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Heart-cutting two-dimensional gas chromatography in combination with isotope ratio mass spectrometry for the characterization of the wax fraction in plant material

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ABSTRACT

Gas chromatography coupled to isotope ratio mass spectrometry after on-line combustion (GC–C-IRMS) and high temperature conversion (GC–HTC-IRMS) is used for compound specific isotope ratio determination. This determination can only be performed successfully if the target solutes are fully resolved from other compounds. A new instrumental set-up consisting of heart-cutting two-dimensional GC based on capillary flow technology and a low thermal mass GC oven in combination with an isotope ratio mass spectrometer is presented. Capillary flow technology was also used in all column and interface connections for robust and leak-free operation. The new configuration was applied to the characterization of wax compounds in tobacco leaf and corresponding smoke samples. It is demonstrated that high accuracy is obtained, both in the determination of $\delta^{13}C$ and $\delta^{2}H$ values, allowing the study of biosynthesis and delivery mechanisms of naturally occurring compounds in tobacco.

1. Introduction

During the last years, interest in isotope ratio mass spectrometry (IRMS) has increased tremendously. IRMS is used for the determination of subtle differences in isotopic ratios of light elements such as C, H, N, O and S. These differences are an indication for the geographical, chemical or biological origin of organic compounds [1,2]. IRMS is therefore extremely useful to differentiate samples that are chemically and biologically identical and can be used for the determination of the origin/source of products and for adulteration detection [3]. Application areas include natural product research [4–6], forensic analysis [7], doping analysis (detection of endogeneous versus exogeneous origin of solutes) [8,9], drug analysis [10] and the analysis of non-radioactive tracers in biomedical experiments [11].

Differences in the natural isotopic abundance are calculated as the ratio of the heavier isotope to the lighter isotope (e.g. $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{2}H/^{1}H$) in the sample relative to an international standard (Vienna PeeDeeBelemnite (VPDB) or Vienna Standard Mean Ocean Water (VSMOW)). The difference in isotopic abundance is expressed as the delta value (δ) in part per thousand (‰) using the following equation (for $^{13}C/^{12}C$, as example):

$$\delta^{13}C_{VPDB} = \frac{R_{sample} - R_{stand}}{R_{stand}} \times 1000$$

whereby R_{sample} is the $^{13}C/^{12}C$ ratio of the sample and R_{stand} is the $^{13}C/^{12}C$ ratio of a standard [12].

Initially, IRMS was mostly used for bulk analysis and was hence limited to the determination of natural isotopic ratios of the entire sample (introduced directly in the combustion or HTC oven). More recently, interest has grown in the use of IRMS as a compound-specific isotope analyzer after gas chromatography (GC) or liquid chromatography (LC) [13,14]. The potential of GC–IRMS has been demonstrated by the analysis of a variety of compounds in very diverse matrices, however, the applicability seems restricted to relative simple matrices in which the target compound(s) is/are well resolved by GC. Co-eluting compounds result in erroneous delta values that are a combination of the co-eluting peaks [15], since, in contrast to classical GC–MS, compounds eluting from the column are transformed into CO_2 or H_2 in the combustion or high temperature conversion reactor and all molecular information is lost. Therefore, chromatographic resolution is extremely important and for the analysis of more complex samples such as extracts of plant material, one-dimensional GC is often not enough

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to obtain isolated, pure peaks. In order to obtain better separation and isolation of individual solutes from a complex profile, multidimensional chromatography offers interesting possibilities. There have been a few research groups who focused on the use of two-dimensional GC in combination with IRMS. Off-line two-dimensional GC with fraction collection was recently applied by Ball et al. [16] for the analysis of lignin phenols. On-line two-dimensional GC hyphenated to IRMS (2D-GC–IRMS) was pioneered by the research group of Mosandl in the late 90s. A (at that time) commercially available 2D-GC instrument, using a 'live switching' device, was coupled to IRMS [17,18]. Both columns were temperature controlled in individual ovens. Good results in terms of precision and accuracy of the delta values were obtained for standard mixtures. A 2D-GC–IRMS approach, using a moving capillary stream switch, was used to analyze technical PCB and PCN mixtures by Horii et al. [19]. For the analysis of C2–C5 hydrocarbons, a 2D-GC set-up based on gas switching valves was used [20]. More recently, also two-dimensional comprehensive GC was coupled to IRMS (GC × GC–IRMS) for the detection of synthetic testosterone [8,9].

The limited number of papers that describe the combination of two-dimensional GC with IRMS indicate that this hyphenation is quite challenging, since the more complex GC configuration needs to be combined with reactors via splitters which are often prone to leaks. For 2D-GC–IRMS, no commercially available instrument is "ready-to-use" and some equipment used in the past [17,18] is no longer commercially available. Combining 2D-GC with IRMS via combustion or HTC reactors also requires specific flow settings, back-flush options for reactor regeneration, etc. These operational parameters have to be taken into account when setting up a 2D-GC–IRMS configuration. Only recently, new technology has become available that allows easier coupling of columns to splitters and Deans switch devices [21,22]. In addition, also the advantages of low thermal mass (LTM) ovens for independent column heating in 2D-GC were demonstrated [22,23].

In this paper, commercially available equipment was combined to a dedicated instrumental set-up for the hyphenation of a heart-cutting two-dimensional configuration based on capillary flow technology and a low thermal mass GC oven with IRMS. Capillary flow technology (CFT) was used in all column and interface connections for robust and leak-free operation. This allows the use of 2D-GC–IRMS for routine analysis. The major challenge was to apply the set-up not only to standard solutions, but also to natural product extracts. This hyphenated system was used for compound specific isotope analysis (both ^{13}C and ^{2}H) of wax compounds in tobacco leaf and in corresponding smoke samples. It is demonstrated that high accuracy is obtained, allowing the study of different delivery mechanisms of tobacco leaf constituents into tobacco smoke and the study of biosynthetic pathways of naturally occurring compounds.

2. Materials and methods

2.1. Chemicals

Hexane (pesticide grade) for sample extraction was from Biosolve (Valkenswaard, The Netherlands). A reference mixture of alkanes and a nicotine reference standard were from A. Schimmelmann (Indiana University, Bloomington, USA) [24].

For the nicotine reference sample, the certified value for $\delta^{13}C$ was −29.98‰ (±0.01‰) and the certified value for $\delta^{15}N$ was −5.82‰ (±0.05‰). In the n-alkane mixture, the certified value for $\delta^{13}C$ was −33.34‰ (±0.02‰) for $C_{24}H_{50}$ and −32.21‰ (±0.03‰) for $C_{28}H_{58}$.

References gases were calibrated against these standards.

2.2. Instrumentation and conditions

The GC analyses were carried out on an Agilent 7890 GC (Agilent Technologies, Wilmington, DE, USA). The GC was equipped with a split/splitless inlet and FID detector. GC parameters (inlet temperature, GC oven, column flows, FID detection) were controlled via Chemstation software. Injection was done using a Gerstel MPS2 injection unit controlled via Maestro software (Gerstel GmbH, Mülheim, Germany). The isotope ratio mass spectrometric analyses were done on a Delta V advantage (Thermo Scientific, Bremen, Germany) equipped with a Conflo IV and a GC–Isolink interface consisting of a HTC oven and a combustion oven with appropriate reactors (Thermo Scientific). All IRMS related parameters were controlled via the Isodat software (Thermo Scientific). For carbon and nitrogen determination the combustion oven was used at 1000 °C. For hydrogen determinations the HTC reactor was used at a temperature of 1420 °C. For nitrogen analysis (on nicotine), the cryo-trap was activated to trap CO_2 formed in the reactor. After 20 measurements, the cryotrap is raised to room temperature to release the trapped CO_2 and to avoid the trap of becoming saturated.

One-dimensional GC–C-IRMS analysis, was performed using a conventional set-up as shown in Fig. 1. A 30 m × 0.25 mm ID × 0.25 μm HP-5-MS column (Agilent Technologies) was used. Stainless steel unions and splitters with graphite/vespel ferrules, including double-hole ferrules, that were used in the original set-up were replaced by three CFT non-purged two-way splitters (Agilent Technologies) in combination with SilTite ferrules (SGE, Ringwood, Australia). In splitter 1, part of the column effluent is split to FID and the other part to the GC–Isolink (1/10 FID/Isolink). Splitter 2 is used to direct the flow either to the combustion oven or to the high temperature conversion (HTC) oven, depending on the position of the switching valve after the reactors. The GC–Isolink is also equipped with a back-flush option to protect the reactors and to extend their lifetime. This back-flush is activated by adding a helium flow after the switching valve, inverting the flow through the reactors. This extra flow is vented via a back-flush exit valve and therefore splitter 3 was installed before the FID. In a typical one-dimensional GC–C-IRMS operation, solvent is detected by FID, while the back-flush is ON (no solvent to GC–Isolink). Solutes can enter the combustion oven (transformation to CO_2 and N_2) by switching the back-flush OFF, while detection on FID is maintained. After elution of the compounds of interest, back-flush can be switched ON again.

For the two-dimensional separation, the GC oven was further equipped with an additional FID detector, a low thermal mass (LTM) oven, an auxiliary pressure control (AUX-EPC) and a CFT based Deans switch device (Agilent Technologies) (Fig. 2). The column used in the first dimension (^{1}D) was a 15 m × 0.25 mm ID × 0.25 μm HP-5-MS column (Agilent Technologies). This column was installed in the GC oven from the S/SL injector to the Deans switch device. The second dimension column (^{2}D) was installed in the LTM oven and was a mid-polar 15 m × 0.25 mm ID × 0.15 μm DB-17-MS column (Agilent Technologies). This column was connected to a three-way purged splitter, allowing to send the sample to both a second FID and the GC–Isolink. Moreover, a two-way splitter was maintained before the second FID to allow the IRMS back-flush option. An overview of the instrumental parameters is given in Table 1. The 2D-GC–IRMS operation is illustrated in Figs. 2A–C and can be summarized as follows:

First, a scouting analysis is made whereby the sample is injected and flow directed toward the first FID detector (configuration in Fig. 2A). The compounds separated on the HP-5-MS ^{1}D column give a complete ^{1}D profile of the sample. Meanwhile the ^{2}D column is flushed (flow from AUX-EPC to the purged 3-way splitter). In this mode, the GC–Isolink back-flush is ON and the reactors are kept clean with flow exiting through the back-flush exit. From this scouting run, heart-cut windows are defined.