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Comprehensive two-dimensional gas chromatography with capillary flow modulation to separate FAME isomers☆

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ABSTRACT

A method to separate FAME and the linoleic and linolenic acids isomers by GCxGC using an apparatus equipped with a capillary flow technology (CFT) based modulator and a FID detector has been developed. Four different column combinations (one conventional and three inverted phase sets) were used in these experiments. The conventional set first involved a DB5-MS non-polar column followed by a highly polar HP-INNOWax column in the second dimension. The inverted phase set comprised of a highly polar BPX-70 column in the first dimension and a non-polar ZB5-MS column for the second dimension. Furthermore, the influence of the length of the second dimension column on FAME isomer separation was studied in the inverted phase sets, along with other parameters like the modulation time and column flow. The best results in terms of the time required for the analysis and number of FAME identified with the inverted set were achieved with the shorter second dimension column. After supercritical fluid extraction, the method was applied to identify FAMEs in broccoli leaves from three different cultivars (Naxos, Nubia and Viola).

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1. Introduction

Fatty acids (FAs) are important nutritional elements for living organisms and indeed, long-chain polyunsaturated fatty acids (PUFA) are essential for human metabolism, especially those of the ω-3 series like α-linolenic acid (C18:3(n3)). These FAs have beneficial effects in a number of diseases, such as coronary heart disease, inflammation, autoimmune disorders, hypertension, hypotriglyceridemia, etc. [1,2].

Gas chromatography with FID or MS detectors is the analytical technique most often employed to study fatty acids. However, single GC using the currently available columns is not always the best option to identify components from natural samples. In conjunction with MS detectors, newer columns permit more fatty acids and isomers to be separated [3–7], as happened with the linoleic acid [5] although sometimes it is not possible to achieve the separation of all the fatty acid isomers as in the case of linolenic acid [6], from complex mixtures with single GC. It must be born in mind that it is generally necessary to prepare non-reactive derivatives of FAs prior to GC analysis, usually fatty acid are converted into their methyl esters (FAMEs) and in some particular cases into their respective steryl esters compounds (fatty acids steryl esters, FASEs), which are more volatile than the free acid components [4–10].

Comprehensive two dimensional gas chromatography (GCxGC), a multidimensional technique that has recently gained much attention, especially in relation to food analysis [11], offers a promising alternative that may resolve some of these limitations in FAME analysis, due to the significant increase in the separation power in comparison with one-dimensional GC, and therefore, the physical separation of compounds in complex and difficult samples because of the two columns employed [11].

In GCxGC, the first column effluent is separated into small fractions according to the resolution of the first-dimension, and then this effluent is subjected to a second dimensional study using another column. In this technique, the interface between the two columns (dimensions) is a modulator, the main function of which is to increase the amplitude of the signal and to facilitate its transfer to the second dimension. This can be achieved with thermal [12–15] or capillary flow modulators [16–18]. Although thermal modulation may provide better resolution, the simplicity of capillary flow modulators coupled with their low cost and robustness makes these modulators a good choice for routine analysis [18,19]. GCxGC has been used to determine FAs in matrices such as veg-

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etable oils, milk, biodiesel, human plasma, etc. [13,20–23]. It should be remarked that only in one case [17], a GCxGC system equipped with capillary flow modulator has been employed to analyze such compounds but in a different matrix and with a longer analysis time.

Although much attention is being given to TOFMS coupled to GCxGC systems, FID has been successfully used for determining fatty acids [13,17,20,23–26]. Moreover this detector is being usually used with GCxGC equipped with capillary flow modulators, not only because of its faster response or its robustness and economical price but also, it allows easy quantification of FAME peaks due to similar responses. The main goal of this work was to explore the usefulness of a GCxGC-FID system equipped with a Capillary Flow Technology (CFT) modulator for the chromatographic separation of fatty acid methyl esters (FAMEs), including the linoleic and linolenic acid isomers, which have never been determined at the same time, as one of the scientific novelties of this manuscript. In the existing literature, they could be found several works where different FAME and some isomers have been determined by single GC [3–7,27,28] or comprehensive GCxGC systems equipped with thermal modulators [13,20–26], several times in shorter chromatographic runs but in most of the cases they were not analyzed so many FAMEs. But, it must be remarked that only in one of this works the four linoleic acid isomers were separated [5] and in another work the eight isomers of linolenic acid were analyzed [6], although two of them were not separated.

For this purpose, we have tested several column sets, with different degrees of orthogonality and column lengths (second dimension). In GCxGC, the column combination plays an important role in the separation, and usually two columns of different compositions are used, separation being dictated by the boiling point properties in the first dimension and the polarity in the second [29]. Most of the times, the first phase is non-polar and the second phase is more polar, considered as a conventional column configuration. However, the use of a polar column in the first dimension and a non-polar one in the second, it is referred to as an "inverted phase" column set up [30].

Finally, and to assess the potential of comprehensive two-dimensional gas chromatography equipped with a CFT modulator to separate and identify FAME in complex matrices, the proposed method was successfully applied to analyze FAME isolated by supercritical fluid extraction (SFE) of broccoli leaves from three different cultivars (Naxos, Nubia and Viola).

2. Materials and methods

2.1. Standard solutions

Standard mixtures of FAMEs (reference 47885-u, mix of 37 FAMEs, see Fig. 1; reference 47791, linoleic acid methyl ester isomer mix, see Table 1 and Figs. 1–3; reference 47792, linolenic acid methyl ester isomer mix, see Table 2 and Figs. 1, 2 and 4) in dichloromethane were purchased from Supelco (Bellefonte, PA, USA). Similarly, 2 kits of 10 saturated (ME10-1KT) and 14 unsaturated (ME14-1KT) individual FAME standards were also obtained from Supelco (Bellefonte, PA, USA). Fatty acids were named using the formula Cx:y(nz;catb), where "x" is the number of carbon atoms, "y" the number of double bonds and "z" the position of the first double bond beginning at the methyl terminal group; "a" and "b" were the conventional positions of the double bonds with *cis*, "c", or *trans*, "t", stereoisomerism, which were omitted in the formula when all the FAs double bonds were *cis*-type.

Standard stock solutions were prepared in dichloromethane (Labscan, Dublin, Ireland) at a concentration of 1000 mg/L. These standard stock solutions were diluted daily with dichloromethane to produce a set of working standards. All standards and stock solutions were kept in the dark at +4 °C and they were stable at least for 1 month.

2.2. Samples

Leaves from broccoli grown at CEBAS-CSIC (Murcia) were freeze dried as was described previously [31]. Briefly, broccoli seeds were pre-hydrated with aerated and de-ionised water for 12 h and germinated for 2 days in vermiculite at 28 °C in an incubator. The seeds were then transferred to a controlled-environment chamber on a 16 h light–8 h dark cycle at air temperatures of 25 and 20 °C, respectively, and with a relative humidity (RH) of 60% (day) and 80% (night). The environmental temperature and humidity were strictly controlled throughout the experiment. After 5 days, the seedlings were placed in 15-L containers and they were supplied with a modified-Hoagland nutrient solution [32]. The solution was prepared and replaced each week. After 15 days, the plants were transplanted to perlite containers (one plant per container) irrigated with modified Hoagland nutrient solution, and these containers were located in a controlled-environment greenhouse where the plants grown until they were harvested 11 weeks after transplanting.

The broccoli leaves used in this work belonged to the Nubia, Viola and Naxos varieties. All the samples were lyophilized to preserve them until they were analyzed in triplicate.

2.3. GCxGC columns

To adequately evaluate the different parameters that could influence the flow modulated GCxGC system, different column combinations were studied to determine which produced the best results. Two different column combinations were tested: (i) non-polar first dimension DB5-MS column (30 m × 0.25 mm × 0.25 μm) and a polar HP-INNOWax second dimension column (5 m × 0.25 mm × 0.15 μm) both from J&W Scientific (Folsom, CA, USA), considered as a conventional orthogonal set and abbreviated as C-5 (C = conventional orthogonal set; 5 = length of the second dimension column); (ii) a polar first dimension BPX-70 column (30 m × 0.25 mm × 0.25 μm: SGE Analytical Science, Victoria, Australia) and a non-polar ZB5-MS second dimension column (10, 5 and 2 m × 0.25 mm × 0.25 μm: Phenomenex, Torrance, CA, USA), referred to as the inverted phase orthogonal sets, abbreviated as I-10, I-5 and I-2, respectively (I = inverted orthogonal set; 10, 5 and 2 = lengths of the second dimension columns).

2.4. Fatty acid analysis by GCxGC-FID

Fatty acid content was determined by flow modulated GCxGC-FID after converting them into their corresponding methyl esters using Morrison's method [33]. Briefly, 2 mL of the extracts were hydrolyzed in sealed tubes with 2 mL of 1 M KOH in methanol at 90 °C for 20 min. After that, 3 mL of BF_3 14% in methanol were added and the mixture was heated again at 90 °C for 20 min. Finally 2 mL of hexane were added, and the tube was stirred for 15 s. The hexane layer was withdrawn, partitioned twice against water and submitted to GCxGC-FID analysis. The GCxGC-FID system consisted of an Agilent 7890A GC apparatus (Agilent Technologies, CA, USA) equipped with an autosampler ALS 7683B (Agilent Technologies, CA, USA) that injected 1 μL at 250 °C in pulsed splitless mode, the injection pulse pressure was set at 31 psi until 0.75 min, the purge flow to split vent worked at 15 mL/min at 0.75 min, meanwhile the septum purge flow and the gas saver flow were set at 3 mL/min and 15 mL/min (after 2 min) respectively. They were coupled to a capillary flow modulator (Agilent G3486A CFT Modulator)

Fig. 1. GCxGC-FID 2D chromatogram of the standard mixture of 45 FAME employing the column set I-2. Columns and chromatographic conditions described in Section 2.3 and Table 3.

Table 1
Chemical composition of the linoleic acid methyl ester isomer mix.

Abbreviation	Compound	Weight (%) in solution[a]
C18:2(n6;t9t12)	*trans*-9, *trans*-12-Octadecadienoic acid methyl ester	50
C18:2(n6;c9t12)	*cis*-9, *trans*-12-Octadecadienoic acid methyl ester	20
C18:2(n6;t9c12)	*trans*-9, *cis*-12-Octadecadienoic acid methyl ester	20
C18:2(n6)	*cis*-9, *cis*-12-Octadecadienoic acid methyl ester	10

[a] Data obtained from the standard mixture brochure (Supelco 47791).

Fig. 2. Close-up GCxGC-FID 2D chromatogram of the elution area of the C18 FAMEs. Columns and chromatographic conditions described in Section 2.3 and Table 3.

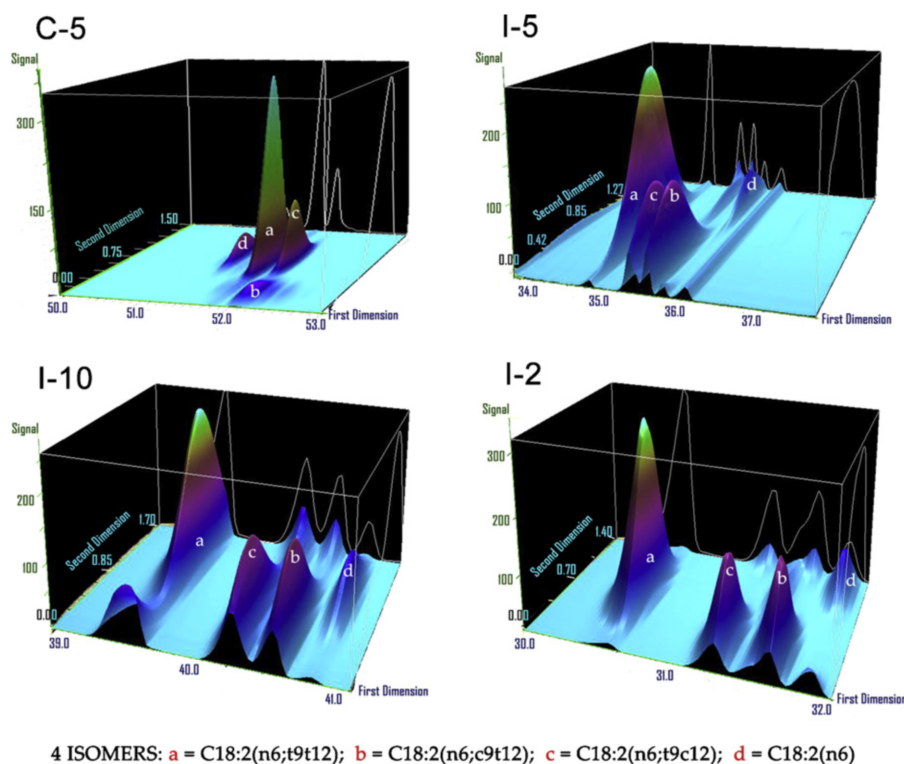

Fig. 3. GCxGC-FID 3D chromatogram of the linoleic acid methyl ester isomer mix employing the columns and chromatographic conditions described in Section 2.3 and Table 3.

Table 2
Chemical composition of the linolenic acid methyl ester isomer mix.

Abbreviation	Compound	Weight (%) in solution[a]
C18:3(n3;t9t12t15)	*trans*-9, *trans*-12, *trans*-15-Octadecatrienoic acid methyl ester	30
C18:3(n3;t9t12c15)	*trans*-9, *trans*-12, *cis*-15-Octadecatrienoic acid methyl ester	15
C18:3(n3;t9c12t15)	*trans*-9, *cis*-12, *trans*-15-Octadecatrienoic acid methyl ester	15
C18:3(n3;c9t12t15)	*cis*-9, *trans*-12, *trans*-15-Octadecatrienoic acid methyl ester	15
C18:3(n3;c9c12t15)	*cis*-9, *cis*-12, *trans*-15-Octadecatrienoic acid methyl ester	7
C18:3(n3;c9t12c15)	*cis*-9, *trans*-12, *cis*-15-Octadecatrienoic acid methyl ester	7
C18:3(n3;t9c12c15)	*trans*-9, *cis*-12, *cis*-15-Octadecatrienoic acid methyl ester	7
C18:3(n3)	*cis*-9, *cis*-12, *cis*-15-Octadecatrienoic acid methyl ester	3

[a] Data obtained form the standard mixture brochure (Supelco 47791) and Hejazi et al. [6].

and a FID detector (operated at 260 °C and 200 Hz data acquisition frequency). The flow of air, hydrogen and nitrogen (make-up) was 450, 20 and 25 mL/min for FID, respectively, and all the gases were supplied by Carburos Metálicos (Barcelona, Spain). The GCxGC parameters selected for each column combination are shown in Table 3.

Two software programs were used for data transformation and visualization: a ChemStation was applied for system control and data acquisition (version B.04.01, Agilent Technologies, CA, USA); and GC Image software was used to generate second and third-dimension chromatograms from the modulated signals (version R1.9, Zoex Corp, Texas, USA).

2.5. Supercritical fluid extraction (SFE)

The supercritical fluid extractor employed in this work was a home-built modular system [4,34]. It was equipped with two intelligent preparative pumps (model PU-1586 from Jasco Corporation, Tokyo, Japan), one of which was used to supply the organic modifier and the other to propel the CO_2. The pump-head of the latter was

Table 3
Optimized values of the most influential GCxGC parameters.

GCxGC parameter	Studied range	Optimal value			
		C-5	I-2	I-5	I-10
Oven temperature program		120 °C to 220 °C at 2 °C/min 220 °C to 260 °C (10 min) at 10 °C/min	120 °C to 230 °C at 2 °C/min 230 °C to 260 °C (10 min) at 20 °C/min	120 °C to 220 °C at 2 °C/min 220 °C to 260 °C (10 min) at 20 °C/min	120 °C to 230 °C at 2 °C/min 230 °C to 260 °C (10 min) at 15 °C/min
1st dimension carrier gas flow rate (mL/min)	0.5–1.0	0.7	0.5	0.6	0.6
2nd dimension carrier gas flow rate (mL/min)	10–25	18	12	22	24
Modulation time (s)	1.40–2.00	1.50	1.40	1.70	1.70

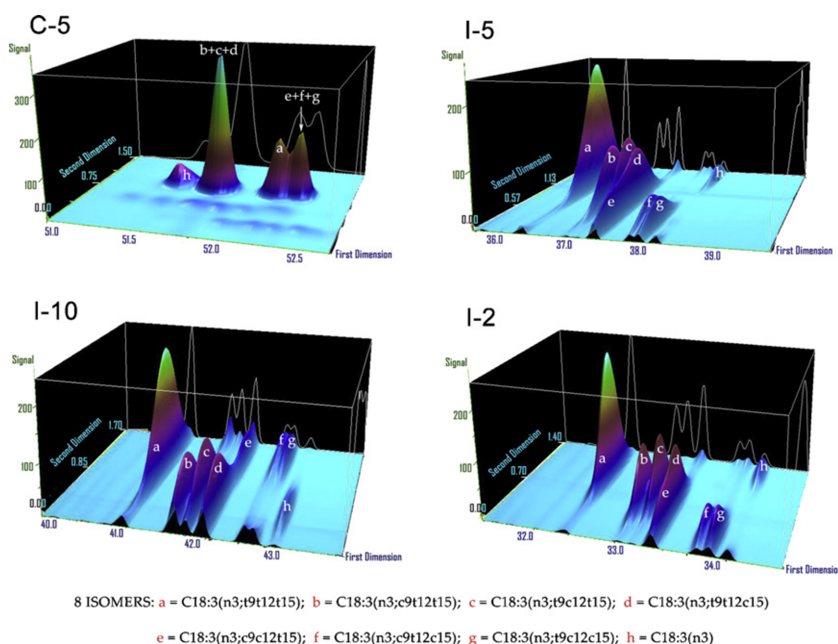

Fig. 4. GCxGC-FID 3D chromatogram of the linolenic acid methyl ester isomer mix employing the columns and chromatographic conditions described in Section 2.3 and Table 3.

cooled at 0 °C using a thermostatic bath (Frigomix U from B. Braun, Melsugen, Germany) and the pressure was controlled with a back-pressure regulator (BP-1580-81, Jasco Corporation, Tokyo, Japan). A SFE procedure previously employed by our research group to isolate FAME from broccoli leaves [4] has been modified to determine the best extraction conditions. Briefly, samples of Broccoli leaves (0.3 g) were placed into the extraction cell (2 mL: Jasco, Tokyo, Japan) and introduced into an oven (CO-2056, Jasco, Tokyo, Japan) equipped with two (V3, V4) 7000 Rheodyne valves (Cotati, CA) and two (V1, V2) NV-5272 NOVA Swiss valves (Cesson, France), which enabled a static or dynamic mode to be employed. After a static extraction time of 10 min, the extracts obtained at 65 °C and 250 bar, with a flow-rate of 3 mL/min and a dynamic extraction time of 60 min, were collected into 20 mL glass vials with a pierced-cap and previously filled with 1 ml of methanol using a Gilson 233XL sampling-injector (Villiers-le-Bel, France). The 233XL device had a collection needle that was programmed to move down to the bottom of the vial. Finally, the broccoli leaf extracts were diluted to 20 mL with methanol.

3. Results and discussion

3.1. Optimization of the GCxGC conditions

In GCxGC, chromatographic separation is fundamentally influenced by four parameters: oven temperature, carrier gas flow in both dimensions and the modulation time. But, after making several preliminary tests, it was found that the carrier gas flow in the two columns and the modulation time showed a strong influence onto the separation of the FAME. This conclusion is in good agreement with Gu et al. [17], who stated that flow modulation depends on the careful selection of these three operational parameters.

An optimization study was carried out by performing several tests with the different standard FAME mixtures (see Section 2.1), similar to that employed to select the best GCxGC conditions to analyze the different column sets.

The starting conditions selected were those previously optimized in the analysis of FAME by conventional GC [35]: injection of 1 μL at 250 °C in pulsed splitless mode and FID operated at 260 °C with a data rate of 200 Hz/0.001 min. The initial oven temperature was programmed from 50 °C (1 min) to 205 °C (17 min) at 6 °C/min, and then to 250 °C at 6 °C/min (20 min). Regarding the modulation time and the carrier gas flow, the starting values recommended in the GCxGC instrument manual were taken as reference values: 1.70 s modulation and 0.7 and 18 mL/min as the first and second dimension carrier gas flows, respectively.

It should be noted that after performing several tests, the variation of injection and FID parameters indicated above did not significantly affect FAME separation and thus, these values were maintained constant for all the column sets.

To study the influence of each of the most important parameters on FAME separation (oven temperature program, carrier gas flows in both dimensions and the modulation time), one was varied while the others remained constant. With this procedure the optimal conditions to separate FAMEs in the standard mixtures were selected. In order to achieve this objective, the criterion used to determine the optimal conditions of FAME separation was to find the best separation between the entire 45 standard FAMEs, but paying special attention in obtaining the best separation and resolution between peaks of the 2 groups of isomer mixtures (linoleic and linolenic acids), as it is the main goal of this work. The parameters which provided the best separation for each column set are summarized in Table 3.

There were no important differences in terms of oven temperature and while the optimal values for the other parameters studied differed slightly (e.g. 1D carrier gas flow and modulation time), the variation was much larger for the 2D carrier gas flow.

3D chromatograms of the standard linoleic and linolenic acids mixtures obtained under the conditions selected for each column set are shown in Figs. 3 and 4.

3.1.1. Comparative studies of the column sets

3.1.1.1. Comparison between conventional and inverted phase column sets. The results obtained with column sets C-5 and I-5, whose columns dimensions were identical, were compared. Given the number of carbons in the saturated FAME, they always eluted from those with the lower to higher number of carbons as expected, irrespective of the column set employed. The elution order of the

same FAME families depends on the column set, with the non-saturated forms being eluted before the saturated ones when using C-5, while contrary occurred for I-5. Regarding the geometric isomers, the trend also differed according to the column set selected (Figs. 2 and 3). The *cis* isomers eluted before the *trans* isomers, and when *cis-trans* isomers appeared they eluted after the *trans* isomers for set C-5. By contrast, the *trans* isomers eluted first when using set I-5, followed by the *cis-trans* and finally the *cis* isomers. When the FAMEs had the same number of carbons and unsaturated bonds, they eluted similarly in both column sets. Those compounds that had a double bond closer to the ester group eluted first, in other words they eluted in a decreasing order according to their respective "n" values.

It was found when using column set C-5 and employing the best chromatographic conditions (Table 3), that there is a couple of FAME which coeluted, C20:2 and C20:3(n3). However, when using column set I-5 these compounds were separated but two other FAMEs coeluted (C20:3(n6) and C21:0).

Finally, the retention times of the FAME were ≈15 min shorter with set I-5 than those obtained with C-5, and more FAME were separated and identified when using I-5 (Figs. 3 and 4). This was particularly evident for the linolenic acid isomers (Fig. 4) and the FAME with more than 22 carbon atoms. It can be concluded that the use of inverted phase column combinations (I-5) was most effective.

3.1.1.2. Comparison between inverted phase column sets. The influence of the second dimension column length, which varied from 2 to 10 meters, on FAME separation was also studied. After examining the chromatograms (Figs. 3 and 4), the elution order was identical in the three column sets. Thus, the trends described in the previous section for set I-5 explain the elution order in sets I-10 and I-2. The retention time of each of the FAME increased by ≈4 min when the 2D column length increased from 5 to 10 m. Moreover, the resolution and peak shape of the FAME improved as the 2D column length decreased, and the analysis time was shorter. Regarding to the separation of the linolenic acid isomers, the column length affects the resolution between isomers C18:3(n3;t9t12c15) and C18:3(n3;c9c12t15) in the second dimension, or between C18:3(n3;c9t12c15) and C18:3(n3;t9c12c15) in the first dimension (Fig. 4).

As happened with column set I-5, there are two FAMEs, C21:0 and C20:3(n6), which coeluted (Fig. 1) when using column combinations I-10 and I-2, and the best chromatographic conditions (Table 3). Although several tests were done, it was not possible to obtain a complete separation of these compounds with any of the studied column sets due to the need to determine the 45 FAME in the same analysis, at most important, because of separating the linoleic and linolenic groups of isomers. It must be commented that these two FAMEs has been previously separated by using GC and GCxGC, as it could be seen in the existing literature [3,28,24]. We have also separated them by changing the chromatographic conditions, but then some of the linolenic and linoleic acid isomers coeluted. They would appear in Table 4 and Fig. 1 as follows: C20:3(n6) + C21:0.

Hence, a decrease in the length of the second dimension column improved FAME separation and subsequently, set I-2 was selected to analyze broccoli leave samples. A 2D chromatogram of the 45 FAME standards that was obtained with the GCxGC conditions for set I-2 (described in Table 3) is shown in Fig. 1. It must be pointed out that the reproducibility of the retention times ($n = 6$) obtained with flow modulation was very good, with a standard deviation on 1D better than 0.02 min and a standard deviation on 2D better than 0.0002 min for the analysis of the FAME. This performance agrees well with the results obtained with similar GCxGC systems equipped with modulators based on CFT [17]. As it could be observed in Fig. 1, the separation and identification of 45 FAME (2 of them coeluted) have been achieved. Although most of the FAME could be separated in the first dimension, it has been demonstrated that without employing GCxGC the separation of some of them, the linolenic acid isomers, was not possible. It has been included a zoomed figure of this chromatogram to show in more detail the area were eluted the C18 FAMEs (Fig. 2). Some conclusions could be obtained when comparing our results with the analysis time and number or type of FAME separated and identified with previous studies that employed single GC and GCxGC equipped with thermal or CFT modulators. GC methods usually are faster than our proposed method [3,27,28,36], in some of them several FAME isomers were separated and identified, although their number was usually smaller than in our method. It must be remarked that with the only exception of the works presented by Bicalho et al. [5] and Hejazi et al. [6], linoleic and linolenic acids isomer mixtures were not studied, and the mixture of both group of isomers have never been simultaneously analyzed. In the first of these publications [5], it was achieved the separation of the four linoleic acid isomers, among other 89 FAMEs (linolenic acid isomers not included) with a HP-88 GC column of 100 m in less than 60 min. Meanwhile, in the second work [6], it was analyzed the linolenic acid mixture with a BPX70 GC column, six compounds were separated and two of them coeluted and the analysis time was significantly longer. So, the main advantages of our GCxGC method are: (i) the possibility of separating compounds that coeluted with single GC, as the linolenic acid isomers; (ii) the analysis in the same chromatographic run of both groups of isomers.

Regarding to the GCxGC methods, the chromatographic runs were sometimes longer [17], similar [37] or shorter [24,25], and as it could be expected, more FAME isomers were studied. For example, Mondello et al. [25] have identified 59 FAME (12 of them belong to C18 group), meanwhile Villegas et al. [26] have analyzed the C18:1 family among other FAMEs, in both works it was employed a GCxGC-FID with a cryogenic modulator. But at the same time, it must be said that from our knowledge, in our work, it is the first time that the eight isomers of linolenic acid have been separated and that the linoleic and linolenic acid isomers have been together studied.

Finally, it should be remarked, that it was found only one publication where a GCxGC equipped with a CFT modulator have been employed for determining fatty acids [17]. In comparison with this work, the chromatographic run was reduced by more than 20 min with our method, we have analyzed more FAME, more isomers and we could also identify FAME with more than 20 carbon atoms, although FAME with less than 10 carbon atoms (C4:0, C6:0 and C8:0) were not determined with any of the sets employed in both works.

3.2. Analysis of broccoli leaves samples

Broccoli belongs to the Brassicaceae family (*Cruciferae*) and it is a potential source of health promoting compounds like glucosinolates, polyphenolic compounds, etc. [38–40]. It must be pointed out that several single GC methods have been developed to analyze FAME in this matrix, mainly in florets, seeds and stems [4,27,28,40]. Intensive broccoli cultivars are associated with the production of considerable by-products, meanly leaves that are usually discarded even though they may have a similar composition to the edible parts of the plant. These by-products could be used as complements in animal diets or as nutraceutical reservoirs, which reduce their environmental impact and at the same time give them certain economic value. For this reason, attention is now being paid to the composition and possible alternative use of such residues [41] and we have decided to check the effectiveness of our method in broccoli leaves.

Table 4
FAME identified in the broccoli leave samples with column set I-2 pattern. GCxGC-FID conditions are summarized in Table 3.

t_{rI} (min)	t_{rII} (s)	FAME	NAXOS		NUBIA		VIOLA	
			t_{rI} (min)	t_{rII} (s)	t_{rI} (min)	t_{rII} (s)	t_{rI} (min)	t_{rII} (s)
7.1	0.45	C10:0						
8.8	1.03	C11:0	8.8	1.04	8.8	1.05	8.7	1.00
11.0	0.37	C12:0	10.9	0.35	11.0	0.37	11.1	0.36
13.5	1.22	C13:0	13.5	1.20	13.5	1.18	13.5	1.18
16.3	0.74	C14:0	16.2	0.70	16.3	0.73	16.3	0.77
17.9	1.17	C14:1(n5)	17.9	1.12	17.8	1.16	17.8	1.09
19.4	0.26	C15:0	19.3	0.24	19.4	0.27	19.4	0.25
21.0	0.46	C15:1(n5)	21.1	0.42	21.0	0.44	21.0	0.48
22.5	1.22	C16:0	22.4	1.29	22.3	1.32	22.4	1.29
23.8	1.17	C16:1(n7)	23.9	1.11	23.8	1.13	23.9	1.20
25.7	0.76	C17:0	25.8	0.70	25.7	0.79	25.7	0.72
27.0	0.50	C17:1(n7)	27.0	0.45	27.1	0.53	27.0	0.54
28.8	0.29	C18:0	28.8	0.36	28.8	0.39	28.8	0.36
29.5	0.40	C18:1(n9;t9)	29.6	0.47	29.6	0.43	29.7	0.47
29.9	1.28	C18:1(n12)						
30.0	1.18	C18:1(n9)	29.8	1.20	30.0	1.19	29.9	1.19
30.3	1.15	C18:1(n7)	30.1	1.28			30.1	1.24
30.8	0.68	C18:2(n6;t9t12)	30.9	0.62	30.9	0.64	31.0	0.72
31.4	0.44	C18:2(n6;c9t12)						
31.6	0.44	C18:2(n6;t9c12)						
31.8	1.17	C18:2(n6)	31.8	1.19	31.8	1.20	31.9	1.18
33.1	0.13	C18:3(n6)	33.1	0.14	33.1	0.13	33.2	0.12
32.4	0.99	C18:3(n3;t9t12t15)						
33.0	0.64	C18:3(n3;t9t12c15)						
33.2	0.73	C18:3(n3;t9c12t15)						
33.4	0.67	C18:3(n3;c9t12t15)						
33.4	0.48	C18:3(n3;c9c12t15)						
33.8	0.38	C18:3(n3;c9t12c15)						
34.0	0.36	C18:3(n3;t9c12c15)						
34.2	0.06	C18:3(n3)	34.2	0.05	34.3	0.04	34.3	0.02
35.1	0.85	C20:0					35.3	0.63
35.8	1.25	C20:1(n9)	35.8	1.19			35.9	1.27
36.1	0.04	C20:2(n6)						
38.0	1.00	C20:3(n6) + C21:0	37.9	0.97	38.0	1.06	38.1	1.04
39.2	1.13	C20:3(n3)	39.2	1.16	39.1	1.09	39.1	1.11
40.0	0.23	C20:4(n6)						
40.2	1.13	C20:5(n3)						
40.9	0.43	C22:0	40.9	0.41	41.0	0.43	41.1	0.40
41.9	0.40	C22:1(n9)	41.9	0.38	41.9	0.41	42.0	0.42
42.3	0.68	C22:2(n6)						
43.7	1.10	C23:0					43.8	1.05
46.4	1.02	C24:0	46.3	1.07	46.3	0.98	46.4	1.00
47.4	0.83	C24:1(n9)	47.3	0.85	47.3	0.80	47.4	0.87
48.8	0.79	C22:6(n3)						

t_{rI}: retention time in the first dimension; t_{rII}: retention time in the second dimension.

Once the GCxGC-FID and SFE conditions were set, the method developed was applied to analyze the FAME in broccoli leaves of three different cultivars (Naxos, Nubia and Viola: $n = 6$). The identification of FAMEs compounds and isomers in real broccoli samples was efficient, quick and simple. It was done by using a pattern constructed with a combination of the three standard FAME mixtures chromatograms, analyzed with column set I-2, and the chromatographic conditions described in Section 2.3 and Table 3. To identify the FAME in the broccoli leave samples, the GCxGC chromatogram was obtained with the GC Image program R1.9 and after loading the pattern obtained previously with the standards; the peaks were matched and subsequently identified.

The presence of 26 FAME was confirmed in Naxos, whereas 24 were identified in Nubia and 28 in Viola broccoli leave samples (see Table 4). The number of FAME determined in broccoli leaves with our method is similar [4,28] or higher [40] than the presented in preceding published works in leaves or other parts of broccoli. It must be pointed out that as it was above commented in Section 3.1, the retention times when using single GC were usually lower [28], but at the same time, none of them have separated and identified so many FAME, including the linoleic and linolenic acid isomers.

From a qualitative point of view, the 4 FAME with the strongest signal in the three broccoli varieties were two saturated fatty acids (palmitic, C16:0 and behenic acid, C22:0) and two unsaturated fatty acids (linoleic, C18:2(n6) and α-linolenic, C18:3(n3)). These results matched perfectly with those ones presented in the literature in broccoli [4,40] and other Brassicaceae varieties like cabbage [36] and cauliflower [42]. The α-linolenic acid was the most intense FAME within the samples studied, which is relevant when taking into account that it belongs to the ω-3 family and that these compounds not only promote cardiovascular health [43] but also, the healthy functioning of many other biological systems [44–46].

4. Conclusions

The identification and separation of a large number of FAMEs, including for the first time the separation of the eight isomers of the linolenic acid, has been achieved in less than 50 min by using GCxGC-FID equipped with a capillary flow modulator, with excellent reproducibility of the retention times. Although the analysis time with the proposed method was longer than in several one-dimensional GC and GCxGC (equipped with thermal modulators methods), it was 20 min faster than the only method where GCxGC

with CFT modulator was employed, and it is required to achieve the separation of the linoleic and linolenic acid isomers. Better FAME separation was obtained when inverted phase column combinations were used, particularly in terms of analysis time, resolution and the number of FAME identified. However, some differences in the FAME elution order were observed according to the type of column set employed. The resolution and analysis time with inverted phase combinations were better when the length of second dimension column was shorter (I-2). The method was successfully applied to analyze FAMEs extracted by SFE from leaves of three different broccoli varieties, identifying more than 24 FAMEs in the samples analyzed. The most intense FAME was α-linolenic acid, which is relevant due to its health promoting effects.

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