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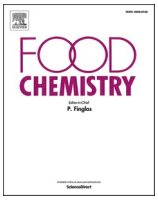

A sterol database: GC/MS data and occurrence of 150 sterols in seventy-four oils

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ABSTRACT

Comprehensive data on the occurrence of sterols in plant oils is currently hardly available since only a few sterols are obtainable as standard compounds. Accordingly, many peaks are rarely labeled in gas chromatograms due to missing or outdated information. This lack of information hampers the progress in sterol research. For this reason, gas chromatography with mass spectrometry in selected ion monitoring mode (GC/MS-SIM) was used to create a database that summarizes the occurrence and semi-quantitative levels of 150 sterols with 27–32 carbon atoms and 0–4 double bonds in 66 different vegetable oils and eight other matrices. The highest number of sterols was detected in rice bran and tamanu oil (40 sterols), eggplant (39 sterols), moringa, chili seed, and amaranth oil (37 sterols). Several sterols were detected in >60 of the 74 matrices. This detailed information in the database will serve users working in food authentication and the biosynthesis of sterols.

1. Introduction

>250 structural variants of sterols were described thus far in the scientific literature (Goad & Akihisa, 1997). Virtually all of these true sterols have in common the tetracyclic sterane backbone consisting of 17 carbon atoms and an obligate OH moiety on C-3 (Moreau et al., 2018). On the first level, the enormous structural diversity emerges from variations in the total number of carbon atoms (i.e. 27 to 32 carbon atoms (Goad & Akihisa, 1997; Schlag et al., 2023) including an alkyl residue of eight to ten carbon atoms on C-17 (hereafter named “side chain”), as well as zero to four double bonds or double bond equivalents, respectively (Sommer & Vetter, 2020). On the second level, sterol structures differ in (I) the (number and) position(s) of double bond(s) on the sterane backbone (e.g. Δ5-, Δ7-, and Δ8-sterols), (II) zero, one or two methyl substituents on C-4, (III) lack or presence of a methyl group on C-14, (IV) (number and) position(s) of the double bonds in the side chain, (V) additional alkyl substituents on the side chain (mainly methyl/methylene or ethyl/ethylene on C-24), and, in a few cases, (VI) a methylene group bridging C-9 and C-10 to give a cyclopropane ring (Mandl et al., 1999; Piironen et al., 2000).

Further variations emerge from the presence of stereocenters, e.g. at C-5, C-21, and C-24 but the respective configurations are only sparsely specified in the scientific literature. For instance, C-24 stereoisomers remain unresolved under typical gas chromatography (GC) conditions (Thompson et al., 1981). The true sterols are accompanied by closely related compounds (both structurally and biosynthetically) such as other tetracyclic (e.g. butyrospermol, tirucallol) and pentacyclic (e.g. amyrins) triterpene-derived compounds with an OH group on C-3 (triterpenoids). Partly, the division between sterols and triterpenoids is equivocal and both groups overlap with each other in GC with mass spectrometry (GC/MS), so they are often listed together (Schwartz et al., 2008; Yang et al., 2001; Zhang et al., 2020).

Although so many sterols are known to exist, scientific publications usually report only data on five to 15 sterols (Fiebig et al., 1998; Schwartz et al., 2008). Especially less abundant and less common representative sterols remain unreported when (silylated) sterols are analyzed by GC with flame ionization detection (GC/FID) (Fiebig et al., 1998). In this context, more reliable peak assignments can be made by GC/MS or high performance liquid chromatography with mass spectrometry (LC/MS). Mainly due to the poor peak resolution, LC/MS is less frequently used for the analysis of total sterols (Abidi, 2001; Carretero et al., 2008; Zhang et al., 2019), so that GC/MS is still the gold standard in sterol analysis. For better sensitivity, different research groups determined silylated sterols by GC/MS operated in the selected ion monitoring (SIM) mode (Cunha et al., 2006; Wang et al., 2019). A refined GC/MS-SIM method was recently introduced by the implementation of a series of internal standards (fatty acid pyrrolidides, FAPs) and the application of retention time locking (RTL) (Schlag et al.,

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2022b).

Here, we used the GC/MS-SIM method of Schlag et al. (2022b) and accompanying developments (Schlag et al., 2023; Schlag & Vetter, 2024) for the analysis of 74 different matrices (mostly oils). Including individual replicates, 112 samples were thoroughly analyzed in duplicate (technical replicates) on sterols. In a first step, GC/MS and GC/MS-SIM data of all detectable sterols was fed in a database. Further structural information was derived by the hydrogenation of double bonds of sterols and by application of solid phase extraction (SPE) using the same GC/MS method which enabled the differentiation of 4-desmethylsterols (4_0-sterols), 4-methylsterols (4_1-sterols), and 4,4-dimethylsterols (4_2-sterols), respectively (point II, see above). Then, the presence of all detected sterols was investigated and relative abundances were reported in the oil samples.

2. Materials and methods

2.1. Short terms of sterols

Because of the relevance of the substitution grade on C-4, this structural feature was addressed in short terms which will be used in the following. These short terms start with an "S" for sterols. When the substitution grade on C-4 could be determined, this was addressed in subscript style by a "$_0$", "$_1$" or "$_2$" for 4_0-, 4_1-, and 4_2-sterols, respectively. In addition, pentacyclic triterpenoids were labeled with a "$_t$" in subscript style. It then follows a number which denotes the (total) number of carbon atoms and, separated by a colon, a second number that displays the number of double bonds and double bond equivalents. Specifically, the presence of a 5th ring (either in form of a cyclopropane ring or a pentacyclic backbone) is equivalent to a double bond. Accordingly, lanosterol, cycloartenol and α-amyrin (both one double bond and one double bond equivalent) have the same molecular formula of $C_{30}H_{50}O$. For instance, the following short terms apply for: cholesterol ($S_0$27:0), β-sitosterol ($S_0$29:1), lanosterol ($S_2$30:2), α-amyrin (S_t30:2) and their corresponding isomers.

2.2. Chemicals and standards

The silylating agent (SILYL-991) was ordered from Macherey-Nagel (Düren, Germany). Palladium on activated charcoal (10%) and pyridine (distilled before use) were obtained from Sigma-Aldrich (Steinheim, Germany). Potassium hydroxide (>85%) was purchased from Carl Roth (Karlsruhe, Germany) while *n*-hexane (HPLC grade) was from Th. Geyer (Renningen, Germany). Ethanol and 2-propanol (BASF, Ludwigshafen, Germany) were distilled before use. Helium (99.999% purity), hydrogen (99.9990% purity), and nitrogen (99.95% purity) were from Westfalen company (Münster, Germany). The internal standard 5α-cholestane (98%) was obtained from Acros Organics (Geel, Belgium), whereas the additional internal standards, i.e. fatty acid pyrrolidides (FAPs) and cholestanyl-*tert*-butyldimethylsilylether (cholestanyl-TBDMS), were prepared as previously described by Schlag et al. (Schlag et al., 2022a, Schlag et al., 2022b). Campesterol (≥90%), α-amyrin (≥98%), and lupeol (≥98%) were from Santa Cruz Biotechnology (Heidelberg, Germany). Spinasterol was obtained from Cayman Chemical (Talinn, Estonia). Cholestanol (≥99%) and stigmasterol (~95%) were from Fluka (Taufkirchen, Germany). Sigma-Aldrich (Steinheim, Germany) delivered cholesterol (≥99%, *sigma grade*) and cycloartenol (≥90%) while β-sitosterol (~99%) and lanosterol (95%) were isolated by Schlag and Vetter (2024) and Rüttler et al. (2022).

2.3. Samples

Bought in local or internet stores, the 74 different individual sample matrices (61 vegetable oils, five vegetable fats, one animal fat, five other vegetable materials, one alga and one truffle sample) were analyzed as two technical replicates, respectively. Independent replicates (n = 2–8), also analyzed in duplicate, were examined for 24 matrices (e.g. native vs. refined, different suppliers, different varieties) to ensure that the sterol proportions found were representative of a given matrix (**Table S1**).

2.4. Sample preparation

Sample saponification and trimethylsilylation procedures were previously described in detail (Schlag et al., 2022a; Schlag et al., 2022b). For quantification, aliquots between 15 and 200 μL from 2 mL sample extract prepared from ~20 mg oil were subjected to trimethylsilylation, depending on the total contents of the different matrices (estimated from the peak areas determined in qualitative measurements). The silylated samples were then diluted in 100 μL of the internal standard solution, which contained 15 ng/μL cholestanyl-TBDMS and 6 ng/μL 5α-cholestane.

2.5. Hydrogenation

About 50 mg stigmasterol or β-sitosterol, 10 mg palladium on activated charcoal (10%), 5 mL 2-propanol and 50 μL pure water were added to a 50 mL two-neck round bottom flask (Schröder & Vetter, 2012). After flushing the flask with about 150 mL of hydrogen, one neck was sealed with a balloon filled with ~300 mL of hydrogen. Under permanent stirring, the solution was heated to 70 °C for about two days. After one day, a 1 mL aliquot was removed and separately analyzed. The catalyst was separated by membrane filtration and the solvents were evaporated. After weighing and re-dissolving in 1 mL *n*-hexane, an 80 μg aliquot was used for silylation.

2.6. Gas chromatography coupled to mass spectrometry (GC/MS)

Sterols were determined after saponification of the sample followed by hexane-extraction (i.e. in the so-called unsaponifiable matter) and trimethylsilylation. Silylated sterols were measured on a 6890 GC/5973 N MSD system (Hewlett-Packard/Agilent, Waldbronn, Germany), equipped with an Optima 5HT column (95%-methyl-5%-phenyl polysiloxane; 30 m, 0.25 mm i.d., 0.25 μm film thickness, Macherey-Nagel, Düren, Germany) using the parameters of Schlag et al. (2022b). The selected ion monitoring (SIM) method was adopted from Schlag and Vetter (2024) except that the lengths of the five time windows were slightly modified: time window 1: 17–30.6 min; time window 2: 30.6–32.7 min; time window 3: 32.7–34.07 min; time window 4: 34.07–34.75 min; and time window 5: 34.75–41.8 min. FAP retention indices (RI_{FAP}) were calculated and sterols were attributed to an assignment level and unknown sterols were identified according to Schlag et al. (2022b). Specifically, sterols available as an authentic reference standard were assigned to level 1 while further major sterols which could be unequivocally verified by GC/MS and literature data were assigned to level 2; further sterols which could be tentatively assigned were assigned to level 3, while the remaining ones were assigned to level 4 (Schlag et al., 2022b). Further classification of unknown 4_1- and 4_2-sterols was based on Schlag et al. (2023) (**Table S2**).

2.7. Semi-quantitative analysis

Sterols were generally measured by GC/MS after being silylated but for reason of simplicity, they will only be mentioned as "sterols", i.e. the form in which they are occurring in the samples. Sterols were semi-quantified using a characteristic ion trace of the GC/MS-SIM chromatogram. The peak area of known and unknown sterols of the quantification ion was corrected with the mean multiplication factor introduced by Schlag and Vetter (2024). Sterol patterns will be reported using the 100% method, i.e. the share (contribution) of the multiplication factor-corrected area of a given sterol to the total area of all sterols. The main sterol from each sample was quantified using an external

polynomial calibration curve of the respective standard (β-sitosterol, spinasterol, α-amyrin, or cholesterol). For the calculation of the total sterol content, the content of the main sterol was equated with the relative proportion in the sterol pattern and was subsequently corrected to 100%. As a quality control for the comparison of measurements on different days, the main sterol β-sitosterol in sunflower oil was quantified with each batch. The quantitative amounts varied <20% in ~six runs.

3. Results and discussion

3.1. Setup of the database – Sterols (data in table S2)

3.1.1. Variety of sterols

Examination of all 74 sample matrices enabled the distinction of 150 sterols whose GC/MS data were fed into **Table S2** of the database, i.e. 109 true sterols, 24 closely related compounds (mostly triterpenoids), and 17 sterols which could not be assigned to the one or the other group. This included GC data in the form of relative retention times (reference compound: 5α-cholestane) and retention indices (reference compounds: fatty acid pyrrolidides; RI_{FAP}), as well as characteristic MS data (molecular ions, M^+, along with diagnostic fragment ions) gained in GC/MS-SIM mode (**Table S2**). Known sterols will be presented with their trivial names while unknown sterols will be labeled as such in combination with the corresponding RI_{FAP} (e.g. unknown_2229).

Eighty-five percent of the sterols (i.e. 128 out of 150) were detected in one or (usually) more of the 74 matrices. The remaining 22 sterols were characterized in two different ways: (i) three stanols were detected after hydrogenation of stigmasterol and β-sitosterol as shown in section 2.4, and the structure of a fourth one could be predicted based on GC retention time extrapolations from the data of the other stanols (section 3.5). (ii) The remaining 18 sterols, including three S32:2 sterols (#148 - #150), were detected in a previous study after additional SPE enrichment of several vegetable oils (Schlag et al., 2023) and re-examined in this study.

In at least six cases it was known that pairs of sterols existed that differed only in the stereochemistry at C-24. These six pairs of diastereomers could not be separated with the present GC method and their GC/MS spectra were identical (Thompson et al., 1981). Therefore, these six pairs were summarized with one number, respectively (#25, #34, #38, #39, #59, #68) in **Table S2**. Only for the sterols found in truffles it could be determined that they showed 24β-configuration (the second compound listed in **Table S2**, see footnote), because they were structurally related to ergosterol (Sommer & Vetter, 2020). Contrarily, (almost) all other sterols in the investigated vegetable oils were assumed to show (predominately) 24α-configuration (i.e. the first isomer listed in **Table S2**). However, at least in the case of campesterol and dihydrobrassicasterol (#25, $S_0$28:1) it is known that both compounds can co-occur in vegetable samples, for example in soybean (Nes et al., 1976; Phillips et al., 2002; Wright et al., 1978).

Finally, the 3,28-diols uvaol and erythrodiol (#141 and #147, **Table S3**), both belonging to the group of pentacyclic triterpenoids (S_t30:2-OH), were detected in three and five matrices, respectively. Known especially from the presence in olive oil, both isomers were also part of the GC/FID method of 16 (18) classic sterols developed by Fiebig et al. (1998). Uvaol and erythrodiol could be easily differentiated from the true sterols due to the late elution and a relatively abundant $[M\text{-}90]^+$ fragment ion at *m/z* 496, which was part in the GC/MS-SIM method because it also represents M^+ of S30:3 sterols. This and further characteristic ions of the 3,28-diols (M^+ at *m/z* 586 and *m/z* 216) in the GC/MS full scan spectra were in accordance with literature data (Li et al., 2007). The high mass of M^+ results from the presence of two silylated OH groups in both isomers.

3.1.2. Variety of sterol subgroups

The highest variety was found within the group of S30:2 isomers (Table 1), which also included the pentacyclic triterpenoids and amyrins (1 db and one double bond equivalent due to the 5th ring, section 2.2). In general, sterols with two double bond equivalents showed the highest frequency in the database ($n = 102$) followed by sterols with one double bond equivalent ($n = 28$). With regard to the number of carbon atoms, the variety decreased in the order S30 (54 sterols) > S29 (37 sterols) > S28 sterols (30 sterols) > S31 (18 sterols) > S27 (8 sterols) > S32 (3 sterols).

Table 1
Number of sterols in dependence of the number of carbon atoms and number of double bonds or double bond equivalents (db).

		Number of double bonds and double bond equivalents (db)					
		0 db	1 db	2 db	3 db	4 db	SUM
Number of carbon atoms	27	2	3	3	0	0	8
	28	2	10	11	6	1	30
	29	3	7	26	1	0	37
	30	2	6	45	1	0	54
	31	0	2	16	0	0	18
	32	0	0	3	0	0	3
	SUM	9	28	104	8	1	150

Fifty-nine sterols (39%) were 4_0-sterols (Fig. 1a), and 39 of them could be structurally identified (corresponding to assignment levels 1 or 2, **Table S2**). The exact molecular structure of the remaining 20 4_0-sterols could not be disclosed (assignment levels 3 or 4, **Table S2**). Another 27% were 4_2-sterols ($n = 40$) and 15% were 4_1-sterols ($n = 22$). Unknown sterols in these two groups were at least divided into subgroups (4_1-sterols: 4-methylsterol or 14-methyl-4-methylsterol, 4_2-sterols: with or without cyclopropane ring) after measurement by a second GC/MS-SIM method (Schlag et al., 2023) (**Table S2,** see right column "comments"). Finally, 21 compounds featured a pentacyclic triterpenoid backbone (14%), while for the remaining eight compounds could be verified that they were sterols but the specific subgroup could not be determined (Fig. 1a). This was mostly owing to their very low abundance in samples or due to their co-elution with more prominent sterols.

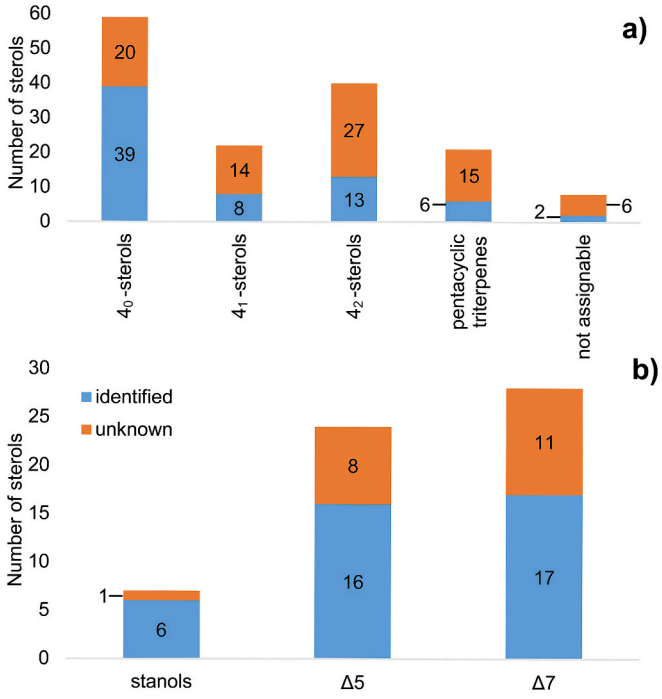

Fig. 1. Number of identified and unknown sterols (a) according to methylation degree (4-desmethylsterols correspond to 4_0-sterols, 4-methylsterols to 4_1-sterols, and 4,4-dimethylsterols to 4_2-sterols) and (b) further classification of 4_0-sterols according to the position of the double bond at ring B.

Within the family of 4_0-sterols, 24 belonged to the most commonly known and frequently predominant group of Δ5-sterols (e.g. β-sitosterol, campesterol, stigmasterol, and cholesterol). Sixteen of the Δ5-sterols could be identified while eight remained unknown (Fig. 1b). Interestingly, the variety of Δ5-sterols was exceeded by Δ7−/ Δ8-sterols (e.g. spinasterol, Δ7-sitosterol, Δ7-campesterol; n = 28, 17 known, 11 unknown, Fig. 1b). This is remarkable since Δ7−/Δ8-sterols are currently only scarcely reported in publications which is mostly due to the lack of reference standards and their (frequently) low abundance which requires the application of sensitive GC/MS methods for their detection (Schlag et al., 2022a). In this context, the GC retention data in **Table S2** will be helpful for a more comprehensive consideration of this group of sterols. Specifically, with regard to the position of the double bond (and all other structural features identical), the GC elution order (DB-5 like column, section 2.6) was Δ5- < Δ8(9)- < Δ7-sterols. Additionally, data of seven stanols (only one remained unknown) was added to the database (Fig. 1b).

3.2. Setup of the database – Occurrence of sterols in the sample matrices (data in table S3)

3.2.1. Sample matrices and sterol abundances

The 74 chosen matrices included the most (a) common edible oils, (b) nut oils, (c) grain oils, (d) fruit oils, (e) residual oils, (f) *Solanaceae*, (g) fats, (h) seasoning, (i) spinasterol containing oils, and (j) "special" samples (here: wakame and truffle) and based on this classification they were grouped (**Table S3**). In a few cases, it could not be unequivocally clarified that the samples were authentic, e.g. due to the lack of literature values. These samples were listed separately under (k) authenticity not secured and (l) not authentic (**Table S4**). When different samples of the same matrix were analyzed only the most representative one was added to **Table S3**.

Also, due to the large number of matrices and sterols examined in this study, not all literature values and references could be listed in this document. Since the sterol pattern is known to vary (slightly) in dependence of subspecies, geography, season, maturity etc. (Whitaker & Lusby, 1989; Yang et al., 2001; Zangenberg et al., 2004), the contribution (%) of individual sterols to the total sterols was scaled as follows: Major sterols (>25%) were designated abundance A. Lower proportions were numbered alphabetically with descending values, with abundance B corresponding with 10–25%, abundance C with 5–10%, abundance D with 1–5%, abundance E with 0.1–1%, and abundance F with <0.1% to the sterol pattern. Additionally, in the case of two co-eluting sterols having the same quantification ion, only the major sterol was evaluated, while both of them were additionally labeled with the letter Z. Compounds which could not be (semi-)quantified (e.g. uvaol, erythrodiol) were marked with an X to display their occurrence (**Table S3**). Given the reported variability from sample to sample (Whitaker & Lusby, 1989; Yang et al., 2001; Zangenberg et al., 2004), these assignments should only be read as indicative.

3.2.2. Major sterols in the sample matrices

As it is known from different classic vegetable oils (Moreau et al., 2018), the sterol pattern of 58% of the analyzed matrices was dominated by β-sitosterol (#68, $S_0$29:1, Fig. 2). In the case of β-sitosterol it turned out to be useful to distinguish matrices with >70% β-sitosterol (abundance A^a, **Table S3**) from those with 60–70% β-sitosterol (abundance A^b), because (only) abundance A^b samples covered some interesting oils (Fig. 2). A third group (27% of the matrices) still featured β-sitosterol at abundance A but additionally one or more other sterols of at least abundance B (>10%) (Fig. 2).

Apart from β-sitosterol, only eleven of the 74 matrices contained another sterol of abundance A (see next paragraph). Overall, β-sitosterol was detected in 67 of the 74 matrices (90%, Table 2). However, further classic Δ5–4_0-sterols like campesterol (#25, $S_0$28:1), stigmasterol (#39, $S_0$29:2), and Δ5-avenasterol (#77, $S_0$29:2) were detected with similar or

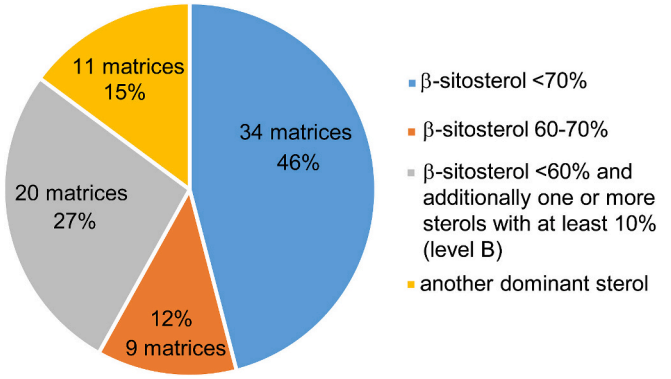

Fig. 2. Percentage amount [%] of matrices containing β-sitosterol at >70%, 60–70%, or < 60% (together with other sterols >10% - level B), and samples with another main sterol.

Table 2
Detection frequency (in parentheses) of the top 15 sterols detected in the matrices, detected as abundance A (contribution >25%) and the samples with the most detected sterols.

No.	Highest frequency	No. of times abundance A (contribution >25%)	Highest sterol variety in oils.
1)	lanosterol/obtusifoliol (72)	β-sitosterol (63)	rice bran oil (40)
2)	Δ5-avenasterol (69)	α-amyrin (3)	tamanu oil (40)
3)	24-methylencycloartanol (69)	cycloartenol (2)	eggplant (39)
4)	campesterol (68)	24-methylencycloartanol (2)	moringa oil (37)
5)	stigmasterol (68)	campesterol (2)	chili seed oil (37)
6)	sitostanol (68)	unknown_2229 (2)	amaranth oil (37)
7)	cholesterol (67)	tirucallol (1)	sea buckthorn oil (36)
8)	β-sitosterol (67)	cholesterol (1)	milk thistle oil (35)
9)	clerosterol (66)	lanosterol (1)	peach seed oil (34)
10)	cycloartenol (66)	brassicasterol (1)	tomato seed oil (34)
11)	citrostadienol (63)	ergosterol (1)	kukui nut oil (33)
12)	stigmasta-5,24(25)-dienol (60)	spinasterol (1)	tea seed oil (*Camellia sinensis*) (33)
13)	campestanol (59)	fucosterol (1)	safflower oil (32)
14)	Δ7-sitosterol (58)	stigmasta-7,25-dienol (1)	mustard oil (31)
15)	Δ7-campesterol (57)	Δ7-sitosterol (1)	pomegranate seed oil (31)

even higher frequencies (Table 2). Also, the two corresponding stanols (no double bond) sitostanol (#76, $S_0$29:0) and campestanol (#34, $S_0$28:0) had similarly high frequencies, as indicated by Schlag and Vetter (2024) (Table 2). This co-occurrence of campesterol/campestanol (68/59 out of 74 matrices) and especially β-sitosterol/sitostanol (67/68) became even more apparent in this study (Table 2). Next to the classic Δ5–4_0-sterols, several Δ7–4_0-sterols (e.g. Δ7-sitosterol, $S_0$29:1), 4_1-sterols (e.g. citrostadienol, $S_1$30:2), and 4_2-sterols (e.g. 24-methylenecycloartanol, $S_2$31:2, and cycloartenol, $S_2$30:2) also reached detection frequencies of >80% in selected samples (Table 2). However, the highest detection frequency of 97% (72/74) was observed for lanosterol/obtusifoliol (both co-eluting sterols could not be distinguished by GC/MS-SIM).

In total, 15 sterols reached abundance A in at least one of the matrices (Table 2, central column). Next to β-sitosterol (abundance A in 63 matrices), α-amyrin, cycloartenol, 24-methylenecycloartanol, unknown_2229 and campesterol were present with abundance A in more than one matrix (and spinasterol, lanosterol, ergosterol, and cholesterol

in only one matrix). The only unknown abundance A sterol was detected in three *Cucurbitaceae* oils (nara oil (*Acanthosicyos horridus*), melon oil, and watermelon oil). However, at least it could be assured that unknown_2229 (M^+ 498, *m/z* 134 (Schlag et al., 2023)) was an isomer of the pentacyclic triterpenoid glutinol (#31; S_t30:2, Table 2, **Table S3**).

3.2.3. Highest diversity of sterols in the sample matrices

Fifteen oils featured >30 sterols (Table 2, right column). Sample matrices with the highest variety of sterols were rice bran oil and tamanu oil (40 sterols, each) (Table 2, right column). A high variety was found especially in less common sample matrices such as eggplant (39 sterols), moringa oil, chili seed oil, and amaranth oil (37 sterols each). Nevertheless, a high variety was also detectable in some more common vegetable oils, e.g. safflower oil (32 sterols) or mustard oil (31 sterols). Vice versa, the lowest number of sterols was detected in wool wax (eleven sterols).

3.2.4. Brief comments on selected sterols in other sample matrices (data in table S3)

Apart from the examples presented above, the sterol patterns were frequently very similar even for samples from completely different matrices (fruit oils, grain oils, nut oils, typical edible oils, **Table S3**). The largest deviations were observed in group (g) (fats) and group (i) (spinasterol containing matrices) (**Table S3**).

The sterol pattern of three vegetable fats (cocoa butter, coconut oil, cupuacu butter) in group (g) was dominated by β-sitosterol (#68, $S_0$29:1). However, α-amyrin (#106, S_t30:2) was the major sterol of shea butter and also in the analyzed mango butter sample. Contrary to that, previous literature studies reported little or no α-amyrin in mango butter but high amounts of β-sitosterol (Dhara et al., 2010; Jin et al., 2016). These deviations produced strong evidence that the mango butter examined was not authentic, but rather a mixture with (or even pure) shea butter. Therefore, the mango butter sample is shown in **Table S4**.

In the case of spinasterol (#70, $S_0$29:2) containing matrices (group i), the glutinol isomer unknown_2229 (#31; S_t30:2) stood out as it was only predominant in nara oil and the two melon oils (melon oil and watermelon oil, Fig. 3a). Botanically, these species are closely related members of *Cucurbitaceae*. However, unknown_2229 was not detected in pumpkin seed oil, which also belongs to the family of *Cucurbitaceae*. Therefore, unknown_2229 is not suited to serve as a general marker for this plant family. However, all members of *Cucurbitaceae* featured a relatively high proportion of stigmasta-7,25-dienol (#93, $S_0$29:2) that was higher than in *Amaranthaceae* (quinoa, amaranth) and argan oil (**Tables S3** and **S4**).

Finally, wool wax was the only examined animal fat (Fig. 3b). Typical for all animal matrices (Moreau et al., 2018), cholesterol (#2, $S_0$27:1) was the predominant sterol. In addition, wool wax contained high shares of lanosterol (#66, $S_2$30:2) and dihydrolanosterol (#40, $S_2$30:1) – abundance A and B, respectively, as well as eight minor sterols (Rüttler et al., 2022). Since the eleven sterols are the typical ones in animal fats (frequently with a high dominance of cholesterol and (rarely) a low variety of other cholesterol-derived sterols) (Nes, 2011), further samples from animals were not analyzed in this study.

3.3. Variations in the sterol pattern in different oils of the same sample matrix

The representativeness of the sterol patterns listed in the database was tested with independent replicates from 24 matrices. These variants included oils from other manufacturers, different sub-varieties, as well as pairs of native and refined oils. No relevant differences were found in 80% of the matrices (19 matrices), which confirmed that the sterol distributions displayed in the database were representative for the examined matrices (**Table S1**).

For instance, the sterol pattern of refined and virgin (cold pressed) rapeseed oil showed no major difference (Fig. 4a, left bars). However,

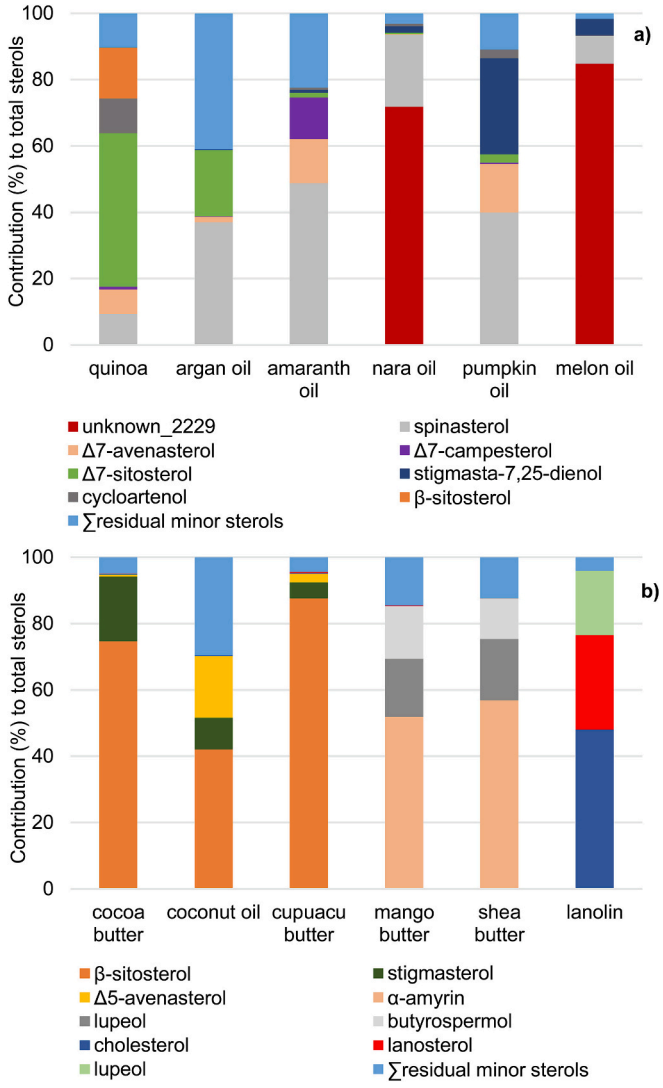

Fig. 3. Sterol distribution [%] of (a) the six analyzed matrices containing spinasterol and (b) the six analyzed fats.

the total sterol content was ~10% lower in the refined sample. Although this could also be a coincidence since the oils were not from the same batch, lower sterol amounts (and the same characteristic sterol pattern) after refining are consistent with previous studies (Verleyen et al., 2002). In contrast, campesterol (#25, $S_0$28:1) was eight times more abundant in a refined avocado oil (Fig. 4a, right bars) than in a native avocado oil from the same supplier (15 vs. 1.8%). This suggested that the refined avocado oil may have been adulterated. This suspicion was reinforced by reports of Green and Wang (Green & Wang, 2020) who found the same campesterol contribution in authentic native and refined avocado oil, but a fourfold higher campesterol content in one unrefined and two extra virgin samples adulterated with soybean oil (20% vs. 5%). The present conspicuous refined avocado oil also contained a higher number of sterols (28 vs. 19) than the native one, which supported the evidence of food fraud (and therefore only data from the native sample was presented in the database). Also, a native moringa oil contained a lower number of sterols than a refined one (27 vs. 37, Fig. 4, central bars). Specifically, the refined moringa oil additionally featured three isomers each of 24-methylenecycloartanol (#130, $S_2$31:2) and 24-methylenedihydrolanosterol (#99, $S_2$31:2). Isomers of these sterols are known to be formed during refining (Strocchi & Marascio, 1993; van Hoed et al., 2006). The detection of the isomers in moringa and rice bran oil but not in avocado oil (Fig. 5) indicated that the type of the oil and/or

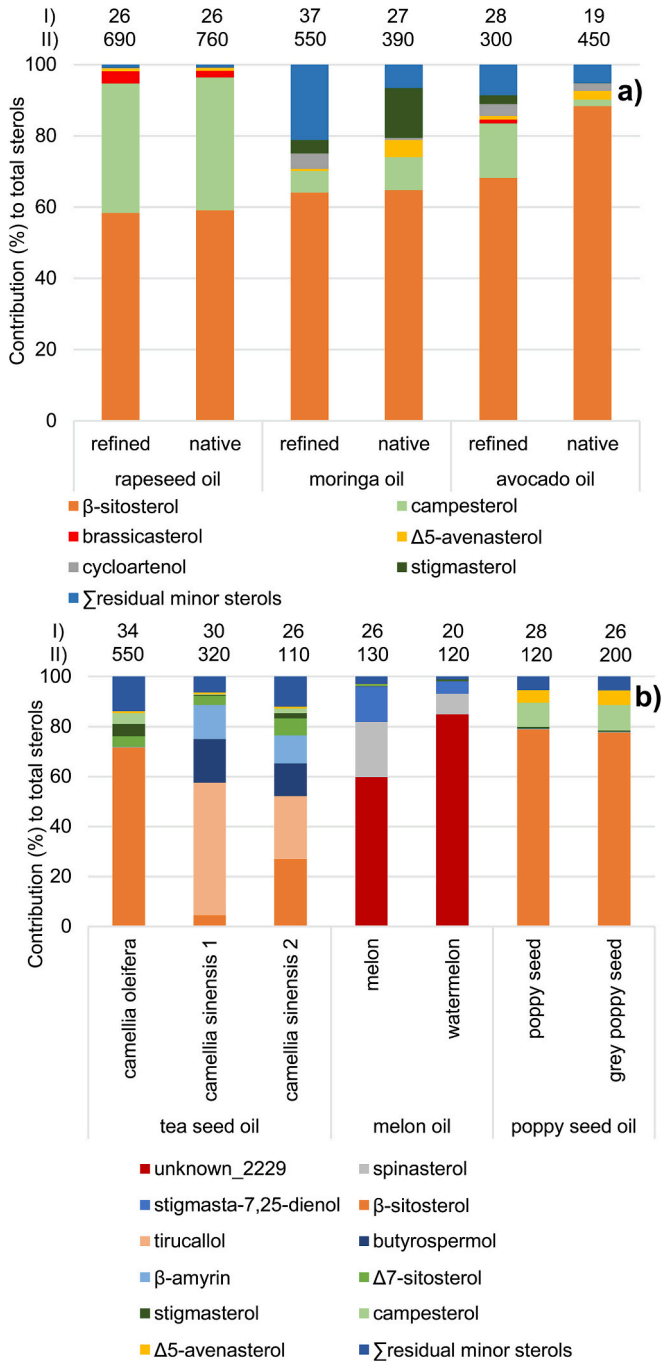

Fig. 4. Sterol variety (I), sterol content in [mg/100 g oil] (II), and sterol pattern of the main sterols in (a) native and refined oils and (b) different subspecies.

the harshness of the refining conditions could also influence the formation of these artefact peaks. Similarly, less stable sterols and also sterol conjugates (e.g. where the OH group on C-3 is esterified) may be more affected than others. For instance, sterol ferulates like γ-oryzanol were more readily degraded and isomerized during refining than other forms (van Hoed et al., 2006). Accordingly, the sterol pattern of refined oils may vary more than the one of native oils.

The same "refining artefact" isomers were detected in one of three neem oils and one tamanu oil which were all labeled as native oils (Fig. 5, "refining artefact" grey dots of isomers within the red square). Since two of the three neem oil samples did not contain these sterols, it was considered unlikely that the conspicuous neem oil sample and the tamanu oil (Fig. 5) were truly cold pressed in terms of quality. Possible

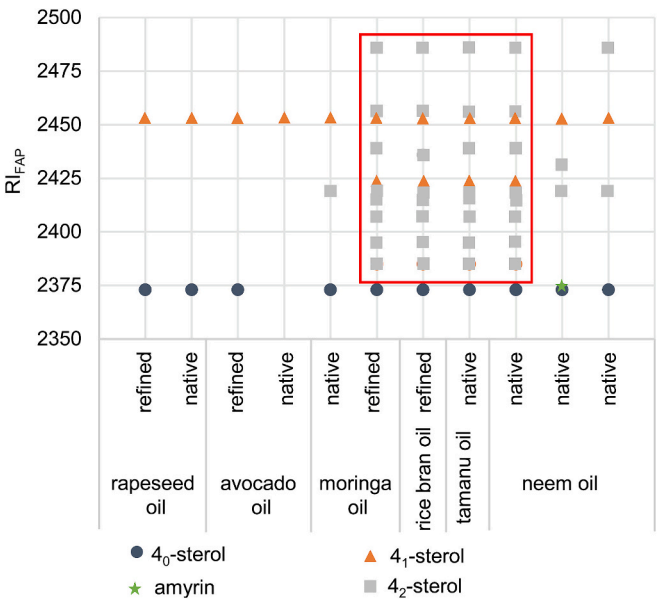

Fig. 5. Fatty acid pyrrolidide retention indices (RI_{FAP}) of the detected sterols in dependence of the processing status (native/ refined). Desmethylsterols (4_0-sterols) were labeled with blue circles, 4-methylsterols (4_1-sterols) with yellow triangles, 4,4-dimethylsterols (4_2-sterols) with grey squares and amyrins with green stars. „Refining artefact" sterols are circled with a red line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

issues could be improper storage or processing, heating, and roasting, but also refining and adulteration with other oils. Since these "refining artefact" sterols should not be present in cold pressed native oils, test on their absence could be used as markers for gentle refining and possibly other processes. Therefore, the sterol pattern of tamanu oil was moved to **Table S4** which summarizes data of oils which were not unequivocally authentic.

Some oils from different subspecies differed greatly in their sterol patterns (e.g. truffles (Sommer et al., 2020)) while others did not. Further factors such as harvest year, temperature, genotype, etc. can also have an impact on the sterol contents and pattern (Yang et al., 2001; Zangenberg et al., 2004). As an example, two poppy seed oils showed virtually identical sterol patterns but the sterol content varied almost by a factor of two (Fig. 4b, right bars). Also, the melon oil and the watermelon oil showed almost the same sterol content and the same dominant sterols (Fig. 4b, central bars). However, unknown_2229, #31, S_t30:2) was more prominent in the watermelon oil (80% of all sterols) while the number of sterols was higher in the melon oil (Fig. 4b).

Larger differences were detected in the sterol pattern of three tea seed oils (also known as camellia seed oil) (Fig. 4b, left bars). For commercial samples labeled "camellia oils", it is not always clear whether they were made from *Camellia oleifera* (i.e. the "true" tea seed oils) or *Camellia sinensis*, but both can be differed by means of their sterol patterns (Zhang et al., 2020). Specifically, tirucallol (#108, $S_2$30:2) was suggested as a marker since it is mainly present in *Camellia sinensis* (Zeng & Endo, 2019; Zhang et al., 2019). According to this feature, one tea oil was assigned to *Camellia oleifera* and the two other ones to *Camellia sinensis* (Fig. 4b, left bars). This classification was further substantiated by differences in the contribution of β-sitosterol, which was >50% in *Camellia oleifera* (sample 1), while it remained below 30% in the *Camellia sinensis* samples 2 and 3 (Fig. 4b). Hence, both tea oils were listed individually as type 1 and type 2 tea seed oils in the database (**Table S3**, e-44 and e-45).

3.4. Hydrogenation of sterols in the laboratory

Complete hydrogenation (saturation) of the Δ5,22-double bonds of stigmasterol (#39, $S_0$29:2) leads to sitostanol (#76, $S_0$29:0). This reaction proceeds via two detectable $S_0$29:1 intermediate products (Fig. 6a), with one being β-sitosterol (#68, $S_0$29:1, Δ5, Fig. 6a, III) according to indications of Wärnå et al. (2006). According to expectations (and the GC/MS data), the second $S_0$29:1 isomer (M^+ 486) featured the double bond in the side chain due to the diagnostic fragment ion at *m/z* 257 which in turn confirmed the presence of a saturated ring system (Goad et al., 1967; Schlag & Vetter, 2024). Our GC/MS data produced strong evidence that this $S_0$29:1 isomer was stigmasta-22-enol (#47, Δ22, Fig. 6a, II). The similar abundance of both $S_0$29:1 intermediates indicated that the two double bonds of stigmasterol (at Δ5- and Δ22-position) were equally hydrogenated (Fig. 7). In GC/MS-SIM mode, (silylated) stigmasta-22-enol ($S_0$29:1) is difficult to distinguish from 4_1-isomers ($S_1$29:1) since both variants show the base peak at *m/z* 129 and *m/z* 215 and *m/z* 213 in the ratio of >1 (Schlag et al., 2022b). However, the GC/MS spectrum of stigmasta-22-enol showed *m/z* 255 and *m/z* 215 in a ratio of >1 which made it possible to differentiate it from 4_1-sterols. In addition, the hydrogenated stigmasterol standard solution contained minute amounts of a second stanol isomer (#27, $S_0$29:0) which eluted much faster than sitostanol from the DB-5 like GC column. A corresponding stanol (coprostanol) had previously been described for cholestanol (both related to cholesterol) (Brooks et al., 1968). In both cases, the hydrogenation of the Δ5-double bond creates an additional stereocenter on C-5. Accordingly, the earlier eluting isomer of sitostanol was most likely 5β-sitostanol. Theoretically, the corresponding 5β-isomer of (5α-) campestanol should also exist. However, a campesterol standard was not available and we could not apply hydrogenation to verify this prediction (**Table S1**).

A similar case of other S28 sterols could be clarified due to the data of Sommer et al. (2020). As expected, two $S_0$28:1 intermediates (dihydrobrassicasterol and unknown_2176b, are located between brassicasterol ($S_0$28:2) and ergostanol ($S_0$28:0). Similarly to stigmasterol and stigmasta-22-enol (ΔRRT = 0.01 min), unknown_2176b eluted shortly after brassicasterol (ΔRRT = 0.02 min). Accordingly, unknown_2176b could be assigned to be brassicasta-22-enol (#12, $S_0$28:1), which verified previous suggestions of Sommer et al. (2020). In the case of stigmasta-22-enol und brassicasta-22-enol (#12, $S_0$28:1) – both with a saturated ring system – there may also exist the corresponding 5β-$S_1$28:1 isomers. However, these could not be detected, most likely due to their insufficient abundance.

Finally, commercial samples of a hydrogenated and an original cotton oil were screened by GC/MS-SIM and GC/MS in full scan mode (Table 3). Tentatively, one S30:1 isomer obtained from citrostadienol (S30:2) and two S31:1 sterols obtained from 24-methylenecycloartanol (S31:2) were detected in the hydrogenated oil. However, only in the case of unknown_2439 and citrostadienol ΔRRT was almost consistent with the other pairs being hydrogenated at the Δ24(24[1])-position. Consequently, unknown_2439 (only detected in the hydrogenated cotton oil) was most likely the Δ7-monounsaturated intermediate product of the hydrogenation of citrostadienol (i.e. 24α-ethyllophenol). This approach may also be used in the future to identify more sterols, especially those formed by hydrogenation.

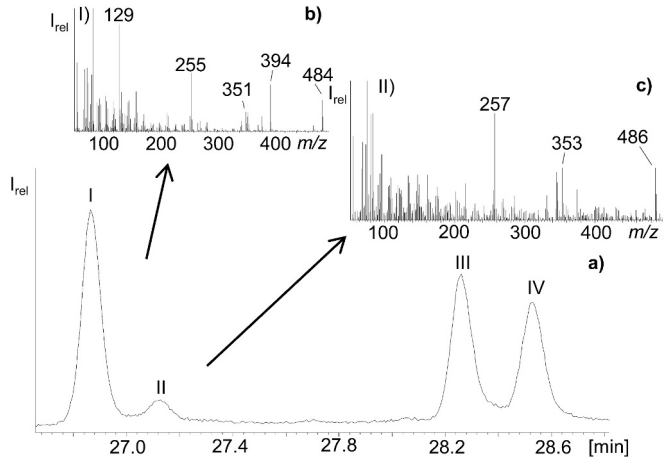

Fig. 6. (a) GC/MS chromatogram of the partially hydrogenated stigmasterol standard containing I) stigmasterol, II) stigmasta-22-enol, III) β-sitosterol, and IV) sitostanol, and mass spectra of (b) stigmasterol and (c) stigmasta-22-enol.

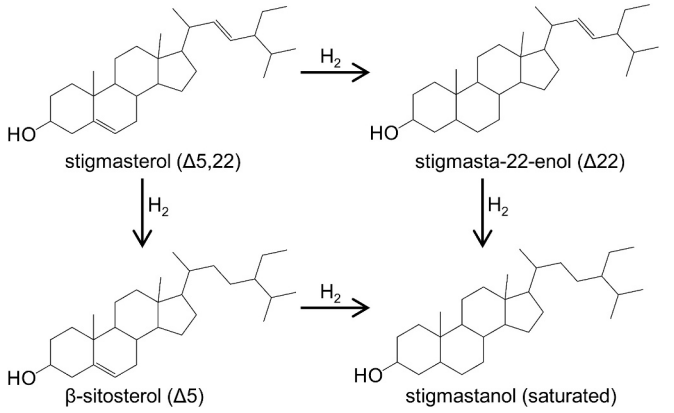

Fig. 7. Structures of the mono-unsaturated hydrogenation products of stigmasterol (β-sitosterol and stigmasta-22-enol) and the fully saturated sitostanol without specification of stereocenters.

4. Conclusions

The database (**Tables S2** and **S3**) was compiled to help researchers in their daily work to assign more sterols in the present and other (edible) oils or other samples. For instance, high amounts of campesta-5,24(25)-dienol were recently detected in red goji berries (Zheng et al., 2024) which enabled the assignment of this very rarely reported sterol in 46 sample matrices, although (apart from red goji berries) only at very low levels (abundances *E*-F).

The data in **Tables S2** and **S3** can be accessed and expanded or adapted depending on individual research tasks. E.g., our database will also serve researchers using LC/MS in analyzing sterols. Despite its lower resolution power, LC/MS is superior to GC/MS in analyzing conjugated sterols (Bezerra and Filho, 2020). In such a context, our database will serve as a tool for selecting the expectable sterols present in the reported matrices. Possible further application fields could be their use in the authentication of oils. Especially data on minor sterols

Table 3
Sterol pairs with different double bond positions, their differences and the corresponding impact on the fatty acid pyrrolidide retention indices (ΔRI_{FAP}).

sterol pair	db Δ-position	difference	ΔRI_{FAP}
β-sitosterol / Δ5-avenasterol	Δ5 / Δ5,24(24[1])	Δ24(24[1])	14
Δ7-sitosterol / Δ7-avenasterol	Δ7 / Δ7,24(24[1])	Δ24(24[1])	13
β-sitosterol / stigmasta-5,24(25)-dienol	Δ5 / Δ5,24(25)	Δ24(25)	34
Δ7-sitosterol / sitostanol	Δ7 / Δ0	Δ7	45
dihydrolanosterol / lanosterol	Δ8(9) / Δ8(9),24(25)	Δ24(25)	42
cycloartanol / cycloartenol	Δ0 / Δ24(25)	Δ24(25)	45
unknown_2439 / citrostadienol	? / Δ5,24(25)		14
unknown_2402 / 24-methylenecycloartanol	? /Δ24(25)		19
unknown_2424b / 24-methylenecycloartanol	? /Δ24(25)		3

(abundances D-F) should be helpful in this context, because they may not only enable the distinction of different varieties but also indicate their origin (Sommer et al., 2020). Similarly, the sterol data in **Tables S2** and **S3** could be useful for the investigation and confirmation of biosynthesis routes of phytosterols. Last but not least, the database can contribute to the identification of unknown compounds, e.g. by the comparison of ΔRRT ratios of different sterol pairs. We would be very happy if researchers could take advantage of the database which shows the knowledge cumulated by us from different methods developed over several years.

CRediT authorship contribution statement

Sarah Schlag: Writing – original draft, Visualization, Investigation, Data curation. **Sabrina Schäfer:** Writing – review & editing, Investigation. **Katrin Sommer:** Writing – review & editing, Investigation. **Walter Vetter:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140778.

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