Investigation of unsaponifiable matter of plant oils and isolation of eight phytosterols by means of high-speed counter-current chromatography

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ABSTRACT

Phytosterols are minor components of plant oils. Due to their beneficial effect on human serum cholesterol level, new products supplemented with phytosterols have been marketed. Commercial phytosterol standards are frequently of insufficient purity, very expensive, only available in (semi-) synthetic form or not available at all. For this reason we aimed to explore the unsaponifiable matter of three plant oils (rapeseed oil, linseed oil and olive oil) in order to study their compositions and to purify several phytosterols. We fractionated ~100 mg of the unsaponifiable matter of the plant oils with high-speed counter-current chromatography (HSCCC) by the combination of an enrichment step and a purification step. In the first part (enrichment step) composition of phytosterols, alkanes, fatty alcohols from 14:0 to 30:0 including isomers, 15-nonacosanone and other ketones as well as further minor compounds in the different fractions was studied by GC/MS. By means of the solvent system *n*-hexane/methanol/aqueous silver nitrate solution (34/24/1, v/v/v) in normal phase mode (tail-to-head) β-sitosterol could be isolated (6.4 mg, purity ≥99%) and several phytosterols (e.g. citrostadienol, cycloeucalenol and erythrodiol) could be enriched. Moreover, the fast eluting hydrocarbons squalene and nonacosane as well as the later eluting phytol (pure, 7 mg) and geranyl geraniol could also be efficiently enriched.

Suited HSCCC fractions from the three plant oils were merged and re-injected into the HSCCC system (purification step). The HSCCC purification step provided 6.9 mg campesterol (≥99%), 2.9 mg brassicasterol (≥99%), 0.3 mg Δ5-avenasterol (≥90%), 9.5 mg cycloartenol (≥90%), 3.7 mg 24-methylene-cycloartanol (≥99%), and ~1 mg of an unknown compound (~80%) isolated from rapeseed and linseed oil. Furthermore, the combined HSCCC enrichment and purification of a hydrogenated sterol standard provided two pure phytostanols (campestanol ≥99% and sitostanol ≥99%) and several by-products.

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1. Introduction

Sterols are tetracyclic minor lipid components found in animals, plants and microorganisms. Several hundred different structures have been identified to date. While cholesterol (**1**) is the major sterol in animals, the most common representatives in the plant kingdom are β-sitosterol (**7**), campesterol (**4**), stigmasterol (**6**), Δ5-avenasterol (**9**) and Δ7-avenasterol (similar to **9** aside from double bond which is shifted from C-5 to C-7) (Fig. 1). The relative abundances of these and further, less frequently distributed phytosterols can be used as a fingerprint for food authentication. For instance, high amounts of brassicasterol (**3**) as well as cycloartenol (**10**) and 24-methylene-cycloartanol (**11**) are characteristic for rapeseed and linseed oil [1]. Phytosterols are bioactive compounds which can promote human health. For instance, intake of

phytosterols (1–2 g per day) was reported to lower the human serum cholesterol (**1**) level by ~10% [2,3]. Therefore, functional food products (e.g. margarine, yoghurt, cheese and bread) supplemented with phytosterols have been marketed in Germany and elsewhere. However, immoderate consumption of phytosterols can be accompanied with negative health effects. For instance, higher intake of phytosterols may cause a decrease in the human blood levels of α-carotene, β-carotene, α-tocopherol and lycopene [4,5]. For this reason, the European Food Safety Authority (EFSA) has recommended a maximum intake level of 3 g phytosterols per day as prudent precaution [6]. However, little is known about the role of individual phytosterols neither with regard to beneficial nor to detrimental health effects. One major drawback is that pure phytosterol standards are barely available. Phytosterols standards are frequently gained from purified plant extracts. The most common purification method of phytosterols is recrystallization which only provides moderate purity and/or bad yield [7]. Recently, high-speed counter-current chromatography (HSCCC) has been used as an alternative for the enrichment and isolation of β-sitosterol (**7**) and sitostanol (**8**) from phytosterol mixtures [8,9] as well as of

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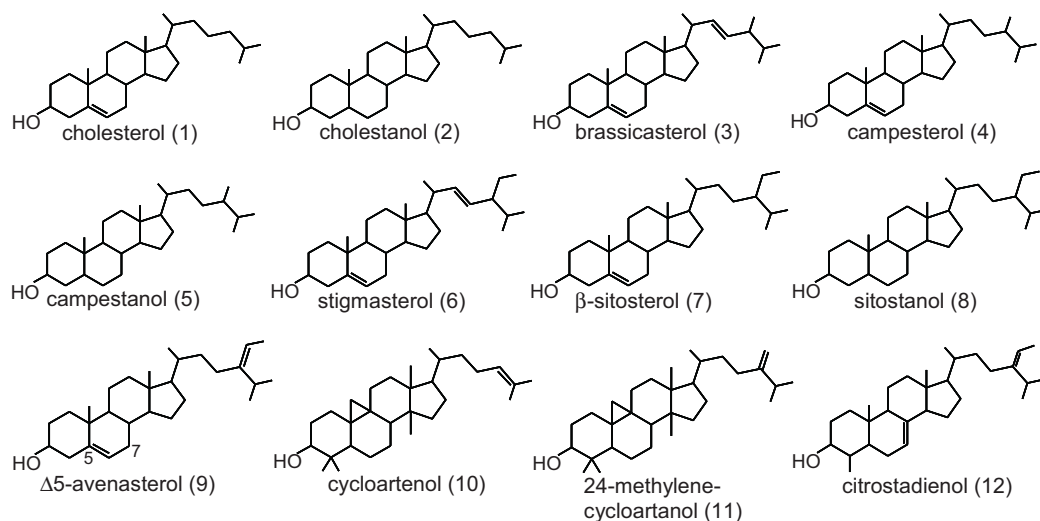*E-mail address:* walter.vetter@uni-hohenheim.de (W. Vetter).

Fig. 1. Chemical structures of selected sterols and stanols.

ergosterol and stigmasterol (**6**) from the orchid *Anoectochilus rox-burghii* [10]. HSCCC is a chromatographic liquid–liquid separation technique initially developed in the 1970s by Ito and Bowman [11]. The separation is based on the different solubility of the analytes in a liquid mobile phase (normally the phase which provides the better solubility) and a liquid stationary phase which are immiscible. Usually, a planetary rotation device is used in order to keep the stationary phase static in the HSCCC-columns (coils). Typically, the rotation speed of the coils is set between 800 and 1200 revolutions per minute (rpm), which refers to as HSCCC. Semi-preparative HSCCC was successfully used since the 1980s for the separation of mixtures and the isolation of natural products from different sample matrices [12].

Based on experience gathering during the previous isolation of β-sitosterol (**7**) from a commercial phytosterol standard mix [9], we combined the high sample capacity and separation power of HSCCC in order to determine the detailed composition of the unsaponifiable matter of three plant oils including phytosterols, alcohols, aldehydes and other rarely studied compounds. In addition we aimed at isolating pure phytosterol and phytostanol (prepared by the hydrogenation of phytosterols) standards by HSCCC. For this purpose, we saponified the plant oils, extracted the unsaponifiable matter (including the phytosterols) and injected concentrated solutions into the HSCCC system. Carefully selected fractions of phytosterols were combined and re-injected into the HSCCC system for compound isolation.

2. Material and methods

2.1. Chemicals, standards and samples

Methanol (purest; distilled before use), silver nitrate (>99.5%) and geranyl geraniol (≥85%), palladium on activated charcoal (10%) were from Sigma–Aldrich (Steinheim, Germany); *n*-hexane (HPLC gradient grade) was from Th. Geyer (Renningen, Germany), the silylation agents (N,O-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), 99:1, v/v) were from Supelco (Bellefonte, PA, USA). Ethanol and 2-propanol (both BASF, Ludwigshafen, Germany) were distilled before use; potassium hydroxide (>85%) was from Carl Roth (Karlsruhe, Germany). Reference standards for compound identification (squalene, >98% purity; *cis*-/*trans*-phytol, ≥95% purity; β-sitosterol standard, ~85% purity) were from Merck (Darmstadt, Germany). Hydrogen (99.9990%) for hydrogenation was from WestfalenGas (Münster, Germany).

Samples of cold-pressed linseed oil (Schneekoppe), native olive oil extra (Cantinelle) and refined rapeseed oil (Bellasan) were bought retail (2009/2010) in a local supermarket in Stuttgart, Germany.

2.2. High-speed counter-current chromatography (HSCCC) apparatus

HSCCC was performed with a PTR Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, Maryland, USA) equipped with a quaternary P580 HPLC pump (Dionex, Idstein, Germany) for solvent delivery [13]. A semi-preparative column (total volume 325 mL) consisting of three serially coupled coils made of PTFE with 1.6 mm internal diameter was used. The revolution radius R was 7.6 cm while the β-value ranged from 0.50 (internal terminal) to 0.75 (external terminal) [13]. Fractions were collected with the help of an Iso Retriever 500 (Teledyne Isco, Lincoln, NE, USA).

In order to obtain a suitable solvent system, shake-flask tests have been done by using 6 mL test tubes with 1.0 mg β-sitosterol standard (containing sitostanol (**8**), β-sitosterol (**7**), campesterol (**4**) and stigmasterol (**6**)) and the solvents [9]. Combinations of *n*-hexane, methanol, and water proved to be best suited. The use of 1% of silver nitrate in water slightly improved the separation [9]. The most suited mixture was selected based on $K_{L/U}$ values of ~0.5 for the phytosterols. This was fulfilled with the system consisting of *n*-hexane/methanol/water (which contained 1% AgNO₃) in the ratio 1.7/1.2/0.05 (v/v/v) [9]. Accordingly, 680 mL *n*-hexane, 480 mL methanol and 20 mL water, which contained 0.20 g silver nitrate, were mixed and thoroughly equilibrated in a separation funnel over night. The two phases were separated before use and degassed for about 5 min in an ultrasonic bath.

The HSCCC separation was performed in normal-phase mode (tail-to-head-mode) according to Schröder and Vetter [9]. The lower phase of the biphasic solvent system was used as stationary phase. The rotation speed of the centrifuge was set to 1010 ± 10 rpm and the flow of the mobile phase was adjusted to 1.0 mL/min. The actual flow according to the fraction volume was ~0.9 mL/min. Extrusion of the stationary phase was in every case 40–45 mL.

2.3. Sample preparation

About 100 g plant oil (100.4 g olive oil, 101.2 g rapeseed oil, or 102.5 g linseed oil) was weighed in a 1 L flask and 450 mL ethanolic KOH (prepared from 50% aqueous KOH and ethanol,

1:9, v/v) were added for saponification. The solution was heated to 80 °C and stirred for 3 h. Then, the solution was cooled and 200 mL *n*-hexane and about 300 mL water was added. The upper phase which contained the unsaponifiable matter was separated, and the procedure was repeated twice with 100 mL *n*-hexane. The hexane extracts were combined and evaporated until dryness. The output weight of extracted unsaponifiable was 0.214 g for olive oil, 0.260 g for rapeseed oil and 0.135 g for linseed oil.

2.4. Hydrogenation

Pre-weighed crude β-sitosterol standard (1 g which contained 56% β-sitosterol (**7**), 3% stigmasterol (**6**), and 34% campesterol (**5**) [9]), 20 mg Pd on C (10%), 10 mL 2-propanol and 0.1 mL pure water were given into a 50 mL two-neck round bottom flask. One neck opening was sealed with a balloon filled with ~300 mL hydrogen. The flask was flushed with about 100 mL hydrogen and the solution was heated at 70 °C under stirring for about one day [14]. The catalyst was removed by membrane filtration and the solvents were evaporated.

2.5. HSCCC separation procedures

After equilibration of the system, between 100 and 130 mg of sample (106 mg unsaponifiables of rapeseed oil, 109 mg unsaponifiables of olive oil, 130 mg unsaponifiables of linseed oil, and 130 mg of the hydrogenated β-sitosterol standard) was dissolved in 4.6 mL *n*-hexane, 3.2 mL methanol and 130 μL water, and was injected by means of a 10 mL sample loop. Due to previous experience we expected lower sample weights in the beginning of the HSCCC runs, bulk fractions in the middle and low fraction weights at the end of the procedure [9]. Based on the complexity of the plant oils, slightly different fractionation schemes were carried out for the three oil samples (HSCCC-1: rapeseed oil, HSCCC-2: olive oil; HSCCC-3: linseed oil) (Fig. 2; see also supporting information). Note that the fraction lengths were not optimized since a detector was not available for monitoring the eluting peaks. All HSCCC fractions (hereafter abbreviated "Fr.") were evaporated to dryness by means of a gentle stream of nitrogen, accurately weighed, and re-dissolved in 1 mL *n*-hexane. From these stock solutions, aliquots corresponding with about 0.07 mg sample were transferred into 1.5 mL vials for silylation. The solvent was removed by a gentle stream of nitrogen. Into each vial 100 μL BSTFA:TMCS (99:1) solution was added, and the closed vial was heated to 70 °C for 20 min [15]. After cooling, 400 μL *n*-hexane was added and the trimethylsilylated sample was subjected to GC/MS analysis in full-scan and SIM mode (see Section 2.6).

Selected fractions of HSCCC-1, HSCCC-2 and/or HSCCC-3 (see Fig. 2) were combined dissolved in 2.3 mL *n*-hexane, 1.6 mL methanol and 65 μL water and re-fractionated using the same solvent system (HSCCC 4–6). The parameters were the same as shown above. HSCCC-4 was carried out to purify 24-methylene-cycloartanol (**11**) and Δ5-avenasterol (**9**), HSCCC-5 delivered pure 24-methylene-cycloartanol (**11**), cycloartenol (**10**), and campesterol (**4**), and HSCCC-6 was carried out to obtain pure 24-methylene-cycloartanol (**11**), cycloartenol (**10**), and brassicasterol (**3**) (Fig. 2).

Fractionation of the hydrogenated crude β-sitosterol standard (HSCCC-7) was carried out twice (Fig. 3) in order to receive higher amounts. Purification of sitostanol (**8**) and campestanol (**5**) was based on the combination of suitable fractions from both fractionations (see Fig. 3).
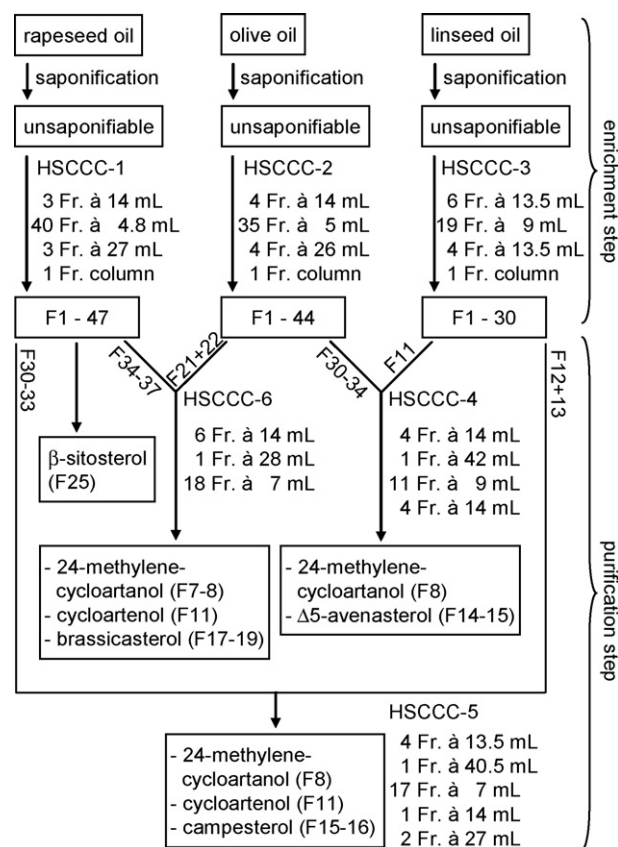

Fig. 2. HSCCC fractionation scheme (enrichment step and purification step) of rapeseed, olive, and linseed oil.
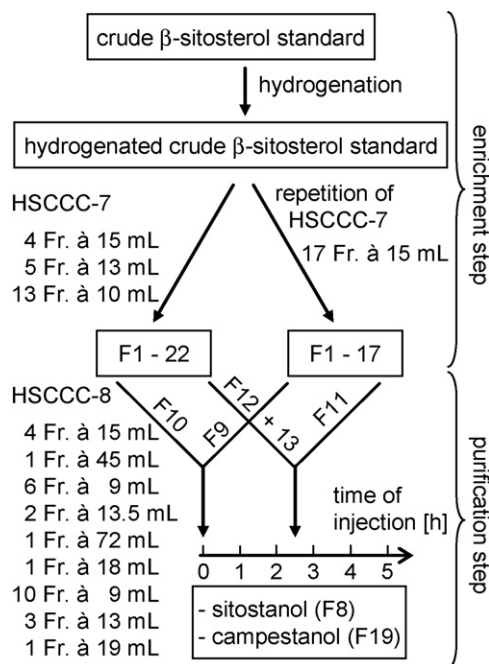

Fig. 3. HSCCC fractionation scheme of the enrichment step (HSCCC-7) and purification step (HSCCC-8) of a hydrogenated β-sitosterol standard. Suitable fractions in the purification step were injected twice during the same run after ~2.5 h as shown in the bottom.

2.6. Gas chromatography coupled to electron ionisation mass spectrometry (GC/EI-MS)

A 5890 series II plus/5972 GC/MS system (Hewlett-Packard/Agilent, Waldbronn, Germany) was used for analysis of trimethylsilylated phytosterols. Separations were performed with a 30 m × 0.25 mm i.d. fused-silica capillary column coated with 0.25 μm DB-5MS (Hewlett-Packard/Agilent, Waldbronn, Germany) [9]. The GC oven programming started at 55 °C (1 min hold time), then the temperature was increased at 20 °C/min to 255 °C, at 1.5 °C/min to 283 °C and finally at 15 °C/min to 300 °C (hold time 5 min) [9]. GC/EI-MS full scan chromatograms (m/z 50–550) were recorded from 7.0 to 35.8 min for peak identification. In selected ion monitoring (SIM) mode we recorded m/z 75, m/z 129, m/z 213 and m/z 215 from 7.0 (end of solvent delay) to 35.8 min (end of the run). In addition, we recorded from 7.0–10.85 min: m/z 143 and m/z 353 (base peak and [M−15]$^+$ of the trimethylsilyl ether of phytol), from 10.85–15.3 min: m/z 69 and m/z 81 (two fragment ions characteristic of squalene and geranyl geraniol-trimethylsilyl ether), from 15.3 to 16.2 min: m/z 217 and m/z 372 (fragment and molecular ion of 5-α-cholestane), from 16.2 to 20.0 min: m/z 458 and m/z 460 (molecular ion of the trimethylsilyl ether of cholesterol and cholestanol), from 20.0 to 20.7 min: m/z 380 and m/z 470 (fragment and molecular ion of the trimethylsilyl ether of brassicasterol), from 20.7 to 21.3 min: m/z 363 and m/z 468 (fragment and molecular ion of the trimethylsilyl ether of ergosterol), from 21.3 to 21.8 min: m/z 472 and m/z 474 (molecular ion of the trimethylsilyl ether of campesterol and campestanol), from 21.8 to 22.6 min: m/z 394 and m/z 484 (fragment and molecular ion of the trimethylsilyl ether of stigmasterol), from 22.6 to 23.9 min: m/z 486 and m/z 488 (molecular ion of the trimethylsilyl ether of β-sitosterol and sitostanol), from 23.9 to 25.8 min: m/z 393 and m/z 408 (fragment ions of the trimethylsilyl ether of cycloartenol), from 25.8 to 27.5 min: m/z 216 and m/z 357 (fragment ions of the trimethylsilyl ether of erythrodiol/uvaol and citrostadienol) and, finally, from 27.5 to 35.8 min: m/z 203 and m/z 232 (fragment ions of the trimethylsilyl ether of oleanyl aldehyde).

3. Results and discussions

3.1. HSCCC fractionation of the unsaponifiable matter of plant oils (enrichment step)

3.1.1. HSCCC-1: fractionation of unsaponifiable matter of refined rapeseed oil (106 mg)

3.1.1.1. Non-phytosterols.
First compounds were already detected with the breakthrough of the solvent front of the mobile phase (i.e. Fr. 3). Weighing of the evaporated fractions indicated output weights between 0.1 and 32.7 mg. Fr. 3 (32.7 mg) showed an intense yellow colour which originated from β-carotene. However, the main constituent (∼50%) of this fraction was nonacosane (**VII**, $C_{29}H_{60}$, Fig. 4) which was identified by virtue of a full match of its GC/EI-MS spectrum with a reference spectrum including M$^+$ at m/z 408. **VII**, a known major constituent of the wax fraction of rapeseed oil [16,17], could also be detected in the unfractionated sample (Fig. 5a). Fr. 3 also contained traces of squalene (**VI**, Fig. 4, ∼2%) as well as two steradienes and one steratriene which were most likely formed by dehydration of sterols in the refining process [18]. The full-scan GC/EI-MS spectra indicated the presence of campesta-3,5,22-triene (**XI**, M$^+$ 380, ∼4%, Fig. 4), campesta-3,5-diene (**XII**, M$^+$ 382, ∼12%, Fig. 4) and stigmasta-3,5-diene (**XIII**, M$^+$ 396, ∼12%, Fig. 4) which are artifacts produced from brassicasterol, campesterol and β-sitosterol. All three peaks were also detected in the unfractionated sample but their identity could not be elucidated without HSCCC fractionation (Fig. 5a). Fr. 3 also contained
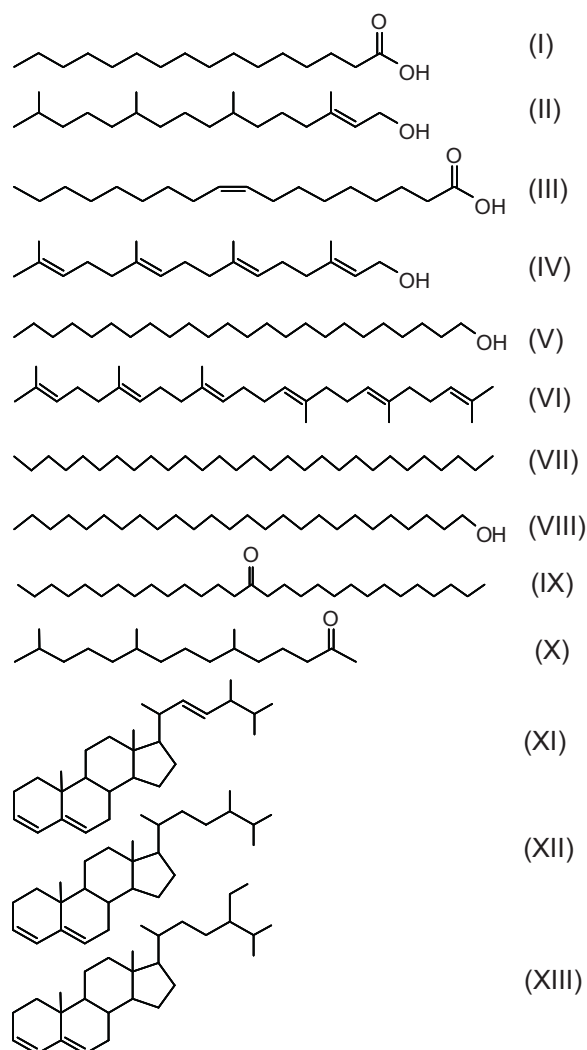

Fig. 4. Chemical structures of palmitic acid (**I**), phytol (**II**), oleic acid (**III**), geranyl geraniol (**IV**), tetracosane alcohol (**V**), squalene (**VI**), nonacosane (**VII**), hexacosane alcohol (**VIII**), 15-nonacosanone (**IX**), 6,10,14-trimethylpentadecan-2-one (**X**), campesta-3,5,22-triene (**XI**), campesta-3,5-diene (**XII**), stigmasta-3,5-diene (**XIII**).

15-nonacosanone (**IX**, $C_{29}H_{58}O$, ∼15%, Fig. 4) which was identified by a full match of the literature mass spectrum and our mass spectra in form of M$^+$ at m/z 422 as well as further characteristic fragment ions at m/z 225, 240 and 241 [19]. The symmetric ketone **IX**, which was also detected in the unfractionated sample (Fig. 5a), is a known wax compound of Brassica oleracea [19]. Rape (Brassica napus) also belongs to the family of cruciferous vegetables (Brassicaceae), and it was no surprise that it also contained **IX**. A further ketone eluted into Fr. 16–19 (99.6–118.8 mL, output weight: ∼2 mg). The GC/EI-MS spectrum indicated the presence of 6,10,14-trimethylpentadecan-2-one (**X**, Fig. 4) due to the M$^+$ m/z 268 and the characteristic fragment ions at m/z 58, 71, 124, 194, 210 and 250 [20]. Injection of an aliquot of the unsilylated fraction resulted in the same retention time and mass spectrum of **X**. However, the purity of **X** was never higher than ∼65%. Due to the low amounts, **X** was not detected without HSCCC fractionation. The output weights of Fr. 4–19 ranged from 0.4 to 1.4 mg. None of these fractions contained phytosterols. Key-compound was the fatty alcohol 30:0 ($C_{30}H_{61}OH$) in Fr. 5 (1.0 mg; purity ∼10%). It was followed by the fatty alcohols 29:0 (two isomers, about equal amounts), 28:0, 27:0 (two isomers, first eluting isomer dominated), 26:0 ($C_{26}H_{53}OH$, **VIII**, Fig. 4, two isomers, traces
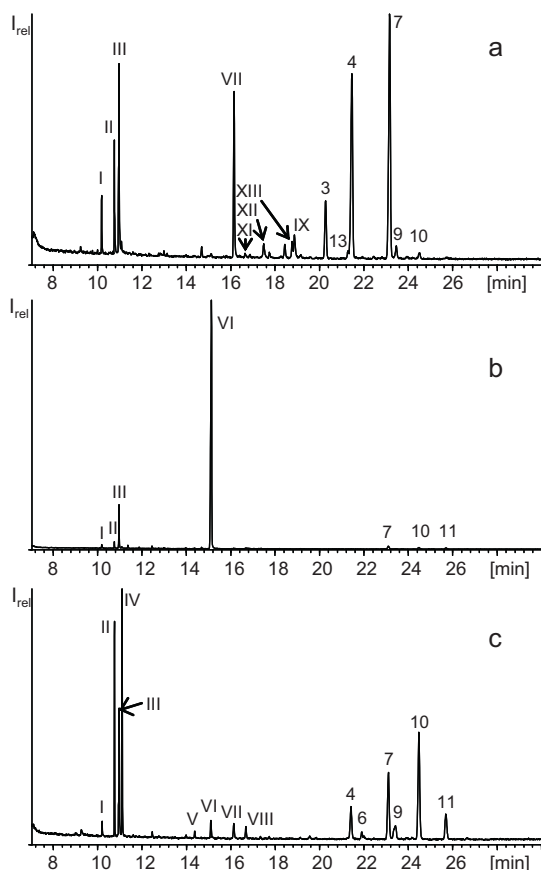
Fig. 5. GC/EI-MS full scan chromatogram of trimethylsilylated of the unsaponifiable of (a) rapeseed oil, (b) olive oil and (c) linseed oil. palmitic acid (**I**), phytol (**II**), oleic acid (**III**), geranyl geraniol (**IV**), tetracosane alcohol (**V**), squalene (**VI**), nonacosane (**VII**), hexacosane alcohol (**VIII**), 15-nonacosanone (**IX**), campesta-3,5,22-triene (**XI**), campesta-3,5-diene (**XII**), stigmasta-3,5-diene (**XIII**), brassicasterol (**3**), unknown phytosterol (**13**), campesterol (**4**), stigmasterol (**6**), β-sitosterol (**7**), Δ5-avenasterol (**9**), cycloartenol (**10**), 24-methylene-cycloartanol (**11**).

of first eluting isomers), 24:0 (**V**, Fig. 4) and 22:0, up to Fr. 23 (all alcohols were identified by means of the mass spectra of the corresponding trimethylsilyl ethers). In general, the last eluting isomer was identified as the straight chain (*n*-alcohol) isomer. The earlier eluting isomer has the hydroxyl-group in the chain (secondary alcohol). 15-Nonacosanol is a well known component of plant wax [21] and its presence, together with nonacosane and 15-nonacosanone (see above) was plausible. In order to identify similarities in the structures of the secondary alcohols we plotted the logarithmic retention time against the number of carbons of the alcohols. This technique has been repeatedly used in the field of fatty acids in order to identify isomers [22,23]. By this measure, structurally related compounds are found on straight lines. In this graph the later eluting isomers (primary alcohols) were located on one line ($R^2 = 0.9997$), as were the first eluting secondary alcohols ($R^2 = 0.9999$, Fig. 6). Both lines were parallel and this indicated that the hydroxyl substituent of the other secondary alcohols were also located in the middle part of the chain.

The elution volume increased with decreasing chain length of the fatty alcohols. Accordingly, further, short chained, fatty alcohols (octadecanol, heptadecanol and hexadecanol) eluted into Fr. 40–46 while portions of hexadecanol as well as pentadecanol, and two isomers of tetradecanol remained non-eluted on the column and were gained afterwards in Fr. 47. None of the fatty alcohols was detected in the sample before the HSCCC fractionation (Fig. 5a). Two compounds (most likely aldehydes) detected in Fr. 5–6 which eluted between **XII** and **3** from the GC column (Fig. 5a) could not be
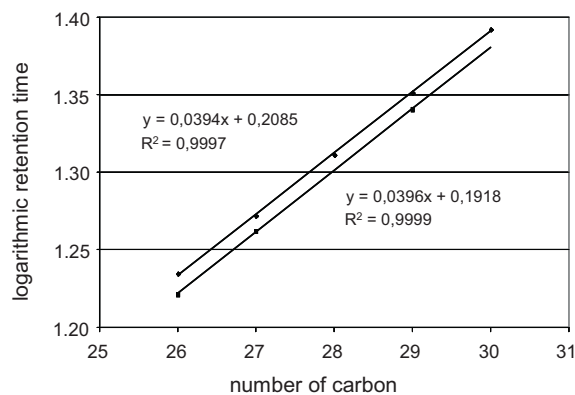

Fig. 6. Logarithmic retention time against the number of carbons from detected fatty alcohols of the unsaponifiable matter of rapeseed oil with equation of the resulting straight line and coefficient of determination (R^2).

identified by means of their mass spectra. Fr. 43–46 (229–324 mL) provided ~2 mg phytol (**II**, the side chain of chlorophyll, Fig. 4). **II** was already visible in the GC/MS chromatogram of the unfractionated sample (Fig. 5a). Fr. 44–45 even showed the presence of two phytol isomers. The more abundant *trans*-isomer (Fr. 44: 92% and 95% in Fr. 45) was accompanied with the smaller peak of *cis*-phytol (8% and 5%, respectively), which was likely formed during the refining process of the crude rapeseed oil. Unfortunately, *cis*- and *trans*-phytol could not be separated with the used HSCCC system.

3.1.1.2. Phytosterols. The first phytosterol (24-methylene-cycloartanol, **11**, Fig. 1) was collected in Fr. 20 (i.e. after 119 mL). **11** was not detected in the unfractionated sample because of its low abundance (Fig. 5a). Cycloartenol (**10**, Fig. 1) first appeared slightly later in Fr. 21 (124 mL) while β-sitosterol (**7**) was first detected in Fr. 22 (129 mL) and up to Fr. 30. Noteworthy, Fr. 25 (143–148 mL) provided neat **7** (6.4 mg, purity > 99%; Fig. 7d). Campesterol (**4**) eluted from Fr. 26–35 (148–196 mL), while stigmasterol (**6**) was not detected in any of these fractions. This is remarkable because **4** and **6** cannot be separated with this HSCCC solvent system [9]. Since **6** is commonly found in many plant oils, rapeseed proved to be suited for the isolation of **4** (see Section 3.2). Fr. 29–36 (162–200 mL) additionally contained comparably small amounts of Δ5-avenasterol (**9**). The highest relative abundance (~15%) of **9** was found in Fr. 33. Fr. 30–41 (167–224 mL) delivered brassicasterol (**3**), which is another major sterol of rapeseed oil (Fig. 5a) [24,25]. The purity of **3** in Fr. 34–38 ranged from >50% to 85%. Fr. 36–43 (196–234 mL) contained about 1 mg of an unknown phytosterol (**13**), but the purity was never higher than 80% (Fig. 7j). These fractions contained another unknown compound whose mass spectrum clarified that it was an isomer of **13** which eluted about one minute later from the GC column (Fig. 7j). The purity of the isomer was never higher than 10%. With the beginning of Fr. 31 (172 mL) several other minor compounds started to elute from the HSCCC system. These compounds shared the GC-retention time range with phytosterols and we expected that these are phytosterols as well. However, their identity could not be determined with mass spectrometry due to their low amounts in the fractions. A summary of the elution profile is shown in the supplementary materials (Table S1).

3.1.2. HSCCC-2: fractionation of unsaponifiable matter of native olive oil extra (109 mg)

3.1.2.1. Non-phytosterols. In agreement with rapeseed oil, the first compounds in the sample from olive oil were detected in Fr. 3 (after 28 mL). The main compound of Fr. 3 was squalene (**VI**, Fig. 4) which
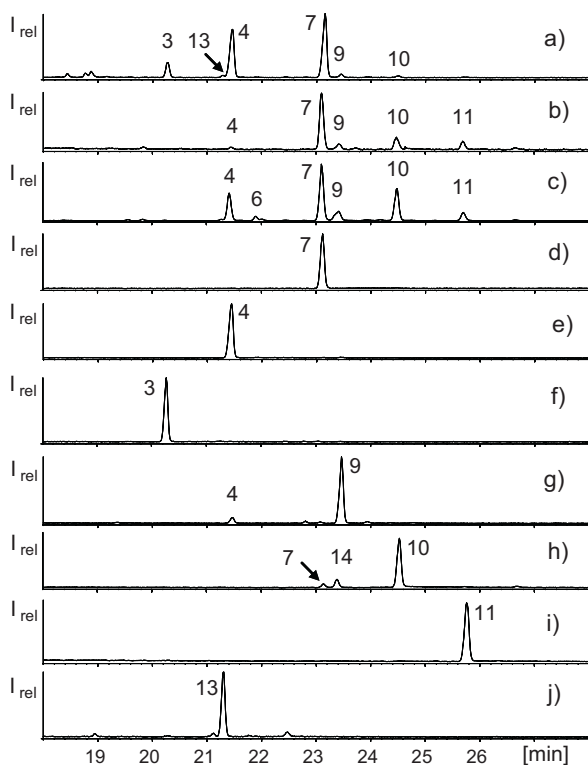
Fig. 7. GC/EI-MS full scan chromatogram of the trimethylsilylated unsaponifiable matter of (a) rapeseed oil, (b) olive oil, (c) linseed oil, as well as purified (d) β-sitosterol (≥99%), (e) campesterol (≥99%), (f) brassicasterol (≥99%), (g) Δ5-avenasterol (≥90%), (h) cycloartenol (≥90%), (i) 24-methylene-cycloartanol (≥99%), and (j) unknown phytosterol (∼80%) extracted from rapeseed oil. The peak numbers present one phytosterol. Peak number 14 is likely an isomer of cycloartenol.

amounted to about 75% (∼83 mg) of total sample (Fig. 5b). The high abundance of **VI** in cold-pressed native olive oils is in accordance with literature reports of 0.08–1.2 g squalene per 100 g olive oil [26,27] or 60–70% in unsaponifiable fraction of olive oil respectively [28]. However, high amounts of **VI** in the unsaponifiable matter of olive oil hamper the determination of phytosterols. We found that the method of Jenske and Vetter, initially developed for the separation of conventional (i.e. non-hydroxylated) fatty acid methyl esters from hydroxyl-fatty acid methyl esters [29], was also suited for the separation of phytosterols from squalene and other hydrocarbons (tested with ∼1 mg unsaponifiable of olive oil). This method can be used to simplify the analysis of phytosterols when high amount of squalene are present. Traces of **VII** were also detectable in these fraction (<0.5%). Fr. 4 contained only traces of **VI** but many further hydrocarbons (due to the similar fragmentation pattern in the mass spectra). The ketone 6,10,14-trimethylpentadecan-2-one (**X**, Fig. 4) was also detected in Fr. 14–17 (101–121 mL, ∼1.5 mg, purity < 50%). Fatty alcohols (22:0–30:0) were found in Fr. 5–22 (56–146 mL), whereas traces of octadecanol (Fr. 40, 231–257 mL) and hexadecanol (Fr. 43, 309–335 mL) occurred in HSCCC fractions after those containing phytosterols. By comparison of *HSCCC-1* and retention times (see above) it can be concluded, that only primary fatty alcohols were present in olive oil. A total of ∼1 mg **II** was detected in Fr. 40–42 (231–309 mL). This amount was comparable with the content of **II** in the unsaponifiable matter of rapeseed oil (see *HSCCC-1*).

3.1.2.2. Phytosterols.
The first eluted phytosterol was 24-methylene-cycloartanol (**11**) (Fr. 17–22; 116–146 mL; purity up to ∼65%) followed by cycloartenol (**10**) (Fr. 19–24; 126–156 mL; purity up to ∼60%) and β-sitosterol (**7**) (Fr. 22–30; 141–186 mL; purity up to ∼90%). It was followed by campesterol (**4**, Fr. 26–32

(161–196 mL), stigmasterol (**6**, Fr. 27–30) and Δ5-avenasterol (**9**, Fr. 27–35, 166–211 mL). While stigmasterol (**6**) could not be separated from campesterol (**4**) (see above), Δ5-avenasterol (**9**) was present at higher amounts and thus was distributed over more HSCCC fractions. Accordingly, the late Fr. 30–34 contained **9** in purities of 50–75%. Noteworthy, Fr. 21–27 (136–171 mL) provided citrostadienol (**12**, Fig. 1, ∼0.5 mg) while GC/MS screening of Fr. 26–29 (161–181 mL) enabled the detection of cycloeucalenol (∼0.1 mg). Retention times and mass spectra were in accordance with data provided by Li et al. [30]. The highest purity of citrostadienol (**12**) and cycloeucalenol was measured in Fr. 23 (∼14%) and Fr. 28 (∼7%) respectively. In the non-eluted fraction (column) traces of erythrodiol was detectable. The full-scan mass spectra and retention time was in accordance with provided data by Li et al. [30]. Due the very small amounts, the three phytosterols were not detected in the GC/MS measurements before the HSCCC-fractionation (see Table S2).

3.1.3. HSCCC-3: fractionation of unsaponifiable matter of cold-pressed linseed oil (130 mg)
3.1.3.1. Non-phytosterols.
The elution scheme resembled that order from HSCCC was observed of unsaponifiable matter of the other oils. Fr. 3 (27–40.5 mL) contained ∼40% squalene (**VI**, Fig. 4) (see *HSCCC-2*) and ∼35% nonacosane (**VII**, Fig. 4) (see *HSCCC-1*) along with β-carotene which added the yellow colour to this fraction. Small peaks of **VI** and **VII** were also detected in the unfractionated sample (Fig. 5c). Fr. 4–11 (40.5–117 mL) delivered fatty alcohols (22:0–30:0) while Fr. 9–10 (90–108 mL) provided 6,10,14-trimethylpentadecan-2-one (**X**, Fig. 4, ∼3 mg, purity up to ∼65%) (see *HSCCC-1*) and Fr. 10–11 (99–117 mL) provided α-tocopherol (identified by the molecular ion *m/z* 502 of α-tocopherol-trimethylsilyl ether and the characteristic fragment ions *m/z* 237, *m/z* 277 and the base peak *m/z* 73 (trimethyl-silylium cation) [31]). Noteworthy, Fr. 16–18 (same elution volume as campesterol) contained traces of docosanol (20:0).

3.1.3.2. Phytosterols.
Fr. 10–13 (99–135 mL) contained up to ∼70% 24-methylene-cycloartanol (**11**) and Fr. 11–15 (108–153 mL) up to ∼75% cycloartenol (**10**). Fr. 12–13 (117–135 mL) contained a compound (purity ∼5%) which shared the GC-retention time with sitostanol (**8**, Fig. 1) and Δ5-avenasterol (**9**). According to the mass spectrum (Fig. 8) it was an isomer of cycloartenol-trimethylsilylether (M+, *m/z* 498). Without HSCCC fractionation these co-elutions would have been overlooked. However, Δ5-avenasterol (**9**) eluted into a different HSCCC fraction than sitostanol (**8**) and the isomer of cycloartenol. The three co-eluting trimethylsilyl ethers of sitostanol (M+, *m/z* 488), the isomer of cycloartenol (M+, *m/z* 498) and Δ5-avenasterol (M+, *m/z* 484) can also be distinguished by their mass spectra (Fig. 8). Further differences in the high-mass range feature the characteristic [M−15]+ ions (elimination of one methyl moiety from the derivatization group) as well as *m/z* 383, *m/z* 398 and *m/z* 431 which were solely detected for sitostanol-trimethylsilyl ether (Fig. 8a). Likewise, the trimethylsilyl ethers of **9** and the isomer of **10** showed characteristic fragment ion at *m/z* 393 as well as *m/z* 296 and *m/z* 386 respectively (Fig. 8b and c). Note however, that these compounds cannot be identified and distinguished when the classic GC/FID method is used for phytosterol determination.

Further phytosterols eluted with the same profile as described above from the HSCCC system: **7** was found in Fr. 13–17 (126–171 mL; purity up to ∼80%), **4** in Fr. 14–19 (135–189 mL; purity up to ∼50%), **6** in Fr. 15–18 (144–180 mL, purity up to ∼12%), **9** in Fr. 15–20 (144–198 mL; purity up to ∼50%), and **3** in Fr. 18–21 (171–207 mL, purity up to ∼10%). In addition, the unknown phytosterol **13** detected in the unsaponifiable matter of rapeseed oil was detected in Fr. 19–24 (180–239 mL, purity up to 60%). After elution
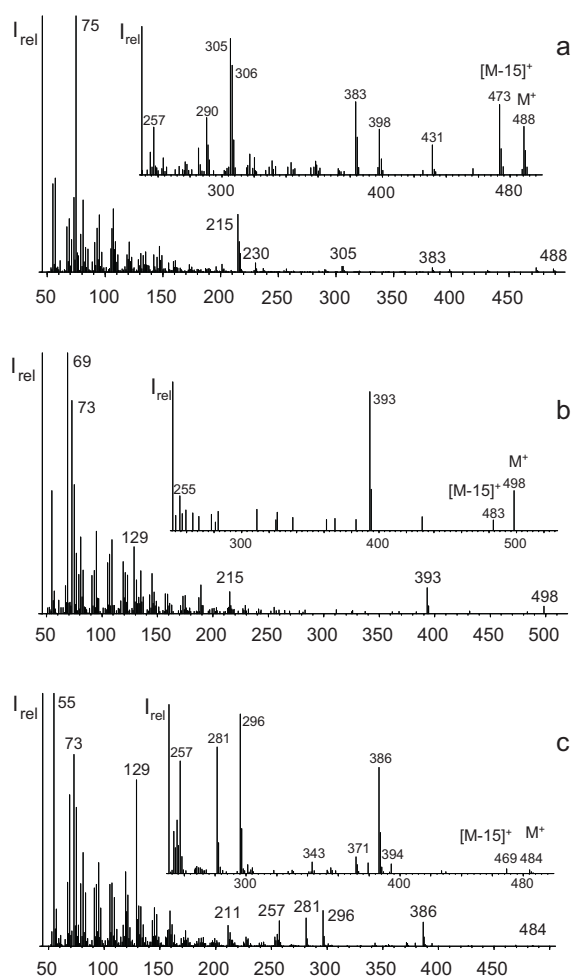
Fig. 8. GC/EI-MS spectra of three coeluting phytosterols as trimethylsilylether of (a) sitostanol, (b) isomer of cycloartenol and (c) Δ5-avenasterol.

of phytosterols it followed octadecanol (Fr. 22–23; 207–225 mL) while 3.5 mg phytol (**II**, 100% purity) was collected in Fr. 26–28 (252–293 mL). Some components (~8.3 mg) were not eluted from the HSCCC-system. This fraction mainly contained geranyl geraniol (**IV**, ~65%), oleic acid (**III**, ~30%), and palmitic acid (**I**, ~3%) (Fig. 4). The three compounds could be detected in the non-fractionated sample as well (Fig. 5c). Small portions of the fatty acids were probably not separated from the unsaponifiable during the initial liquid–liquid fractionating due to their high abundance (see Section 2.3). Geranyl geraniol (**IV**) is a known compound of the unsaponifiable matter of linseed oil [32] and structurally related to phytol (**II**). **IV** eluted later than **II** from the HSCCC system due the presence of three additional double bonds (Fig. 4) (see Table S3).

3.2. Mixing of different HSCCC fractions for the generation of pure phytosterols (purification step)

Despite the wide elution distribution range, phytosterols were not obtained in pure form after the enrichment step (HSCCC 1–3, Fig. 2). Yet, it provided opportunities for an efficient purification step by means of the selection and mixing of suited fractions from different oils in order to isolate two phytosterols in one additional run (Fig. 2). For instance 24-methylene-cycloartanol (**11**) eluted early from the HSCCC system while Δ5-avenasterol (**9**) eluted late (see above). Thus, suitable fractions could be combined and both phytosterols could be purified in one additional HSCCC run (Fig. 2).

3.2.1. HSCCC-4: purification of 24-methylene-cycloartanol (**11**) and Δ5-avenasterol (**9**)

Fr. 11 of HSCCC-3 (linseed oil) and Fr. 30–34 of HSCCC-2 (olive oil) were combined (Fig. 2). The early fraction from linseed oil and the late fractions from olive oil showed no overlap. HSCCC-4 was carried out with 9 mg sample including ~4 mg 24-methylene-cycloartanol (**11**) and ~1 mg Δ5-avenasterol (**9**). Fr. 8 (116–125 mL) provided ~2.8 mg **11** in a purity of >99% (Fig. 7i). Accordingly, ~70% of the injected **11** could be isolated. Likewise, ~0.3 mg **9** was obtained in Fr. 14–15 (170–188 mL) with a purity of ~90% (the main by-product was ~5% campesterol (**4**), Fig. 7g). About 30% of the injected **9** could be isolated.

3.2.2. HSCCC-5: purification of 24-methylene-cycloartanol (**11**), cycloartenol (**10**) and campesterol (**4**)

Early fractions from the unsaponifiable matter of linseed oil (HSCCC-3, Fr. 12–13) and late fractions from rapeseed oil (HSCCC-1, Fr. 30–33) were combined (Fig. 2). This sample weighed 46 mg and contained ~3 mg 24-methylene-cycloartanol (**11**), ~20 mg cycloartenol (**10**), and ~12 mg campesterol (**4**). Fr. 8 (108.5–115.5 mL) delivered ~0.7 mg pure **11** (purity ~97%; ~25% of the injected amount), while Fr. 11 (129.5–136.5 mL) contained ~9.1 mg **10** (purity ~90%; ~50% of the injected amount, Fig. 7h) with ~6% isomer of cycloartenol (**14**) and low amounts (~2%) of β-sitosterol (**7**) and Fr. 15–16 (157.5–171.5 mL) provided ~6.9 mg pure **4** (>99%; ~55% of the injected amount, Fig. 7e).

3.2.3. HSCCC-6: purification of cycloartenol (**10**), 24-methylene-cycloartanol (**11**) and brassicasterol (**3**)

Medium Fr. 21–22 of olive oil (HSCCC-2) and the late Fr. 34–37 of rapeseed oil (HSCCC-1) were combined (Fig. 2) to give about 8 mg sample. The sample contained ~1 mg cycloartenol (**10**), ~0.5 mg 24-methylene-cycloartanol (**11**), and ~4 mg brassicasterol (**3**). Fr. 7–8 (84–119 mL) contained ~0.2 mg pure **11** (>99%), Fr. 11 (133–140 mL) gave ~0.4 mg **10** (purity ~85%, together with ~5% isomer of cycloartenol (**14**)), while Fr. 17–19 (175–196 mL) provided ~2.9 mg pure **3** (>99%, Fig. 7f) which corresponded with ~70% of the injected amount.

3.3. Hydrogenation and HSCCC fractionation of crude β-sitosterol standard and purification of sitostanol (**8**) and campestanol (**5**)

3.3.1. HSCCC-7

Hydrogenation of about 1 g β-sitosterol standard (see Section 2.4; Fig. 9a [9] produced sitostanol (**8**) from both **6** and **7** as well as campestanol (**5**) from **4**. Traces of cholestanol (**2**) which was produced from cholesterol (**1**) were also detected. After the hydrogenation the native phytosterols **1**, **4**, **6**, and **7** were not detectable in the unfractionated sample (Fig. 9b). In addition to these anticipated products, each phytostanol was accompanied with two small additional peaks in the GC/MS chromatograms (Fig. 9b). Such small by-products of the hydrogenation had been described before as isomers [14]. According to Wärna et al., the hydrogenation process may also lead to the by-products sitostane, sitostanone, campestane and campestanone [33].

The first eluted compounds (Fr. 5, 60–73 mL, 6.9 mg) contained one main (71%) and three minor compounds (2.5–14.5%). Two compounds shared the same molecular ion at *m/z* 400 and *m/z* 414, respectively. The mass difference corresponded with that between **4** and **7**. The phytosterols **4** and **7** differ in the substituent at C-24 (i.e. methyl on **4** and ethyl on **7**). Accordingly, the two compounds eluting first from the GC system were hydrogenation products of **4** (M⁺ *m/z* 400) while the latter two were formed from **6** (M⁺ *m/z* 412) and **7** (M⁺ *m/z* 414). The same mass (compared to the phytosterol) indicated that the simultaneous hydrogenation of the double bond (+2H) and oxidation of the hydroxyl group to ketone (−2H) resulted
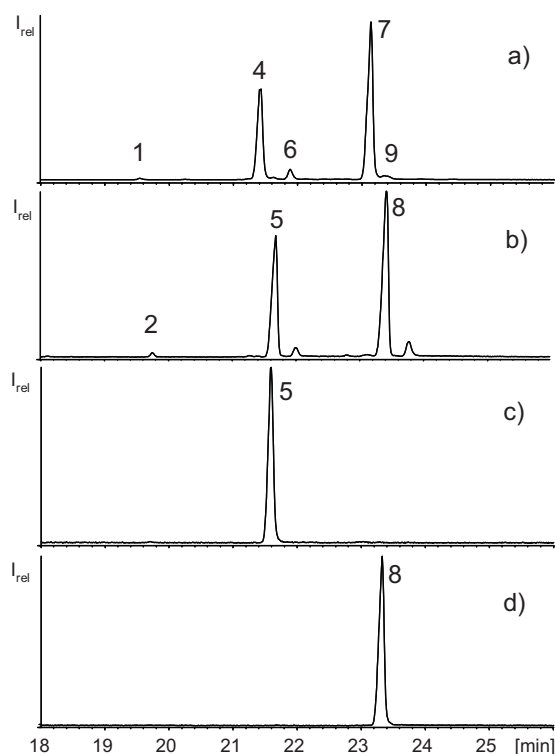
Fig. 9. GC/EI-MS full scan chromatogram of the trimethylsilylated (a) crude β-sitosterol standard, (b) hydrogenated β-sitosterol standard and purified (c) campestanol (**5**) and (d) sitostanol (**8**).

Fig. 10. Recovery, output weight and purity in parenthesis of isolated phytosterols before (black bar), after first HSCCC-fractionation (grey bar) and second HSCCC-fractionation (light grey bar) of the unsaponifiable of (a) rapeseed oil, (b) olive oil and (c) linseed oil. Recovery before HSCCC was set to 100%.

in the same molecular weight. Verification was obtained from the measurement of an aliquot without silylation. The GC-retention time and mass spectra in the differently treated fractions showed no difference and thus confirmed the absence of a hydroxyl group in the products. Fr. 6 (73–86 mL, 6.3 mg) provided the corresponding two products produced from campesterol (**4**) (M⁺ *m/z* 400, 61%) while the compound isobaric with **7** dropped to 29%. Further minor compounds contributed with 1–6% to Fr. 6. The compounds in Fr. 6 were also detected in Fr. 5 except for one minor peak. The first peak in the GC/MS chromatogram was isobaric with **1** (M⁺ *m/z* 386, 1%). Fr. 7 (86–99 mL, 0.4 mg) contained the same compounds as Fr. 6 but with different relative abundances. In Fr. 8 (99–112 mL, 0.3 mg) eluted equal amounts of two further ketones which shared the molecular ion of *m/z* 398 but none of the compounds was found in the previous fractions. Fr. 9 (112–125 mL, 5.8 mg) contained 91% sitostanol (**8**) and ∼0.5% campestanol (**5**). In addition, we observed two further compounds with molecular ions at *m/z* 398 (8%) and *m/z* 384 (∼1%). **8** was detected until Fr. 14 (167.5 mL, 1.0 mg) and **5** until Fr. 17 (201.5 mL, 0.1 mg). The highest purity of **8** (∼96%) was determined in Fr. 10 (125–133.5 mL, 37.9 mg) while **5** was most relevant (purity 92%) in Fr. 13 (150.5–159 mL, 13.3 mg). Traces of **7** and **4** (∼0.2% and ∼0.3% of the original amount) were detected in Fr. 14 (up to 2% of **7**) and Fr. 16 (up to 37% of **4**) after HSCCC fractionation. Cholestanol (**2**) eluted from Fr. 13 (105.5 mL, 13.3 mg, purity: ∼2%) to Fr. 17 (201.5 mL, 0.1 mg, purity: ∼19%). The highest purity of **2** (39%) was achieved in Fr. 16 (176–184.5 mL). Fr. 16–17 also contained traces of **1** (0.2 and 0.1 mg) with a purity of 3.5 and 19%, respectively (see Table S4).

This HSCCC fractionation was repeated (Fig. 3) and the same compounds were detected and the elution order and volume was comparable with the first injection. For instance, the highest purity of sitostanol (**8**) was achieved in Fr. 9 (120–135 mL, 55.7 mg, 95%) and campestanol (**5**) in Fr. 11 (150–165 mL, 19.8 mg, 92%).
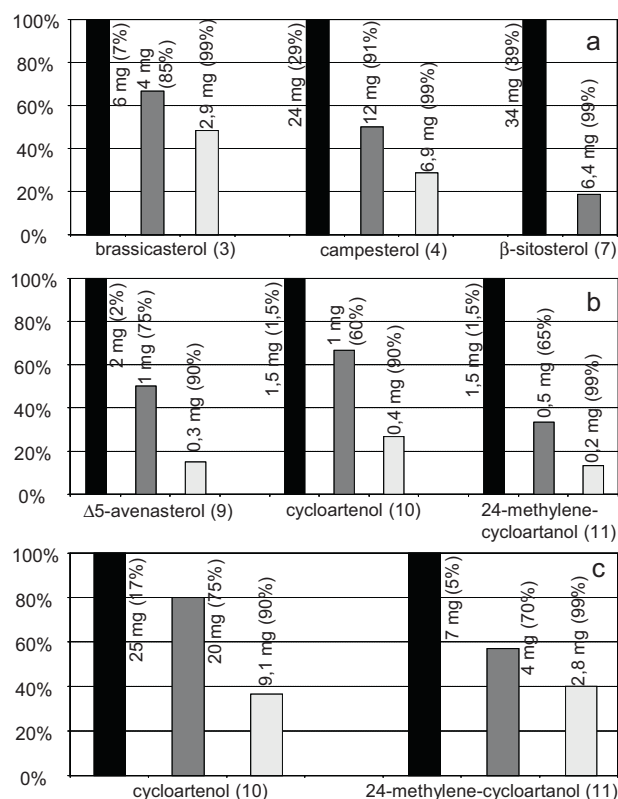
3.3.2. HSCCC-8

Purification of sitostanol (**8**) and campestanol (**5**) was carried out by combining suitable fractions from the two enrichment steps (Fig. 3). For the purification of **8** one fraction of each injection (purity: 95%) and for **5** two fractions of first injection and one fraction of repetition were combined and used (Fig. 3). The purity of campestanol was in every fraction ∼80% or higher. Both phytostanols showed an overlapping HSCCC elution range. However, enriched fractions of both compounds (without the present of the second one) could be selected during the enrichment step (Fig. 3). Accordingly, the fractions enriched with sitostanol were first injected into the HSCCC-system, and after two and half hours, the enriched campestanol fraction was injected into the same run. By this measure both phytostanols could be purified in one HSCCC run (Fig. 3). Fr. 8 (123–132 mL, 37.5 mg, Fig. 9d) contained pure **8** and Fr. 9 (132–141 mL, 34.6 mg) provided a purity of 98%. Pure **5** was obtained in Fr. 19 (303–316 mL, 15.8 mg, Fig. 9c) while its purity in Fr. 18 (294–303 mL, 21.9 mg) was 96%. The impurity in the latter fraction (4%) originated from **8**.

3.4. HSCCC fractionation characteristics

Initially, we injected 100–130 mg unsaponifiable matter of rapeseed oil, olive oil, linseed oil (corresponding with 40–100 g oil) and hydrogenated crude β-sitosterol standard into the HSCCC system. Enrichment and purification of the phytosterols was performed in two steps (Figs. 2 and 3). From the enrichment step to the purification step, the amount recovered decreased while the purity increased (Fig. 10). For instance, **3**, **4** and **7** were enriched from rapeseed oil. The fractions collected in the enrichment step (HSCCC-1) contained 67% and 50% of the amount of **3** and **4** at the start while the purity increased from 7% to 85% and from 29% to 91%

Table 1
Elution volume [mL] of identified hydrocarbons, ketones, fatty acids and fatty alcohols in the unsaponifiable matter of rapeseed oil (RS), olive oil (O), and linseed oil (LS) after HSCCC fractionation and the total output weight [mg]. **I**: palmitic acid, **II**: phytol, **III**: oleic acid, **IV**: geranyl geraniol, **VI**: squalene, **VII**: nonacosane, **IX**: 15-nonacosanone, **X**: 6,10,14-trimethylpentadecan-2-one, **XI**: campesta-3,5,22-triene, **XII**: campesta-3,5-diene, **XIII**: stigmasta-3,5-diene, α-Toc: α-tocopherol.

Elution volume [mL]	Weight [mg]			Hydrocarbons/ketones/fatty acids			Fatty alcohols		
	RS	O	LS	RS	O	LS	RS	O	LS
0–50	34.1	85.1	31.4	**VI, VII** **IX, XI** **XII, XIII**	**VI** **VII**	**VI** **VII**			
50–100	7.7	3.0	10.6				24:0 26:0[a] 26:0[b] 27:0[a] 27:0[b] 28:0 29:0[a] 29:0[b] 30:0	24:0 25:0 26:0[b] 27:0[b] 28:0 29:0[b] 30:0	24:0 25:0 26:0[b] 27:0[b] 28:0 30:0
100–150	23.1	7.0	55.4	**X**	**X**	**X**	22:0 24:0	22:0 23:0 24:0	22:0 23:0 24:0 α-Toc
150–200	36.1	6.9	15.8						20:0
200–250	3.5	1.6	0.8				18:0 **II**	18:0 **II**	18:0 **II**
250–300	1.2	0.8	3.6	20:1 18:0		18:0	16:0 17:0 **II**	**II**	**II**
300–350	3.3	2.3	8.3	**I** 18:2 **III**	**I** 18:2 **III** 18:0	**I** **III**	14:0[a] 14:0[b] 15:0 16:0 **IV**	16:0 **IV**	**IV**

[a] Secondary alcohol.
[b] Primary alcohol.

respectively (Fig. 10a)). The purification step (HSCCC-5 and HSCCC-6) delivered pure **3** and **4** (99%). The amount isolated from the initial sample was 50% for **3** and 30% for **4**. In addition, 33% of **11** could be purified from linseed oil (Fig. 10c). For other phytosterols the purity (~90% for **9** and **10**) and/or the yield (<20% for **7** and **11**) was lower (Fig. 10). In order to gain suited yields, the fractions selected for re-fractionation (purification step) should at least contain 50% of the original amount in the sample. This was fulfilled for all phytosterols except **11** (HSCCC-2). Noteworthy, the remaining share of the compounds is not lost, and additional HSCCC fractionations may be conducted with them. For example, fractions with enriched **7** (HSCCC-1: Fr. 24, 26–27; 14.7 mg; HSCCC-2: Fr. 25–26; 2.0 mg and HSCCC-3: Fr. 14–15; 16.8 mg, in total 33.5 mg; purity ~85%) already contain about 85% **7**, i.e. a better purity than the commercial standard (~60%) previously used for its isolation [8,9]. Due to the different phytosterol composition in different oils, selected fractions can be used and combined (Fig. 2). Both the abundance of a given phytosterol and the presence of other phytosterols in the unsaponifiable matter were important features to be taken into account for the successful purification. For instance, campesterol could be only isolated from rapeseed oil, because this oil did not contain stigmasterol (which cannot be separated from campesterol with the present HSCCC system). Besides these isolations, minor phytosterols such as citrostadienol **12** (~14%), cycloeucalenol (~7%), and erythrodiol (traces) could be enriched. Moreover, a total of 1 mg of the unknown phytosterol **13** was obtained from fractions of rapeseed oil and linseed oil with purities of up to 80%.

In the course of fraction evaluation of the enrichment step we also observed large amounts of different fatty alcohols. Due to the different chain lengths, they were widely distributed in the fractions. The longest fatty alcohol, 30:0, was detected in all studied plant oils (Table 1). All of the analyzed plant oils contained the primary fatty alcohols 22:0 to 29:0, expect 29:0 (not found in the linseed oil) and 25:0 and 23:0 (not found in the rapeseed oil). In addition, the medium chained fatty alcohols octadecanol (18:0) and hexadecanol (16:0) were detectable in the three plant oils. Rapeseed oil additionally contained the odd-chained heptadecanol (17:0) pentadecanol (15:0) along with tetradecanol (14:0). Small traces of docosanol (20:0) were only detected in linseed oil (Table 1). The fatty alcohols 21:0 and 19:0 could not be detected in any sample. Since the elution range of the fatty alcohols overlapped with the much higher concentrated phytosterols the latter could impede the detection of the fatty alcohols. However, the purity of the minor compounds fatty alcohols was generally low. Even so, the first HSCCC fractionation was accompanied with the separation of the major compounds in the unsaponifiable matter (i.e. the phytosterols and squalene) so that a strong enrichment of the fatty alcohols was achieved. Due the different distribution of each compound in each HSCCC fractions, the purification step (a 2nd HSCCC of selected fractions) would be suitable to gather pure fatty alcohols. However, their isolation would be inefficient due to their low amounts found in the unsaponifiable matter. Two further alcohols, phytol (**II**) (side chain of chlorophyll) and geranyl geraniol (**IV**) were detected in all plant oils (Table 1). **II** eluted late in the HSCCC system and **IV** was not eluted at all due to its additional double bonds. Thus, it was found in the last fraction which collected the compounds remaining on the column.

4. Conclusions

The study shows that HSCCC is a strong tool for investigating the unsaponifiable matter of plant oils. Especially the two-step design based on the combination of an enrichment step and purification step proved to be valuable for the isolation of pure and natural phytosterols. The enrichment step can be used for a detailed study of the composition of the unsaponifiable matter of plant oils. This evaluation provided a thorough view on the many

compounds present in the unsaponifiable matter because many of the minor compounds could only be detected after the HSCCC fractionation. In addition, the evaluation of the fractionation scheme allows the selection and combination of fractions enriched with different phytosterols which can be used in the purification step. By this measure we isolated between 0.3 and 9.5 mg of different phytosterols with purities of >90% to >99%). Some of the isolated phytosterols are scarcely commercially available (e.g. Δ5-avenasterol, 24-methylene-cycloartanol or campestanol) or only as synthetic or semi-synthetic compounds. Next to these compounds, we could also isolate (the fast eluting) hydrocarbons squalene and nonacosane as well as (the slow eluting) phytol (pure, 7 mg) and geranyl geraniol. In addition, 37.5 mg sitostanol (>99%) and 15.8 mg campestanol (>99%) could be isolated from the hydrated commercial crude β-sitosterol standard. The HSCCC-method opens the possibility to isolate pure phytosterols which may be useful for assessing the bioactivity of individual, natural phytosterols.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.03.033.

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