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High temperature multidimensional gas chromatographic approach for improved separation of triacylglycerols in olive oil

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Heart-cut multidimensional gas chromatographic (H/C MDGC) methods under suitable flow and high temperature (T) program conditions were developed to separate olive oil triacylglycerols (TAGs). Different column sets were selected for further evaluation, each with relatively short non-polar first dimension (^{1}D) and mid-polar second dimension (^{2}D) columns of high T limits (350 °C). The ^{1}D separation displayed three major groups of peaks in an area ratio of approximately 5:33:62 (of increasing retention), using flame ionisation detection (FID). Four groups of minor peaks, with 2 of them located between the major peaks, were also detected. The H/C fractions of the minor peaks, and sub-sampled regions across the major peaks eluting from the ^{1}D outlet, were cryotrapped at the ^{2}D inlet. The trapped TAGs then underwent temperature programmed ^{2}D separation. Each of the 'H/C' zones generally gave 2–5 – and in some cases more – separated peaks of TAGs on the ^{2}D column, under suitable flow condition and phase polarity that resulted in improved separation. Six sub-sampled H/Cs from various regions of the individual peaks from the ^{1}D column were simultaneously trapped and released to ^{2}D, resulting in apparently more than 22 individual TAG peaks. According to their different retention times, different TAGs were revealed within each of the 3 major groups, using H/C sub-sampling. A comprehensive sampling strategy that covers most of the ^{1}D peaks further revealed the presence of more TAGs in the olive oil sample. This tandem column strategy was able to resolve more components than that usually observed on a single column.

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1. Introduction

Triacylglycerols (TAGs) are the main constituents of vegetable oils and animal fats, arising from esterification of the three hydroxyl groups of glycerol. Based on the number of fatty acids present and the specificity of the enzyme involved in the synthesis of the particular fat or oil, a large number of different TAGs with a variety of functional groups and chemical structures can be found [1,2].

Analysis of TAGs in lipids gives information on the original composition of components in the lipid sample and may be preferential to studying fatty acid composition [3]. Their structures are important in terms of nutritional, biochemical and technological aspects [4]. The number of TAGs that can be detected in a given oil sample is dependent on the degree of separation achieved, and the mode of detection used [5]. Structurally, most TAGs differ according to the number of carbons, the degree of unsaturation, and variation of positions, of each acyl group on the glycerol backbone. This makes their separation as well as identification somewhat difficult. TAGs may be analysed by different methods; analytical techniques mainly employed in the analysis of TAGs in oils are high performance liquid chromatography (HPLC), capillary gas chromatography (CGC), supercritical fluid chromatography (SFC) and thin layer chromatography (TLC) [2,6,7]. More commonly employed techniques are HPLC [5,8,9] and high temperature GC (HTGC) [10–12] coupled to mass spectrometry (MS), using different modes of operation.

In reversed-phase HPLC (RP-HPLC), elution of TAGs follows the increase in their equivalent carbon number (ECN) [4,6]. Equivalent carbon number is defined as ECN = CN − (2 × DB), where CN is the number of carbon atoms and DB is the number of double bond(s) in the fatty acyl chains. TAGs with the same ECN value, called critical pairs, such as oleoyl-linoleoyl-linolenoyl-glycerol (OLLn) and palmitoyl-linoleoyl-linolenoyl-glycerol (PLLn), co-elute. These critical TAG pairs can be separated if they differ in their theoretical carbon number (TCN), on columns with smaller particle size (3 or 5 μm). TCN is an analogue of ECN calculated for unsaturated TAGs from CN and retention factor (k) relationship of the saturated TAGs and used to predict the separation of critical pairs. However, TAGs

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with the same unsaturation and carbon number, such as linoleoyl-linoleoyl-linoleoyl-glycerol (LLL) and OLLn, still have a tendency to co-elute [6]. Moreover, the separation and identification of regioisomers and enantiomers of TAGs are other challenges [4,13].

In CGC, TAGs are eluted mainly in order of increasing molecular mass on non-polar stationary phases whereas on medium polarity and polar stationary phases, which are commonly employed in HTGC analysis of TAGs, elution occurs according to both molecular mass and degree of unsaturation in the fatty acyl groups on the TAG molecules [14]. On medium polarity and polar columns, retention times of TAGs with the same chain length of fatty acyl group but different degree of unsaturation increase with increasing numbers of double bonds.

Although from the above discussion TAG elution seems to follow a predictable order, detection and identification of TAGs in samples is difficult. This may be because oil and fat samples contain varying concentration and numbers of TAGs than present in the TAG standard mixture used to represent the sample in method development [10]. In HPLC, a single column packed with silver-ion-modified octyl and sulfonic co-bonded silica (mixed mode separation) demonstrated an improved selectivity for TAGs separation with rapid analysis time [15]. However, the inadequacy of one dimensional (1D) chromatographic separation for the detection and identification of TAGs in complex samples has led to the use of two dimensional (2D) chromatographic separations. Beccaria et al. reported the identification of more than 250 TAGs in menhaden oil sample using off-line comprehensive 2D HPLC [16]. While 2D separation is becoming a separation technique of choice by using HPLC, less attention has been paid to TAG analysis using multidimensional GC (MDGC).

MDGC conventionally employs two columns providing different selectivity, although alternative use of a single column with thermal sensitivity is also possible [17]. It is superior in terms of enhanced separation and peak capacity as well as improved detection limit, *e.g.* as a result of the cryogenic refocusing effect [18] which has been previously applied in the area of food analysis [19]. Analysis of TAGs in an extracted coffee bean sample by comprehensive two dimensional GC (GC × GC) has been recently reported [20] showing difficulty in separation within the TAG group. This is due to the limitation of the short ^{2}D column applied in GC × GC, as well as the high T elution of compounds resulting in insufficient separation. Development of heart-cut multidimensional GC (H/C MDGC), where a longer ^{2}D column with adjustable flow and T program can be applied, offers an alternative approach. In MDGC separation can be improved by manipulation of gas flows in each column and more than one H/C can be cryotrapped to reduce the number of analyses for ^{1}D target region analysis [21].

Olive oil is prone to adulteration. Separation and detection of its TAG components can assist with authentication of the oil. Reports from different studies indicate that 39 TAGs, existing in concentrations ranging from <1% to >50%, have been identified in olive oil. The TAGs are largely derived from eight different fatty acids [6]. In this study, olive oil was selected as a sample to develop a H/C MDGC method. Effects of chromatographic conditions such as stationary phases, column geometries, T programs and flow rates on the separation performance (analysis time and resolution) were investigated. A suitable condition was further applied to perform comprehensive multiple H/C MDGC of TAGs.

2. Experimental

2.1. H/C MDGC analysis

A gas chromatographic instrument (7890A, Agilent Technologies, Mulgrave, Australia) equipped with dual flame ionisation

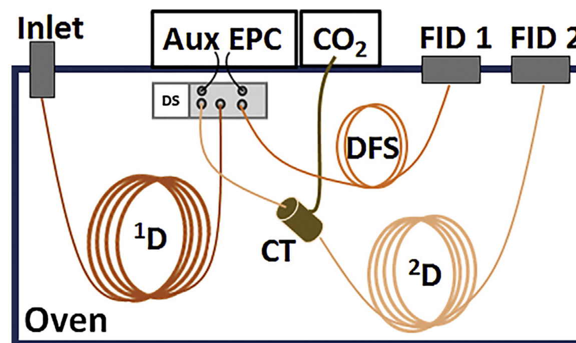

Fig. 1. Configuration of the H/C MDGC system. AUX EPC: pneumatic auxiliary port with electronic pressure control; DS: Deans switch; ^{1}D: ^{1}D column; ^{2}D: ^{2}D column; CT: cryotrap; CO_2: compressed liquid carbon dioxide supply; FID: flame ionisation detector; DFS: deactivated fused- silica capillary tubing.

detectors (FID) was used for this experiment. The GC was also equipped with an Agilent Deans Switch (DS) which has one inlet, and two further outlet column channels plus two inlet channels to control the switching flow. Two sets of columns with relatively short lengths were used for TAGs separation. An Rtx-65 column, which is a mid-polar phase, was used either as ^{1}D or ^{2}D column in each set with the other column comprising a non-polar phase. Column set I: ^{1}D SLB-5MS (15 m × 0.25 mm × 0.25 μm); ^{2}D Rtx-65 (11.5 m × 0.25 mm × 0.1 μm); restrictor – deactivated fused-silica (DFS, 1.75 m × 0.15 mm). Column set II: ^{1}D HP-5 (10 m × 0.32 mm × 0.25 μm); ^{2}D Rtx-65 (11 m × 0.25 mm × 0.1 μm); restrictor – DFS (1.45 m × 0.15 mm). A speciality Rtx-65TG phase suited to triglycerides is a higher T version of the Rtx-65 phase, though was not used here.

As shown in Fig. 1 the applied H/C MDGC configuration comprise the following elements: (1) ^{1}D column connected between the GC inlet and the DS inlet; (2) ^{2}D column connected between one of the DS outlets and FID 2; and (3) a short restrictor column connected between the other DS outlet and FID 1. The ^{2}D column passes through a cryofocusing trap (CT) near the column inlet. Liquid CO_2 was provided as an on-demand flow to CT, which expands as a coolant gas to trap TAGs sampled or heart-cut from the ^{1}D column to the ^{2}D column. CO_2 was supplied to the CT at least 2 min prior to the first H/C event, with target regions from ^{1}D effluent selected based on retention times detected at FID 1. The H/C regions of TAGs remain trapped at CT until all TAGs were eluted from the ^{1}D column. The CT CO_2 supply was stopped in order to release TAGs to the ^{2}D column, which can be at the prevailing oven T, or the oven can be cooled before the CO_2 supply is terminated.

The oven T program used was as follows: from different ^{1}D start T (80 °C or 250 °C), the T was ramped up to 340 °C (15 °C/min), held for 25 min until all TAGs elute from the ^{1}D column, and then cooled down (at 60 °C/min) to the ^{2}D start T (80 °C or 250 °C; 0.5 min hold). Heart-cut sampling is conducted at required times. This is referred to as the first T program. The trapped components were then released from the CT, and a second T program was applied by ramping up the oven T again to 340 °C (15 °C/min) and then held until the TAGs eluted from the ^{2}D column. The inlet and detector T were set at 300 and 350 °C, respectively.

Constant flow mode was used throughout the experiment in both columns, with two constant flow programs used. The flow for the ^{1}D separation (1st flow program) elutes TAGs from the ^{1}D column during the 1st T program, while the flow for the ^{2}D separation (2nd flow program) provides separation of TAGs on the ^{2}D column during the 2nd T program. Thus the ^{1}D and ^{2}D column flows in each program are different, as summarised in Table 1, with the overall flow for the ^{2}D separation preferably lower than that for ^{1}D. The 1st flow program provided a preliminary separation on the ^{1}D column.

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