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Separation of phytosterol oxidation products by combination of different polarity gas chromatography capillary columns

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Abstract

The number of characterized phytosterol oxidation products (POPs) from both ring- and side-chain structures has increased during recent decades, resulting in difficulties in the separation of POPs on different gas chromatography (GC) capillary columns. The main objective of this study was to separate a mixture of 29 purified and characterized oxidation products from sito-, campe- and stigmasterol using GC capillary columns with different polarity. For the first time in the area of POPs analysis, the separation efficiency of the combination of two capillary GC columns with different polarities was investigated. A non-polar 5% phenyl coated (DB5-MS) and a mid-polar 35% phenyl coated (DB35-MS) column was combined with a pressfit connector. The main improvement was enhanced base line separation for many of the analyzed POPs, compared with the separations achieved using the individual columns. However, three pairs of POPs co-eluted: 24-hydroxysitosterol/campesterol-5β,6β-epoxide, stigmasterol-5β,6β-epoxide/campesterol-5α,6α-epoxide and stigmasterol-5α,6α-epoxide/campestanetriol.
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Keywords: Campesterol; Coupled columns; Column polarity; Phytosterol oxidation products; Sitosterol; Stigmasterol

1. Introduction

Many studies regarding the separation of cholesterol and cholesterol oxidation products (COPs) with different analytical methods have been reported during recent decades. A mixture of the most commonly produced COPs can be separated using capillary gas chromatography (GC) columns, or on an HPLC system [1–3]. Phytosterols are structurally closely related to cholesterol and the oxidation process follows similar pathways [4,5]. Since there are several phytosterols that have the potential to be oxidized, there are numerous possible POPs both from the ring structure and side-chain structure [6–8]. Consequently, the analytical system has to be very powerful and efficient. The most commonly reported capillary GC columns for the analyses of POPs are non-polar types, i.e. 100% dimethylpolysiloxane or 5% phenyl + 95% dimethylpolysiloxane [9].

Lampi et al. [10] investigated the formation of POPs during the heating of rapeseed oil at 180 °C for 12 h. The oxidation products were separated by using a DB5 capillary GC column (60 m × 0.32 mm, 0.10 μm). However, the column used in this study separated neither stigmasterol-5α,6α-epoxide from 7β-hydroxysitosterol nor unoxidized sitosterol from 7β-hydroxycampesterol. The authors suggested that further improvement of the method should include optimization of the GC separation and the possibility to use GC–mass spectrometry (MS) for the separation and quantification of POPs.

The efficiency of a non-polar capillary GC column, DB5-MS (50 m × 0.20 mm, 0.33 μm) was recently demonstrated and the separation of a large number of both COPs and POPs were reported [9]. The analytical time was more than 60 min and co-eluted POPs were; unoxidized brassicasterol/7α-hydroxycampesterol, 7α-hydroxystigmasterol/unoxidized campesterol, 7α-hydroxysitosterol/unoxidized stigmasterol, 7β-hydroxystigmasterol/unoxidized sitosterol, 7β-hydroxysitosterol/stigmasterol-5β,6β-epoxide, sitosterol-5β,6β-epoxide/campestanetriol and sitosterol-5α,6α-epoxide/stigma-

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stentriol. The author suggested that further improvement of the separation was necessary in order to obtain a more efficient separation of complex mixtures of POPs and COPs by capillary GC.

Recently, Grandgirard et al. [11] reported the separation of several POPs on two different capillary GC columns of the same dimensions (DB1-MS and DB5-MS). Relative retention times and elution patterns for many POPs and COPs on both the GC columns, along with co-elution and poor base line separation for some oxidation products on both the GC columns were reported. The authors also observed that minor changes in the temperature programme improved the separation of co-eluted peaks. However, the improvement was followed by the co-elution of other products. The authors suggested that by combining data from the two systems a more efficient separation could be obtained with an additional number of identified peaks.

The aim of this study was to investigate the separation efficiency of two different polarity capillary GC columns, individually and in combination. The non-polar column was a DB5-MS column and the mid-polar column was a DB35-MS column, both having similar dimensions (25 m × 0.2 mm, 0.33 μm). The POPs included in this study were 29 purified and characterized oxidation products from sitosterol, campesterol and stigmasterol.

2. Experimental

2.1. Chemicals and materials

The unoxidized standard sample of stigma sterol was purchased from Sigma Aldrich (Stockholm, Sweden) and the unoxidized standard sample of mixed sitosterol and campesterol was purchased from Research Plus (Bayonne, NJ, USA). All other solvents or chemicals were, unless otherwise stated, of analytical grade and purchased from VWR (Stockholm, Sweden).

2.2. Preparation of phytosterol oxidation products

The analytical procedure containing the thermal oxidation, purification, separation and identification of the different POPs used in this study has been described in detail elsewhere. Briefly, the oxidation products were produced from a thin film of unoxidized sterols using thermal oxidation (120 °C for 72 h). The POPs were then separated with a two step preparative TLC containing different mobile phases. Finally, the purity of the oxidation products was investigated with GC–flame ionization detection (FID), GC–MS and for some products with nuclear magnetic resonance (NMR) [6,7].

2.3. GC separation

The GC–FID analyses were performed separately on a DB5-MS column (J&W Scientific Folsom, CA, USA), a DB35-MS column (J&W Scientific Folsom, CA, USA), having similar dimensions (25 m × 0.2 mm, 0.33 μm), or a combination of these two columns joined together by a press-fit connector (NTK Kemi, Sweden). The two columns were connected in the order of decreasing polarity, i.e. DB35-MS was connected to the injector side and the DB5-MS column was connected to the detector side. The GC equipment was a CP 3800 (Varian, Stockholm, Sweden) fitted with an on column injector and Star Chromatography Workstation software (Version 5.52). The detector temperature was set at 320 °C and the injector temperature was programmed at 65 °C for 0.5 min and raised to 290 °C (180 °C/min). The samples were injected by an autosampler, CP8400 (Varian). The initial oven temperature was 65 °C (0.5 min), programmed at a rate of 90 °C/min to 290 °C (10 min) and then with 0.3 °C/min to 305 °C (17 min). The carrier gas (He) was programmed at a constant flow of 0.8 ml/min for both the individual and for the two combined columns.

3. Results and discussion

The most abundant phytosterols in nature are sitosterol, campesterol and stigmasterol and a large number of oxidation products can be formed from all these sterols. In order to study the separation efficiency of two GC columns of same dimensions, 29 POPs were analyzed on the columns, both individually and in combination. To our knowledge, the combination of two capillary GC columns having different polarity has not been studied previously for the separation of POPs.

The first system investigated was a non-polar DB5-MS capillary column (25 m × 0.33 μm, 0.2 μm) that contained 5% phenyl-dimethylpolysiloxane. This capillary column is commonly used in the separation of COPs and POPs [12]. With the GC conditions used in this study the total analytical time for the investigated POPs was around 30 min. The oxidation product with the shortest retention time was 7α-hydroxycampesterol (14.7 min) and the POPs with the longest retention time was 7-ketositosterol (28.1 min) (Table 1). However, due to the numerous POPs (29 compounds) and low separation efficiency, many of the peaks were not separated satisfactorily. Peaks that overlapped or had poor base line separation were: unoxidized stigmasterol/7α-hydroxysitosterol, 7β-hydroxystigmasterol/7β-hydroxycampesterol/unoxidized sitosterol, campesterol-5β,6β-epoxide/4-campesten-6β-ol-3-one, 4-campesten-6β-ol-3-one/campesterol-5α,6α-epoxide, campesterol-5α,6α-epoxide/stigmasterol-5β,6β-epoxide, stigmasterol-5β,6β-epoxide/7β-hydroxysitosterol, campestanetriol/sitosterol-5β,6β-epoxide, sitosterol-5β,6β-epoxide/4-campesten-6β-ol-3-one, stigmastentriol/sitosterol-5α,6α-epoxide, 25-hydroxystigmasterol/24-hydroxystigmasterol and sitostanetriol/7-ketocampesterol (Table 1).

The second column investigated was the mid-polar capillary GC column, DB35-MS (25 m × 0.33 μm, 0.2 μm) containing 35% phenyl-dimethylpolysiloxane. The total

Table 1
Retention times (Rt) and relative retention times (RRT) of phytosterol oxidation products (POP) separated on two different GC columns, individually and in combination

Systematic name	Trivial name	DB5-MS		DB35-MS		DB-35MS/DB-5MS	
		Rt (min)	RRT[a]	Rt (min)	RRT[a]	Rt (min)	RRT[a]
(24*S*)-Ethylcholest-5,22-dien-3β-ol	Unoxidized stigmasterol	16.5	1.6	19.0	1.7	42.4	1.7
(24*S*)-Ethylcholest-5,22-dien-3β,7α-diol	7α-Hydroxystigmasterol	14.9	1.5	15.2	1.3	35.6	1.5
(24*S*)-Ethylcholest-5,22-dien-3β,7β-diol	7β-Hydroxystigmasterol	17.8	1.8	18.8	1.6	43.4	1.8
(24*S*)-Ethylcholest-5,22-dien-3β-ol-7-one	7-Ketostigmasterol	25.3	2.5	34.2	3.0	69.1	2.8
(24*S*)-5α,6α-Epoxy-24-ethylcholest-22-en-3β-ol	Stigmasterol-5α,6α-epoxide	20.3	2.0	25.0	2.2	54.4	2.2
(24*S*)-5β,6β-Epoxy-24-ethylcholest-22-en-3β-ol	Stigmasterol-5β,6β-epoxide	19.9	2.0	24.4	2.1	52.6	2.2
(24*S*)-Ethylcholest-22-en-3β,5α,6β-triol	Stigmastentriol	22.2	2.2	25.4	2.2	55.7	2.3
(24*S*)-Ethylcholest-5,22-dien-3β,24-diol	24-Hydroxystigmasterol	23.7	2.4	26.1	2.3	58.5	2.4
(24*S*)-Ethylcholest-5,22-dien-3β,25-diol	25-Hydroxystigmasterol	23.6	2.4	25.8	2.2	57.9	2.4
(24*R*)-Methylcholest-5-en-3β-ol	Unoxidized campesterol	16.0	1.6	18.3	1.6	41.0	1.7
(24*R*)-Methylcholest-5-en-3β,7α-diol	7α-Hydroxycampesterol	14.7	1.5	14.8	1.3	34.7	1.4
(24*R*)-Methylcholest-5-en-3β,7β-diol	7β-Hydroxycampesterol	17.6	1.8	18.6	1.6	42.9	1.8
(24*R*)-Methylcholest-5-en-3β-ol-7-one	7-Ketocampesterol	24.6	2.4	33.1	2.9	66.9	2.7
(24*R*)-5α,6α-Epoxy-24-methylcholestan-3β-ol	Campesterol-5α,6α-epoxide	19.7	2.0	24.7	2.1	52.6	2.2
(24*R*)-5β,6β-Epoxy-24-methylcholestan-3β-ol	Campesterol-5β,6β-epoxide	19.2	1.9	23.6	2.0	51.0	2.1
(24*R*)-Methylcholestan-3β,5α,6β-triol	Campestanetriol	21.6	2.2	24.4	2.1	54.4	2.2
(24*R*)-Methylcholest-5-en-3β,24-diol	24-Hydroxycampesterol	18.6	1.9	19.3	1.7	45.2	1.8
(24*R*)-Methylcholest-4-en-6α-ol-3-one	4-Campesten-6α-ol-3-one	23.1	2.3	31.2	2.7	63.8	2.6
(24*R*)-Methylcholest-4-en-6β-ol-3-one	4-Campesten-6β-ol-3-one	19.4	1.9	25.4	2.2	53.3	2.2
(24*R*)-Ethylcholest-5-en-3β-ol	Unoxidized sitosterol	18.0	1.8	20.8	1.8	46.3	1.9
(24*R*)-Ethylcholest-5-en-3β,7α-diol	7α-Hydroxysitosterol	16.4	1.6	16.7	1.4	39.1	1.6
(24*R*)-Ethylcholest-5-en-3β,7β-diol	7β-Hydroxysitosterol	19.9	2.0	20.9	1.8	48.1	2.0
(24*R*)-Ethylcholest-5-en-3β-ol-7-one	7-Ketositosterol	28.1	2.8	37.6	3.3	75.3	3.1
(24*R*)-5α,6α-Epoxy-24-ethylcholestan-3β-ol	Sitosterol-5α,6α-epoxide	22.3	2.2	28.1	2.4	59.3	2.4
(24*R*)-5β,6β-Epoxy-24-ethylcholestan-3β-ol	Sitosterol-5β,6β-epoxide	21.8	2.2	26.9	2.3	57.3	2.3
(24*R*)-Ethylcholestan-3β,5α,6β-triol	Sitostanetriol	24.5	2.4	27.7	2.4	61.0	2.5
(24*R*)-Ethylcholest-5-en-3β,24-diol	24-Hydroxysitosterol	21.1	2.1	21.9	1.9	51.0	2.1
(24*R*)-Ethylcholest-4-en-6α-ol-3-one	4-Sitosten-6α-ol-3-one	26.3	2.6	35.4	3.1	71.7	2.9
(24*R*)-Ethylcholest-4-en-6β-ol-3-one	4-Sitosten-6β-ol-3-one	22.0	2.2	28.8	2.5	59.9	2.4

The columns were of same dimensions and the carrier gas flow was 0.8 ml/min (He) in all analyses.
[a] RRT = Rt of a POP/Rt of internal standard (5α-cholestane). For further analytical details, see Section 2.

elution time with this system was almost 40 min. Similar to the first column investigated, 7α-hydroxycampesterol had the shortest retention time (14.8 min) and 7-ketositosterol the longest (37.6 min) (Table 1). POPs that overlapped or had poor base line separation were, unoxidized campesterol/7β-hydroxycampesterol, 7β-hydroxystigmasterol/unoxidized stigmasterol, unoxidized sitosterol/7β-hydroxysitosterol, campestanetriol/stigmasterol-5β,6β-epoxide, stigmasterol-5β,6β-epoxide/campesterol-5α,6α-epoxide, 4-campesten-6β-ol-3-one/25-hydroxystigmasterol/24-hydroxystigmasterol and sitostanetriol/sitosterol-5α,6α-epoxide (Table 1).

When the two above mentioned GC columns were compared, the column with higher polarity (DB35-MS) clearly indicated fewer overlapping peaks (7 compared with 11). It was also observed that the retention time for all the POPs was longer on the more polar column, which might be one reason for the improved separation. The elution pattern for the first five oxidation products for campesterol on both the columns was the same (first 7α-hydroxy followed by unoxidized sterol, 7β-hydroxy, 24-hydroxy and 5β,6β-epoxide). However, after the first 5 products the elution order shifted. In the system with the non-polar column, 4-campesten-6β-ol-3-one was eluted before campesterol-5α,6α-epoxide and campestentriol. In the more polar system, the elution pattern was campestentriol before campesterol-5α,6α-epoxide and 4-campesten-6β-ol-3-one was last. The last POPs eluted were 4-campesten-6α-ol-3-one followed by 7-ketocampesterol (Table 1). The same elution pattern was reported for oxidation products from sitosterol. For stigmasterol the elution pattern was the same for both systems except that in the non-polar system unoxidized stigmasterol was eluted before 7β-hydroxystigmasterol and in the mid-polar system the order was the reverse.

Data from the present study using a single capillary column in the separation process were in line with previously published papers [9,10]. Dutta [9] reported data on the separation of a mixture of POPs and COPs on a DB5-MS column (50 m × 0.20 μm, 0.33 μm). The total analytical time was 60.1 min (7-ketositosterol) and the POPs that co-eluted were: unoxidized brassicasterol/7α-hydroxycampesterol, 7α-hydroxystigmasterol/unoxidized campesterol, 7α-hydroxysitosterol/unoxidized stigmasterol, 7β-hydroxystigmasterol/unoxidized sitosterol, 7β-hydroxysitosterol/stigmasterol-5β,6β-epoxide and sitosterol-

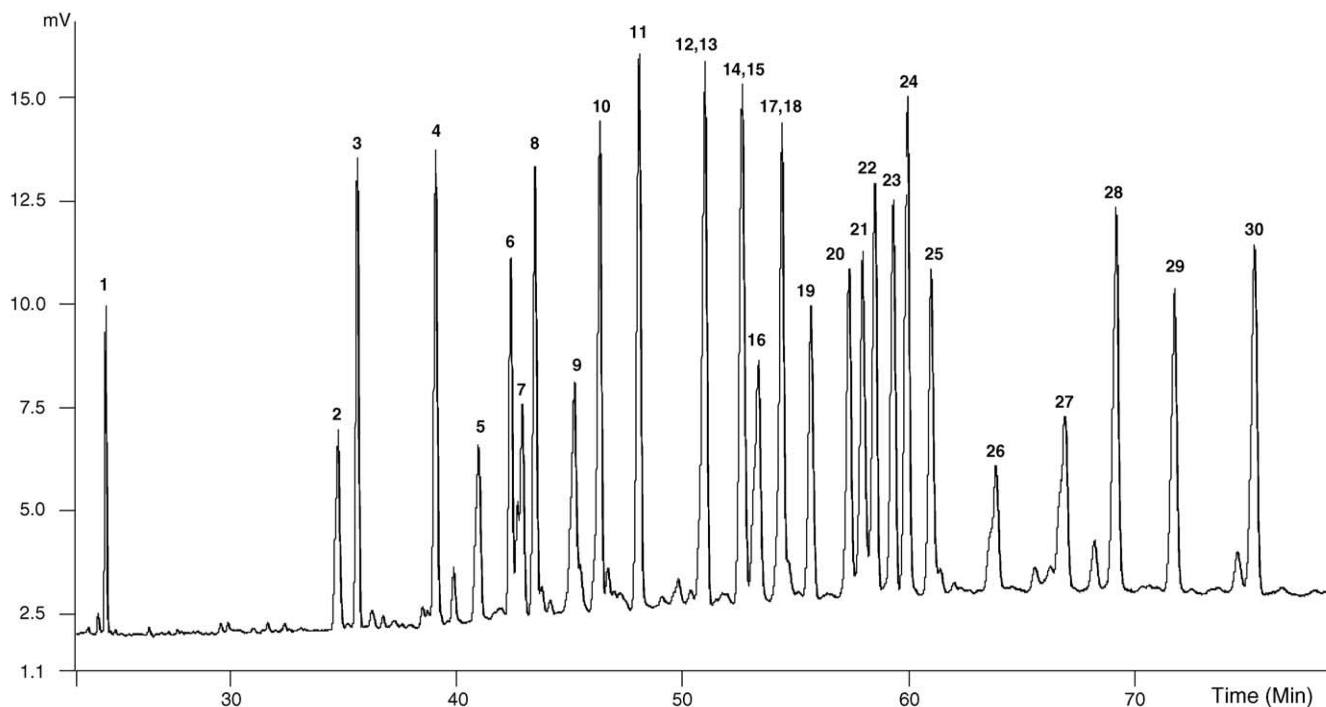

Fig. 1. GC separation of a number of POP on a combination of two capillary columns of different polarities. The dimensions of the columns were 25 m × 0.2 mm × 0.33 μm and the carrier gas flow was 0.8 ml/min. For identification of the peaks, see Table 2. For further analytical details, see Section 2.

5β,6β-epoxy/campestanetriol. Poor base line separation was reported for campesterol-5α,6α-epoxide/7β-hydroxysitosterol, campestanetriol/sitosterol-5α,6α-epoxide and sitosterol-5α,6α-epoxide/7-ketocampesterol.

In order to improve the separation of the POPs, a combination of the columns described above (DB35-MS and DB5-MS) was combined with a pressfit connector. The more polar column, DB35-MS, was connected to the injector because the results from the single column analysis indicated that the efficiency was higher on the mid-polar column. In addition, the only reference available in the sterol area [13] dealing with a combination of capillary columns also reported that the more polar capillary column was connected to the injector side of the GC instrument.

The combination of the two capillary columns improved the separation and adequate base line separation was observed for many of the POPs (Fig. 1 and Table 2). The combined columns used in the present study increased the total analytical time to over 75 min. As for the analysis on the single columns 7α-hydroxycampesterol had the shortest retention time (34.7 min) and 7-ketositosterol the longest (75.3 min) (Table 1). The peaks that co-eluted were 24-hydroxysitosterol/campesterol-5β,6β-epoxide (nos.12 and 13 in Fig. 1), stigmasterol-5β,6β-epoxide/campesterol-5α,6α-epoxide (nos. 14 and 15 in Fig. 1) and stigmasterol-5α,6α-epoxide/campestanetriol (nos. 17 and 18 in Fig. 1). In addition, poor base line separation was reported for unoxidized stigmasterol and 7β-hydroxycampesterol (nos. 6 and 7 in Fig. 1). However, as described in the methodology section, during the analytical procedure used in this study the unoxidized sterols were removed by SPE chromatography and the quantification of 7β-hydroxycampesterol was possible by GC. Almost base line separation was reported for 25-hydroxystigmasterol/24-hydroxystigmasterol ($R_s = 1.4$) (nos. 20 and 21 in Fig. 1) and 24-hydroxystigmasterol/sitosterol-5α,6α-epoxide ($R_s = 1.3$) (nos. 21 and 22 in Fig. 1).

The elution pattern of the oxidation products was different to that obtained in the analysis performed on the single columns used in this study. However, as for the single column systems, the first POPs eluted was 7α-hydroxycampesterol followed by 7α-hydroxystigmasterol and the four POPs with longest retention time were 7-ketocampesterol, 7-ketostigmasterol, 4-sitosten-6β-ol-3-one and finally 7-ketositosterol (nos. 27–30 in Fig. 1). Due to interaction between the column material and the oxidation products, the elution order and retention times of the products shifted between the single columns and the columns in combination. POPs that co-eluted or had poor base line separation in all systems were 24-hydroxystigasterol/25-hydroxystigmasterol (nos. 21 and 22 in Fig. 1).

The combination of capillary columns to improve the separation of lipids has previously been reported [13]. The authors used the selectivity of two individual columns of the phases 5% phenyl and 14% phenyl-coated columns in order to separate steradienes, tocopherols, alkanes, stanols and un-

Table 2
Peak identification of the separated POP in Fig. 1

Oxidation product	Peak no.	Rt (min)
5α-Cholestane	1	I.S.
7α-Hydroxycampesterol	2	34.7
7α-Hydroxystigmasterol	3	35.6
7α-Hydroxysitosterol	4	39.1
Unoxidized campesterol	5	41.0
Unoxidized stigmasterol	6	42.4
7β-Hydroxycampesterol	7	42.9
7β-Hydroxystigmasterol	8	43.4
24-Hydroxycampesterol	9	45.2
Unoxidized sitosterol	10	46.3
7β-Hydroxysitosterol	11	48.1
24-Hydroxysitosterol	12	51.0
Campesterol-5β,6β-epoxide	13	51.0
Stigmasterol-5β,6β-epoxide	14	52.6
Campesterol-5α,6α-epoxide	15	52.6
4-Campesten-6β-ol-3-one	16	53.3
Stigmasterol-5α-6α-epoxide	17	54.4
Campestanetriol	18	54.4
Stigmastenetriol	19	55.7
Sitosterol-5β,6β epoxide	20	57.3
25-Hydroxystigmasterol	21	57.9
24-Hydroxystigmasterol	22	58.5
Sitosterol-5α,6α-epoxide	23	59.3
4-Sitosten-6β-ol-3-one	24	59.9
Sitostanetriol	25	61.0
4-Campesten-6α-ol-3-one	26	63.8
7-Ketocampesterol	27	66.9
7-Ketostigmasterol	28	69.1
4-Sitosten-6α-ol-3-one	29	71.7
7-Ketositosterol	30	75.3

The POP were separated on a combination of two capillary columns with the same dimensions coupled with a pressfit connector (Rt = retention time). For further analytical details, see Section 2.

oxidized sterols. The authors observed that in a temperature programmed separation the exact elution order of the analytes depends on the order of the columns connected in series.

The use of combined GC columns has also been reported from other research areas. Lindig [14] coordinated a proficiency testing for the analysis of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Some of the participating laboratories used a combination of columns in their analytical systems. Fricke et al. [15] used a combination of a non-polar CpSil 5 capillary column and a chiral column in the investigation of the enantiomeric composition of β-caryophyllene in several liverworts.

The results from this study clearly indicate that a combined mid-polar and non-polar capillary column in the analysis of POPs improve the efficiency of the separation. Enhanced base line separation in general was the main improvement in this study. In order to analyze and quantify POPs in food products an efficient separation of the oxidation products is vital. We have conducted successfully analyses of levels of POPs in oxidized vegetable oils by using the described combined system and these results will be published elsewhere. The combination of capillary GC columns is a new potential area in the separation of POPs, and the use of columns of other polarities and further optimization of GC parameters might improve the separation further.

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