SpA, Cernusco sul Naviglio, Milan, Italy
[d] Agilent Technologies, Wilmington, DE, USA

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ABSTRACT

In this study, the first capillary flow technology reverse-inject differential flow modulator was implemented with different column configurations (lengths, diameters and stationary phase coupling) and detector combinations (mass spectrometry – MS and flame ionization detection – FID) to evaluate its potential in the quantitative profiling and fingerprinting of medium-to-highly complex essential oils. In particular, a parallel dual-secondary column dual-detection configuration that has shown to improve the information potential also with thermally modulated GC × GC platforms (MS identification reliability and accurate FID quantitation), was tested. Several system performance parameters (separation measure $S_{GC\times GC}$, modulation ratio M_R, separation space used and peak symmetry) were evaluated by analyzing a mixture of volatiles of interest in the flavor and fragrance field. The systems demonstrating the best chromatographic performance were selected for quantitative profiling of lavender and mint essential oils and fingerprinting of vetiver essential oil. Experimental results demonstrate that careful tuning of column dimensions and system configurations yields improved: (a) selectivity; (b) operable carrier gas linear velocities at close-to-optimal values; (c) ^{2}D separation power by extending the modulation period and (d) handling of overloaded peaks without dramatic losses in resolution and quantitative accuracy.

1. Introduction

Analysis of natural complex mixtures of volatiles is one of the most important fields of application of gas chromatography (GC) and related techniques [1]. GC is usually applied (a) to characterize sample composition, (b) to quantify informative analytes or (bio)-markers such as toxic compounds, regulated substances (e.g. volatile suspected allergens) or potent odorants (e.g. key-aroma compounds), and (c) to detect adulterations.

A common compositional characteristic of plant volatile fractions is the variable nature and abundance of constituents [from traces (ng/g) to some percent (g/100 g)], which mainly consists of secondary metabolites (mono- and sesquiterpenoids, volatile phenols, etc.) and groups of chemicallycorrelated components such as alcohols, carbonyl derivatives, acids and esters, and volatile phenolic derivatives. Post-harvest treatments and/or technological processing further increase chemical complexity because of the thermal-induced or biologically catalyzed reactions that impacts on native constituents. These compounds sometimes show similar chromatographic retention behavior and are characterized by MS fragmentation patterns with several common isobaric ions (fragments) that make their mono-dimensional characterization and quantitation challenging.

When the Giddings' definition of sample dimensionality [2] is applied to samples of vegetable origin (essential oils, extracts and volatiles fraction), "*the number of independent variables that must be specified to identify the components*" is generally very high and very frequently exceeds that of the analytical system. In such cases, it is necessary to adopt multidimensional analytical

* Corresponding author at: Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via Pietro Giuria 9, I-10125 Torino, Italy.
E-mail address: chiara.cordero@unito.it (C. Cordero).

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platforms (multiple analytical dimensions) to obtain resolved, rational and informative separation patterns.

Moreover, when GC is adopted in the context of modern *omics* investigations to study the complex biological phenomena of plant cross-talking and food sensory perception, such as in plant volatilomics [3] or food sensomics [4], the analytical information must be reliable, quantitative and extended to all detectable and chromatographically resolved entities to give the correct informative role to each single chemical.

In this context, comprehensive two-dimensional gas chromatography (GC × GC) coupled with mass spectrometry (MS) is the technique of choice for the detailed analysis (quali-quantitative profiling) of medium-to-high complexity mixtures of volatiles of plant origin. Compared to one-dimensional systems, GC × GC applies different separation selectivity in two chromatographic dimensions thus providing higher separation power, unmatched peak capacity [5–7] and meaningful 2D elution patterns that facilitate analyte identification and sample fingerprinting.

Thermal modulators, and in particular those implementing a cryogenic device [8], are widely used in this field because of the sample complexity (e.g. dimensionality), and in some cases, for the pre-eminent informative role of highly volatiles (C_2–C_4 compounds) [9,10] that require a very efficient band focusing to avoid break-through phenomena. These modulators can provide a peak capacity gain (G_n) that, under optimized conditions, can be 10–20% below the theoretically achievable maximum [7]. A peak capacity gain approximately of one order of magnitude higher compared to 1D-GC has been obtained and is substantially related to the very efficient re-injection of eluting bands into the secondary column. Commercial modulators, adopting liquid nitrogen as cryo-fluid, produce under optimized conditions, re-injection bands of 20 ms width at half-height [7]. Additionally, thermal modulators are connoted by a great flexibility in terms of tuning of modulation parameters. Loading capacity, modulation period (P_M), cryo-focusing temperature (obtained by varying the cold-jet volumetric flow per unit time), hot-jet pulse temperature and duration, above all, can be optimized to match for sample components relative abundance and differential selectivity between the two chromatographic dimensions. However, thermal modulation has also some drawbacks mainly related to the high costs in term of hardware and operations and seemingly complex optimization [8,11,12] that address its application mainly to research and development studies and limits its adoption for routine quality controls and high-informative throughput screenings [13].

Differential-flow modulators (FM), and in particular those based on the original device from Seeley et al. [14,15], can be considered an interesting alternative because of their simple but effective design, their low operational costs and hardware robustness. When operating in a fully flexible configuration [16,17], the accumulation loop can be adjusted in terms of length and diameter to avoid its overloading when extended re-injection periods are applied to obtain a secondary column volumetric gas-flow compatible with MS detection.

The first commercial differential flow-modulation device for GC × GC was introduced by Firor in 2006 (R.L. Firor, Application Brief 5989-6078EN, Agilent Technologies, 2007). The device was fabricated using diffusion bonded capillary flow technology (CFT) microfluidic plates and was based on the forward fill/flush (FFF) dynamics described by Seeley et al. [15]. Several authors demonstrated its effectiveness in some application fields: bacteria fatty acids methyl esters fingerprinting [18], hydrocarbon compounds in light cycle oils (LCO) profiling [19], gasoline and kerosene analysis [20] and volatiles profiling from roasted almonds [21] although some drawbacks for samples with widely variable abundance of components were emphasized.

It was evident that highly concentrated peaks overloaded the accumulation loop by producing in consequence a solid streak in the second dimension at given first dimension (^{1}D) times. In such a situation it is almost impossible to resolve fully the major components from trace analytes eluting in the same ^{1}D region.

More recently, a second generation of differential flow modulation was presented [22]; this new configuration, adopts a reverse fill/flush (RFF) injection dynamic instead of the FFF of the first generation. Advantages include: (a) higher efficiency of band re-injection with improved ^{2}D peak-widths and symmetry, (b) adjustable collection channel volume, (c) better handling of the overloading phenomenon without dramatic loss of separation power and resolution [22,23].

In the present study the effectiveness of the RFF differential flow modulator for GC × GC for the detailed analysis (profiling) and fingerprinting of medium-to-highly complex samples of interest in the flavor and fragrance field is investigated. In particular: a model mixture of volatiles and essential oils of different complexity (mint, lavender and vetiver essential oils) were chosen as challenging examples. Keeping constant the accumulation loop volume and the dynamics of the modulator operation (e.g. RFF), column dimensions (^{1}D and ^{2}D column lengths and diameters), column configuration (stationary phase chemistry combination and film-thickness) and detection (MS and flame ionization detection – FID) were varied. System effectiveness was tested in terms of:

(a) Separation power through the separation measure ($S_{GC\times GC}$) parameter and number of separated peaks above a fixed threshold.
(b) Selectivity exploitation and occupation of the available separation space.
(c) Quantitation reliability with FID predicted response factors (PRF) [24].
(d) Fingerprinting effectiveness for complex samples.

2. Experimental

2.1. Essential oils (EO) samples, pure reference compounds and solvents

Pure standards of *n*-alkanes (from *n*-C9 to *n*-C25) for Linear Retention Indices (I_S^T) calibration and for Internal Standardization (ISTD) were from Sigma–Aldrich (Milan, Italy). Pure standards of volatiles of interest in the flavor and fragrance field listed in Table 1 and those adopted for external calibration and FID Predicted Response Factors quantitation accuracy assessment were from Sigma–Aldrich (Milan, Italy) or from authors' laboratory.

Solvents (cyclohexane and dichloromethane) were all HPLC-grade from Sigma–Aldrich (Milan, Italy).

Mentha × *piperita* L. EO (peppermint) was prepared in agreement to the method of the European Pharmacopeia [25] and kindly supplied by Dr. Franco Chialva (ChialvaMenta, Pancalieri, Turin Italy). *Mentha spicata* L., *Lavandula angustifolia* Mill. EO (lavender) and *Lavandula angustifolia* Mill. × *Lavandula latifolia* Medik (lavandin Grosso) were purchased from the market.

Chrysopogon zizanioides (L.) Roberty (formerly known as *Vetiveria zizanioides* (L.) Nash) EOs from different geographical origins (i.e., *Haiti*, *Brazil*, *Bourbon* and *Java* type) were kindly provided by Prof. Massimo Maffei (University of Turin, Italy).

2.2. Calibration solutions and EO samples dilutions

Standard stock solutions of reference analytes for performance evaluation (volatiles model mixture – VMM), identity confirmation