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Comparison of steradiene analysis between GC and HPLC

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Abstract

Steradienes are sterol dehydration products formed in the vegetable oil refining process during the bleaching and deodorization process. The 3,5-stigmastadiene content of vegetable oils was quantified by GC as well as by HPLC. A good repeatability, as indicated by the low relative standard deviation of 1.11 and 2.46%, and accuracy levels of 113.9 and 107.7% were obtained for the GC and HPLC analytical method respectively. Although a good comparison between both analytical methods was observed, GC data consistently gave on average a higher 3,5-stigmastadiene content of 19.6% compared to HPLC data. Probably there is an overestimation of the steradiene content analyzed by GC due to peak overlap with other hydrocarbons as the FID detector is not as selective compared to the UV detector applied in the HPLC analysis.

Keywords: 3,5-Stigmastadiene; Gas chromatography; High performance liquid chromatography; Steradienes; Vegetable oils

1. Introduction

Steradienes are steroidal hydrocarbons formed by a sterol dehydration reaction, which is acid catalyzed or promoted by elevated temperatures. In the vegetable oil refining process, steradienes are formed during the bleaching process on the surface of the acid activated bleaching earth or during the deodorization process promoted by the elevated temperature (Biederman, Grob, Mariani, & Schmidt, 1996; Grob & Bronz, 1994; Grob, Giuffre, Biedermann, & Bronz, 1994; Homberg, 1975; Kaufmann & Hamza, 1970; Kaufmann, Vennekel, & Hamza, 1970; Lanzon, Albi, Cert, & Gracian, 1994). 3,5-Stigmastadiene derived from the dehydration of β-sitosterol is the most abundant steradiene detected in vegetable oils (Cert, Lanzon, Carelli, & Albi, 1994; Fiebig, 1999; Lanzon et al., 1994; Schulte, 1994).

Crude oils obtained by pressing or solvent extraction have a steradiene content lower than 0.01 mg/kg (Cert et al., 1994; Bruhl & Fiebig, 1995). Upon refining, steradienes are formed during the bleaching and deodorization process and their analysis can define whether an edible oil is crude or refined (Cert et al., 1994; Grob & Bronz, 1994). Depending on the refining conditions, commercially refined vegetable oils contain a steradiene level ranging between 1 and 100 mg/kg (Dobarganes, Cert, & Dieffenbacher, 1999; Schulte, 1994). Besides steradienes, other sterol transformation products are formed during the refining process as well. Among these are sterol condensation products or disteryl ethers (Schulte & Weber, 1987, 1991; Weber, Bergenthal, Bruhl, & Schulte, 1992) and oxidized sterols (e.g. 7-hydroxy-, 7-keto- and 5,6-epoxysterols) (Kaufmann, Vennekel, & Hamza, 1970; Homberg, 1975; Dutta, 1989) which require a long and tedious analysis procedure.

The steradiene analysis is a possible method to detect adulteration of non-refined and specialty oils and fats. For example, the steradiene content is an important quality characteristic for virgin olive oils. Virgin olive oils obtained by cold pressing have a 3,5-stigmastadiene content lower than 0.01 mg/kg (Cert et al., 1994; Dobarganes et al., 1999; Ranalli, Ferrante, De Mattia, & Costantini, 1999) whereas refined olive oils usually have a 3,5-stigmastadiene level ranging between 0.3 and 0.9 mg/kg (Cert et al., 1994; Dobarganes et al., 1999). Steradiene analysis is also used to detect the addition of bleached cocoa butter equivalent fats to cocoa butter, which is generally not bleached (Crews, Calvet-Sarrett, &

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Brereton, 1997, 1999) and identification of 3,5-cholestadiene in anhydrous butterfat allows the detection of adulteration with refined animal fat (Roderbourg, 1979).

Analytical methods for the steradiene analysis are well documented. The AOCS Official Method Cd26–96 (1990) and the IUPAC method (Dobarganes et al., 1999) analyze the steradiene content of a vegetable oil by GC. After saponification of the triacylglycerols and extraction of the non-saponifiables with petroleum ether, steradienes are isolated from other non-saponfiable components by silica gel column chromatography. Upon gas chromatographic separation of steradienes interferences with other hydrocarbons might occur.

Steradienes can also be analyzed by reversed phase HPLC according to the DGF Standard Method (Fiebig, 1999). Therefore, steradienes are isolated from triacylglycerols and polar lipids by silica gel chromatography upon elution with petroleum ether. The collected steradiene and hydrocarbon fraction is injected on a reversed phase column. Detection of steradienes can be done by UV detection at 235 nm as steradienes contain a conjugated double bound. This UV detection is very sensitive and highly selective for conjugated dienes, thus avoiding interference of other components.

The objective of this study was to compare the steradiene analysis by GC and HPLC. Both methods were evaluated and their advantages and disadvantages will be discussed.

2. Experimental

2.1. Materials

Several vegetable oil samples of different types of oil (cacao butter, canola-, coconut-, corn-, palm-, olive-, rapeseed-, soybean- and sunflower oil) were obtained from Extraction De Smet (Edegem, Belgium). All samples were stored in the refrigerator at 4 °C for maximally 4 months until analysis.

Analytical grade solvents (*n*-hexane, petroleum ether, acetonitrile, *tert*-butylmethylketone) were purchased from Merck (Darmstadt, Germany). The internal standard for steradiene quantification, analytical grade 3,5-cholestadiene and silica gel for column chromatography were purchased from Sigma (Bornem, Belgium). Due to the similar structure, 3,5-cholestadiene is the preferred internal standard used for quantification of phytosterol steradienes and no response factors have been determined.

2.2. Analysis of steradienes by gas chromatography

2.2.1. Sample preparation

Steradiene analysis was carried out according to the AOCS (1990) Official Method Cd26–96. Briefly described, vegetable oil samples (20 g) were spiked with 1 ml of internal standard (20 μg 3,5-cholestadiene/ml *n*-hexane) and saponified with 75 ml of a 10% alcoholic potassium solution by gentle boiling for 30 min. After saponification, the mixture was transferred by use of 100 ml water into a separating funnel and extracted with 100 ml of petroleum ether. The mixture was vigorously shaken for 30 s and left to separate. The lower aqueous phase was transferred into a second separating funnel and extracted again with 100 ml petroleum ether. The combined petroleum ether extracts were washed with 100 ml volumes of ethanol/water (1/1) until neutral pH. Generally, three washings were required to reach neutral pH. The extract was dried over anhydrous magnesium sulfate and evaporated. With the aid of 2×1 ml portions of *n*-hexane, the residue was transferred on a silica gel column (15 g silica, column 45×2.5 cm internal diameter) and chromatographic elution was done with *n*-hexane at a flow rate of 1 ml/min. The first 30 ml fraction thus obtained was discarded and the following 40 ml fraction containing the steradienes was collected and evaporated. After evaporation, the residue was dissolved in 0.2 ml of *n*-hexane, transferred in a 2 ml vial and stored in the refrigerator at 4 °C until analysis within 7 days of preparation.

2.2.2. Gas chromatographic conditions

Gas chromatographic separations were performed by a HP 6890 series gas chromatograph (Hewlett Packard, Avondale, PA) on a capillary column Alltech EC5 30 m× 0.25 mm, 0.25 μm (Alltech, Deerfield, IL) using helium as a carrier gas at a pressure of 110 kPa and a split ratio of 15:1.

A temperature program with injection at 235 °C with hold for 6 min and rising at 2 °C/min to 285 °C and continuing the oven heating at a speed of 15 °C/min to 310 °C with 3 min hold, was found optimal to separate the different components. Detection was done by flame ionization with the detector temperature set at 360 °C. Identification of 3,5-stigmastadiene was performed according to the relative retention time reported in the AOCS Official Method Cd26–96.

2.3. Analysis of steradienes by high performance liquid chromatography

2.3.1. Sample preparation

Steradiene analysis by HPLC was carried out according to the DGF Standard Method (Fiebig, 1999). Briefly described, vegetable oil samples (500 mg) were weighed in a small beaker and spiked with 1 ml of internal standard (20 μg 3,5-cholestadiene / ml hexane). The oil was transferred on a silica gel column (5 g silica, 45×2.5 cm internal diameter) with the aid of 2×2 ml portions of *n*-hexane. Chromatographic elution was done with *n*-hexane and the first 20 ml fraction obtained

contained the steradienes and was evaporated. After evaporation, the residue was dissolved in 0.5 ml of *n*-hexane and stored in the refrigerator at 4 °C until analysis within 7 days of preparation.

2.3.2. HPLC conditions

Reversed-phase HPLC analysis of steradienes was performed on a Kontron chromatograph (Serlabo, Brussels, Belgium) equipped with a reversed phase column (Lichrospher RP 18, particle size 5 μm, 250×4.6 mm internal diameter, Merck, Darmstadt, Germany). The solvent mixture acetonitrile / *tert*-butyl methyl ether (7/3 v/v) was pumped at a flow rate of 1 ml/min and detection of steradienes was done by UV adsorption at 235 nm. Peaks identified according to Fiebig (1999).

2.4. Evaluation of comparison

Repeatability of the analytical method for 3,5-stigmastadiene analysis was checked by repeated analysis of a bleached sunflower oil sample. Accuracy of the analytical method for 3,5-stigmastadiene analysis was evaluated by participation in a ring trial. Four samples with varying 3,5-stigmastadiene content were analyzed by GC and HPLC.

3. Results and discussion

Analysis of steradienes by GC is a widely reported method (AOCS Official Method Cd26–96; Dobarganes et al., 1999). The gas chromatographic analysis of steradienes allows the simultaneous quantification of 3,5-stigmastadiene together with steradienes derived from other sterols. A typical chromatogram obtained for the steradiene analysis of olive and corn oil is presented in Figs. 1 and 2, respectively. 3,5-Stigmastadiene, 3,5,22-stigmastatriene and 3,5-campestadiene had a retention time relative to 3,5-cholestadiene of 1.13, 1.17 and 1.24 respectively.

During the dehydration reaction 3,5-stigmastadiene is produced together with minor amounts of the 2,4-stigmastadiene isomer and both substances originate in a single signal upon analysis on a low polar column (Dobarganes et al., 1999; Grob & Bronz, 1994).

The isolation of steradienes from the non-saponifiable fraction by silica gel chromatography is the most critical step in the analysis. Incomplete isolation of the steradiene fraction might give interference in the detection due to the coelution of steradienes with other hydrocarbons, squalene and isomers of squalene, as the FID detector is not selective (Guinda, Lanzon, & Albi, 1996; Lanzon et al., 1994; McGill, Moffat, Mackie, & Cruickshank, 1993; Schulte, 1994). As illustrated in Fig. 2, which represents the gas chromatogram of a bleached corn oil sample, several other unidentified components are eluting in the steradiene area as well, which might give interference.

Recently, an analytical method for the quantification of steradienes by HPLC analysis has been reported (Fiebig, 1999). For the separation of steradienes by HPLC, RP-C18 columns are especially useful and isocratic elution is sufficient as steradienes elute in a short area of the chromatogram. Analysis of steradienes by HPLC allows the simultaneous identification of 3,5-stigmastadiene, 3,5,22-stigmastatriene and 3,5-campestadiene with a retention time relative to 3,5-cholestadiene of 1.06, 1.11 and 1.18 respectively. A typical HPLC chromatogram for the steradiene analysis of soybean oil is presented in Fig. 3.

HPLC analysis of steradienes does not completely resolve all components eluting in the steradiene area. Upon analysis of a severely bleached soybean oil sample, which contains considerable amounts of 3,5-campestadiene, 3,5,22-stigmastadiene and 3,5-stigmastadiene a baseline separation of the different steradienes is not obtained (Fig. 3). Schulte (1994) also reported this incomplete separation of steradienes by HPLC analysis upon injection of several steradienes. In practice only 3,5-stigmastadiene is calculated, as it is the major steradiene present in most vegetable oils. Doubling of the column length by connecting two 250 mm columns improved the resolution but could still not baseline separate all major steradiene components and was

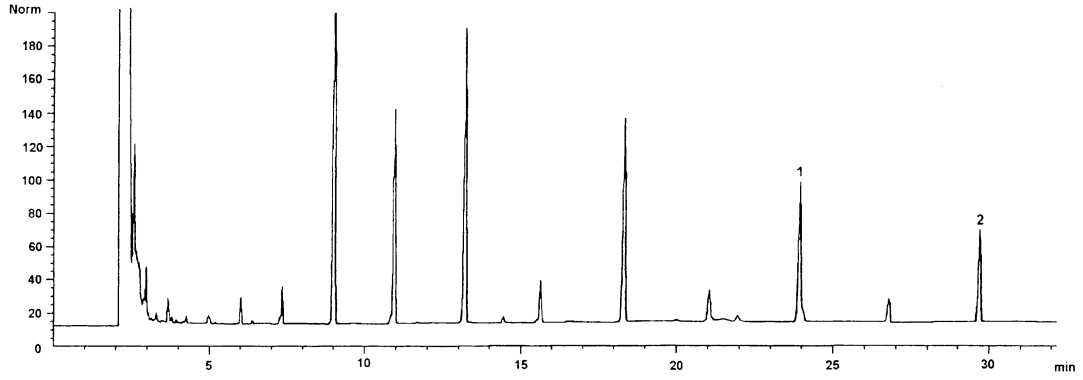

Fig. 1. Gas chromatogram for the steradiene analysis of olive oil. (1) 3,5-Cholestadiene, (2) 3,5-stigmastadiene.

therefore not selected. For the analysis of rapeseed oil special care needs to be taken as coelution of the internal standard 3,5-cholestadiene with 3,5,22-brassicastatriene was observed (Bruhl & Fiebig, 1995). According to Bruhl and Fiebig (1995) the detection limit for steradiene quantification ranged between 0.04–0.08 mg/kg and 0.06–0.09 mg/kg for the GC and HPLC method respectively.

3.1. Repeatability

Both analytical methods had an acceptable repeatability as indicated by the low standard deviation of 0.74 ± 0.01 mg/kg and 0.61 ± 0.02 mg/kg for the GC and HPLC analysis, respectively (Table 1). The average of the 3,5-stigmastadiene content analyzed by HPLC was little lower compared to the average of the GC data and showed a little higher standard deviation. The relative standard deviation obtained was in accordance to literature reports (Dobarganes et al., 1999; Fiebig, 1999).

3.2. Ring trial

Interlaboratory studies on the quantification of the 3,5-stigmastadiene content in vegetable oils indicate a very large deviation (Dobarganes et al., 1999; Fiebig, 1999). Results are listed in Table 2 and indicate the large deviation in the minimum and maximum 3,5-stigmastadiene

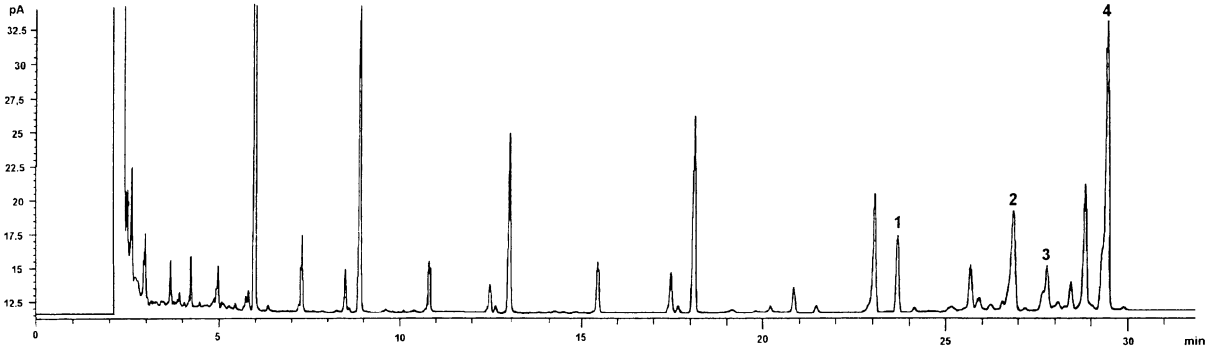

Fig. 2. Gas chromatogram for the steradiene analysis of sunflower oil. (1) 3,5-Cholestadiene, (2) 3,5-campestadiene, (3) 3,5,22-stigmastadiene, (4) 3,5-stigmastadiene.

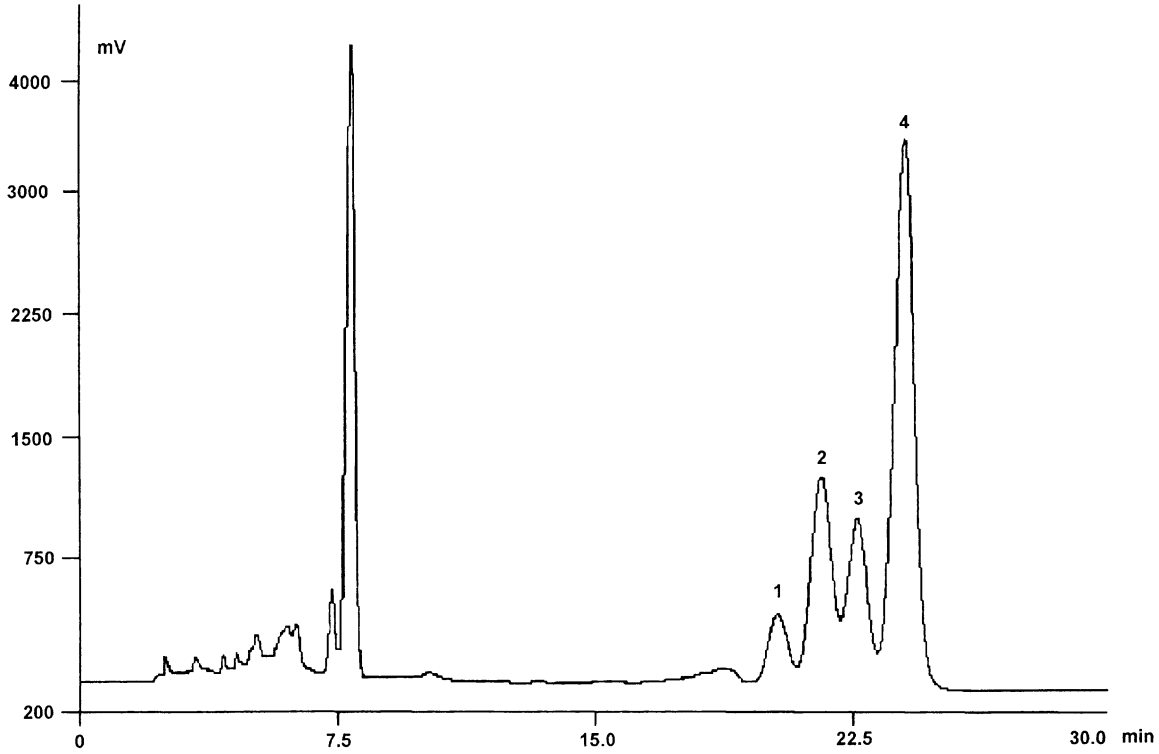

Fig. 3. HPLC chromatogram for the steradiene analysis of soybean oil. (1) 3,5-Cholestadiene, (2) 3,5-campestadiene, (3) 3,5,22-stigmastadiene, (4) 3,5-stigmastadiene.

content reported by the different laboratories. Several laboratories were eliminated as their results were regarded as outliers.

The 3,5-stigmastadiene content obtained by GC and HPLC analysis in our laboratory is comparable to the mean of the 3,5-stigmastadiene value reported by the different laboratories. Little difference in the 3,5-stigmastadiene content analyzed by GC and HPLC was obtained. The good agreement with the mean values from the collaborative study was considered as an indication of the good accuracy of 113.9% for the GC and 107.7% of the HPLC method applied.

3.3. Analysis of commerical oil samples

The 3,5-stigmastadiene content of 30 different vegetable oils was analyzed according to the GC and HPLC method for steradiene analysis (Table 3). Several samples were obtained without knowing their refining conditions. Crude and cold pressed oils had the lowest steradiene concentration. No steradienes could be detected in cold pressed rapeseed oil. Crude coconut oil and crude palm oil had a higher 3,5-stigmastadiene content, indicating these oils have been heat treated during the extraction or pressing process. Little 3,5-stigmastadiene concentrations were detected in olive oil.

Table 1
Repeatability of the 3,5-stigmastadiene content analyzed by GC and HPLC for a bleached sunflower oil

	GC	HPLC
Mean[a] (mg/kg)	0.74	0.61
STD[b] (mg/kg)	0.01	0.02
RSD[c] (%)	1.11	2.46

[a] Mean of five replicates spread over 1 week.
[b] Standard deviation.
[c] Relative standard deviation.

Table 2
Interlaboratory study for the quantification of 3,5-stigmastadiene content (mg/kg) in vegetable oils

Sample	Our analysis[a]		Reference
	GC	HPLC	Mean (range)[b]
1	0.58	0.52	0.54 (0.2–1.0)
2	4.04	3.82	3.02 (1.59–7.05)
3	30.16	29.14	28.22 (16.22–54.23)
4	72.22	70.39	67.04 (34.83–111.48)
Accuracy (%)[c]	113.9	107.7	

[a] Mean of two replicates.
[b] Mean value obtained after elimination of outliers, sample 1 analyzed by 18 laboratories, 3 laboratories were eliminated; sample 2 analyzed by 18 laboratories, 4 laboratories were eliminated; sample 3 analyzed by 19 laboratories, 1 laboratory was eliminated; sample 4 analyzed by 19 laboratories, 6 laboratories were eliminated, range varies between minimum–maximum.
[c] Mean of samples 1,2,3 and 4.

The majority of the bleached vegetable oil samples analyzed had a medium steradiene content ranging between 0.5 and 5 mg/kg (eg. bleached red palm olein, bleached sunflower oil). Deodorized oils (e.g. corn and soybean oil) had the highest steradienes concentration of ± 20 mg/kg. Although a certain steradiene content is removed by distillation during deodorization, more steradienes are formed due to the temperature applied in the deodorization process (Grob et al., 1994).

Comparing the GC and HPLC 3,5-stigmastadiene data for the 30 samples analyzed, a good correlation coefficient (R^2 of 0.9975) between the steradiene content analyzed by GC and HPLC was obtained. This perfect correlation was observed for low steradiene levels (0.5–5 mg/kg) as well as for high steradiene levels (± 20 mg/kg).

The 3,5-stigmastadiene content obtained by GC was consistently higher (19.6%) than the corresponding HPLC data. For the 30 samples analyzed, a mean difference of 0.3 mg/kg between the GC and HPLC analysis was obtained. This observation is in accordance to literature (Schulte, 1994). Probably there is an overestimation of the steradiene content analyzed by GC due

Table 3
3,5-Stigmastadiene content (mg/kg) of several vegetable oils analyzed by GC and HPLC

Sample	GC	HPLC
Olive	0.55	0.43
Olive	0.75	0.51
Olive	0.61	0.42
Olive	0.49	0.29
Olive	0.62	0.39
Olive	0.66	0.41
Olive	0.64	0.42
Olive	1.37	0.90
Olive	0.92	0.56
Olive	0.79	0.54
Almond	0.58	0.39
Cacao butter (refined)	0.66	0.58
Coconut oil (crude)	2.85	2.71
Coconut oil (deodorised)	15.47	14.36
Corn (bleached)	20.96	18.75
Corn (bleached)	5.38	4.96
Palm oil (crude)	0.80	0.65
Palm oil (refined)	5.96	5.20
Peanut (refined)	2.36	2.21
Rapeseed (crude)	0.07	Nd[a]
Rapeseed oil (cold pressed)	0.05	Nd
Red palm olein (bleached)	3.07	3.60
Safflower	0.54	0.37
Soya (bleached)	3.25	2.98
Soya (bleached)	5.54	5.01
Soya (refined)	21.26	20.47
Sunflower (bleached)	0.74	0.61
Sunflower (refined)	0.98	0.94
Sunflower (refined)	0.96	0.85
Walnut	1.49	1.32

[a] Not detectable.

to peak overlap with other hydrocarbons or squalene isomers as the FID detector is not as selective compared to the UV detector applied in the HPLC analysis (Schulte, 1994). This difference between GC and HPLC analysis is observed in analytical data on tocopherol quantification as well. Comparing data for the tocopherol analysis, De Greyt, Petrauskaite, Kellens, and Huyghebaert (1998) also reported a consistently higher tocopherol content in the GC data compared to the HPLC data. The analytical difference was explained by a possible peak overlap in the GC chromatogram.

4. Conclusion

The 3,5-stigmastadiene content of vegetable oils can be analyzed by GC as well as by HPLC. A good repeatability and accuracy was obtained for both analytical methods. Although an acceptable comparison between both analytical methods was observed, GC data consistently gave on average a higher 3,5-stigmastadiene content of 19.6% compared to HPLC data. Therefore, further research towards an identification of interfering compounds in the GC chromatogram should be undertaken.

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