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Online LC-GC-Based Analysis of Minor Lipids in Various Tree Nuts and Peanuts

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ABSTRACT: As information on free sterols/stanols and steryl/stanyl esters in nuts is lacking, the compositions and contents of these lipid constituents in ten different nut types were analyzed. The applied approach was based on online liquid chromatography-gas chromatography and enabled the simultaneous analysis of free sterols/stanols and individual steryl/stanyl fatty acid esters, and additionally of tocopherols and squalene. Total contents of free sterols/stanols ranged from 0.62 mg/g nut in hazelnuts to 1.61 mg/g nut in pistachios, with sitosterol as the predominant compound. Total contents of steryl/stanyl fatty acid esters were in the range of 0.11−1.26 mg/g nut, being lowest in Brazil nuts and highest in pistachios. There were considerable differences between the various nut types not only regarding the contents, but also the compositions of both classes. The levels of tocopherols were highest in pine nuts (0.33 mg/g nut); those of squalene were remarkably high in Brazil nuts (1.11 mg/g nut).

KEYWORDS: *tree nuts, peanuts, free sterols, steryl esters, tocopherols, squalene, online LC-GC*

INTRODUCTION

Although tree nuts and peanuts are rich in fat and energy, their regular consumption is associated with several health benefits, such as a reduced incidence of coronary heart disease, cancer, type-2 diabetes, inflammation, and several other chronic diseases.[1−3] These positive health effects are attributed to their high amounts of unsaturated fat, dietary fiber, proteins, and minerals, but also to the presence of several other phytochemicals. Besides phenols, squalene, and vitamins, tree nuts and peanuts are also rich in phytosterols. Phytosterols are steroidal alcohols playing important roles in the regulation of cell membrane fluidity and permeability and occur in free form, as steryl esters, steryl glycosides, or acylated steryl glycosides.[4,5] They are well-known for their cholesterol-lowering effect but were also suggested to possess other health benefits such as anti-inflammatory or anticarcinogenic properties.[6−8] There is evidence that the gastrointestinal hydrolysis of steryl/stanyl esters by digestive enzymes depends on the type of ester. In vitro and in vivo studies reported an impact of the sterol/stanol and fatty acid moiety on the hydrolysis rate, which, in turn, could influence the cholesterol-lowering effect of dietary phytosterols.[9,10] Therefore, quantitative data on the occurrence of individual intact steryl/stanyl fatty acid esters in plants are essential; however, so far this substance class has been less studied, because of the lack of appropriate analytical methods.

Past research concerning nuts has been focused on the analysis of the contents and compositions of total phytosterols, commonly determined after alkaline or after a combination of alkaline and acidic hydrolysis. Several studies analyzed either total phytosterols in several kinds of nuts or investigated for example the effects of genotype or growing year and location on phytosterols in a single type of nut.[11−21] However, due to the hydrolysis steps, information on the distribution and composition of the naturally occurring individual steryl conjugates was lost. Previously, free sterols and steryl esters have been studied in terms of lipid class analysis in seven different nut types by applying a method based on automated thin-layer chromatography-flame ionization detection (TLC-FID).[22] However, only total amounts, but not the individual compositions of free sterols and steryl esters have been analyzed. A further study investigated the lipid class compositions of almonds, hazelnuts, and walnuts via quantitative TLC; sterol compositions of the isolated free sterol and steryl ester fractions have been determined via gas chromatography (GC).[23] Total amounts of free sterols and steryl esters as well as the sterol compositions of both fractions have also been analyzed in two different peanut cultivars by fractionation of the lipids via preparative liquid chromatography (LC) and TLC.[24] The contents and profiles of free sterols in nine walnut cultivars have been investigated by Verardo et al.;[25] however, steryl esters have not been included in that study.

As comparable data on contents and compositions of individual intact steryl/stanyl esters in nuts are still lacking, the primary objective of the present study was the analysis of these compounds in ten different commercially important nut types. For analysis, a recently published approach based on online liquid chromatography-gas chromatography (online LC-GC) should be applied.[26] This methodology enables the rapid and simultaneous analysis of intact steryl/stanyl esters, of free sterols/stanols, and of other minor lipids, such as tocopherols and squalene, in one run. Representative structures of the analyzed minor lipid classes are shown in Figure 1.

MATERIALS AND METHODS

Sample Material. A total of 30 prepackaged nut samples (i.e., three samples of almonds, Brazil nuts, cashew kernels, hazelnuts,

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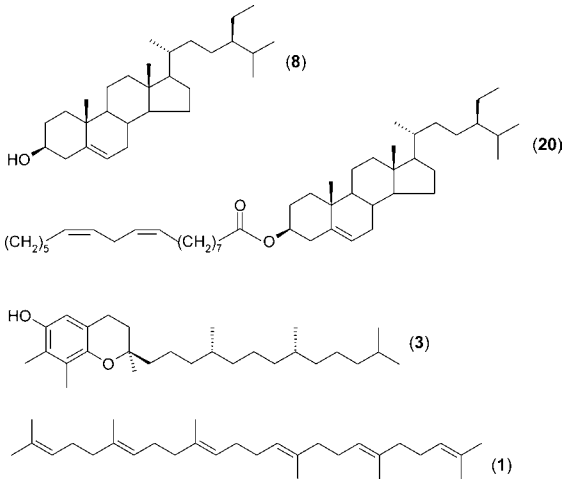

Figure 1. Major representatives of the analyzed minor lipid classes: (8) sitosterol, (20) sitosteryl-18:2, (3) γ-tocopherol, and (1) squalene. The numbers correspond to those shown in Figure 2.

macadamia nuts, peanuts, pecan nuts, pine nuts, pistachios, and walnuts, respectively) were commercially obtained. The nuts were raw and unsalted, except for the peanut samples, which were dry roasted; the suppliers and the information available regarding cultivar, harvest year, and origin are summarized in Table 1. Information on the commercially employed storage conditions of the materials after harvesting was not available. Until analysis, the samples were stored in the original packaging at room temperature in the dark.

Chemicals. Cholesteryl palmitate (≥98%), 5α-cholestan-3β-ol (∼95%), stigmasterol (∼95%), sitostanol (∼95%), squalene (∼95%), (±)-α-tocopherol (95%), (+)-γ-tocopherol (≥96%), (+)-δ-tocopherol (90%), dichloromethane (95%), 2-propanol (CHROMASOLV, for HPLC), pyridine (99.8%), and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) were obtained from Sigma-Aldrich (Steinheim, Germany). *n*-Hexane (AnalR NORMAPUR and HiPerSolv CHROMANORM) was purchased from VWR International (Darmstadt, Germany). Methyl *tert*-butyl ether (MTBE) was supplied by Evonik Industries AG (Essen, Germany) and was distilled prior to use. A mixture of steryl/stanyl fatty acid esters (Vegapure 95E) was provided by Cognis GmbH (Illertissen, Germany). Another steryl fatty acid ester mixture was synthesized from rapeseed oil sterols and soybean oil fatty acids according to previously described procedures.[27] The calculated GC-purity was 80 area% and the mixture consisted of 24.8% sitosteryl-18:2, 20.8% campesteryl-18:2, 14.5% sitosteryl-18:0/18:1, 12.0% campesteryl-18:0/18:1, 6.3% brassicasteryl-18:2, 4.8% sitosteryl-16:0/16:1, 4.0% campesteryl-16:0/16:1, 3.7% brassicasteryl-18:0/18:1, 2.6% sitosteryl-18:3, 2.1% campesteryl-18:3, 1.2% brassicasteryl-16:0/16:1, 0.9% brassicasteryl-18:3, and 2.5% others.

Determination of Dry Matter and Lipid Extraction. After manual removal of the shell, the nuts were chopped with a knife, frozen in liquid nitrogen, and ground in a coffee mill to a fine powder. The ground nut samples were stored in plastic bags at −18 °C until analysis.

Table 1. Characteristics of the Investigated Nut Samples

	no.	cultivar	origin	harvest year	supplier
almond	1	–[c]	Italy	2011	Stolzenberg Nuss GmbH, Germany
	2[a]	Non Pareil	U.S.	2011	ReformKontor GmbH & Co.KG., Germany
	3	Non Pareil	U.S.	2011	ReformKontor GmbH & Co.KG., Germany
Brazil nut	1[a]	–	–	–	Seeberger GmbH, Germany
	2[a]	–	Brazil	2011	Stolzenberg Nuss GmbH, Germany
	3[a,b]	–	Bolivia	–	Flores Farm GmbH, Germany
cashew kernel	1	–	U.S.	–	Seeberger GmbH, Germany
	2[b]	–	Indonesia	–	Flores Farm GmbH, Germany
	3	–	–	2011	ReformKontor GmbH & Co.KG., Germany
hazelnut	1[a]	Ennis	U.S.	2010	Stolzenberg Nuss GmbH, Germany
	2[a]	–	France	2010	Unicoque, France
	3[a]	Runde Römer	Italy	2011	ReformKontor GmbH & Co.KG., Germany
macadamia	1	–	South Africa	–	Stolzenberg Nuss GmbH, Germany
	2[b]	–	–	2011	ReformKontor GmbH & Co.KG., Germany
	3[b]	–	Kenia	–	Flores Farm GmbH, Germany
peanut	1	–	China	2011	PENNY Markt GmbH, Germany
	2	–	Israel	2011	real,-Handels GmbH, Germany
	3	–	U.S.	2011	Eurofood Handelsgesellschaft mbH, Germany
pecan nut	1[a]	–	South Africa	–	Stolzenberg Nuss GmbH, Germany
	2[a]	–	–	–	real,-Handels GmbH, Germany
	3[a,b]	–	Peru	–	Flores Farm GmbH, Germany
pine nut	1[b]	–	Turkey	2011	ReformKontor GmbH & Co.KG., Germany
	2[b]	–	Turkey	–	ReformKontor GmbH & Co.KG., Germany
	3[b]	–	Italy	–	Alnatura Produktions- and Handels GmbH, Germany
pistachio	1[a,b]	–	–	–	Kaufland Warenhandel GmbH & Co. KG, Germany
	2	–	Iran	–	Seeberger GmbH, Germany
	3	–	–	–	ReformKontor GmbH & Co.KG., Germany
walnut	1[a]	Serrs	Chile	2011	Stolzenberg Nuss GmbH, Germany
	2[a]	–	Chile	2011	Stolzenberg Nuss GmbH, Germany
	3[a]	Franquette	France	2011	Valcadis, France

[a]Kernels with testa. [b]Organic farming. [c](–) No data available.

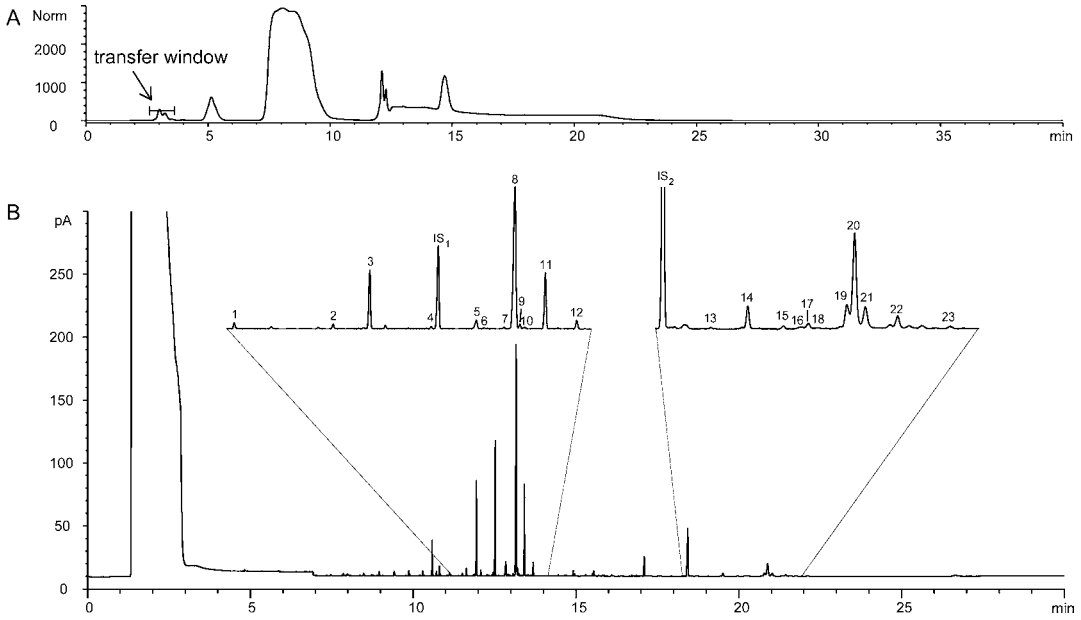

Figure 2. Online LC-GC analysis of free sterols/stanols and steryl/stanyl esters in walnut. (A) LC-UV chromatogram at 205 nm and (B) GC-FID chromatogram of the transferred LC-fraction. (1) squalene, (2) δ-tocopherol, (3) γ-tocopherol, (4) α-tocopherol, (5) campesterol, (6) stigmasterol, (7) clerosterol, (8) sitosterol, (9) Δ^5-avenasterol, (10) sitostanol, (11) cycloartenol, (12) 24-methylene cycloartanol + citrostadienol, (13) campesteryl-16:0/16:1, (14) sitosteryl-16:0/16:1, (15) cycloartenyl-16:0/16:1, (16) campesteryl-18:0/18:1, (17) campesteryl-18:2, (18) campesteryl-18:3, (19) sitosteryl-18:0/18:1, (20) sitosteryl-18:2, (21) sitosteryl-18:3, (22) cycloartenyl-18:2, (23) 24-methylene cycloartanyl-18:2, (IS_1) 5α-cholestan-3β-ol, and (IS_2) cholesteryl-16:0 (For conditions, see Materials and Methods).

The contents of dry matter were determined by drying 5 g of ground nuts in preweighed dishes at 103 ± 2 °C until constant weight (approximately 8 h).[28]

For lipid extraction, 500 μL cholesteryl palmitate (1.0 mg/mL in *n*-hexane/MTBE (3:2, v/v)) and 500 μL 5α-cholestan-3β-ol (1.0 mg/mL in MTBE) were added as internal standards (IS) to 2 g of ground nuts. After addition of a magnetic stir bar, the oil was extracted with 20 mL of *n*-hexane/dichloromethane (1:1, v/v) under stirring at room temperature for 1 h. After filtration, the extraction vessel and the filter were washed, the solvent was removed by rotary evaporation, and the residue was dried at 103 ± 2 °C to constant weight. A total of 50 mg of the obtained oil was dissolved in 5 mL of *n*-hexane. A volume of 250 μL of the solution was transferred into a 1.5 mL vial, and the solvent was removed by a gentle stream of nitrogen. The residue was silylated at 80 °C for 20 min using 75 μL of pyridine and 150 μL of BSTFA/TMCS (99:1, v/v). After silylation, the reagents were removed by a gentle stream of nitrogen and the residue was dissolved in 250 μL of *n*-hexane/MTBE/2-propanol (96:4:0.1, v/v/v) and subsequently used for online LC-GC analysis.

Analysis by Online LC-GC-FID. The applied online LC-GC system consisted of a 1220 Infinity LC, which was coupled to a 7890A GC equipped with a FID via a 1200 Infinity Series 2-position/6-port switching valve (Agilent Technologies, Waldbronn, Germany). The valve was fitted with a 200 μL sample loop.

LC-analyses (20 μL injection volume) were carried out on a 250 mm × 2 mm i.d., 5 μm, Eurospher-100 Si column (Knauer, Berlin, Germany) at 27 °C, using *n*-hexane/MTBE/2-propanol (96:4:0.1, v/v/v) as eluent with a flow of 0.25 mL/min. Ultraviolet detection was performed at 205 nm. The transfer of the LC-fraction was performed by switching the valve 3.7 min after injection. Evaporation of the solvent was performed via a programmable multimode inlet in the solvent vent mode. The injector was set to 50 °C hold for 0.5 min and was then heated at 900 °C/min to 350 °C. Vent flow was adjusted to 1000 mL/min with a vent pressure of 4 psi until 0.5 min. Purge flow to split vent was started at 0.5 min with 2.5 mL/min. A stainless steel transfer line was installed between the valve and the inlet. This line was pressure-controlled to avoid a pushing back of solvent vapors into the transfer line. The pressure was set to 5 psi hold for 0.3 min followed by a ramp of 10 psi/min to 20 psi. GC-separations were carried out on a 30 m × 0.25 mm i.d., 0.1 μm film, Rtx-200MS fused-silica capillary column (Restek GmbH, Bad Homburg, Germany). Hydrogen was used as carrier gas with a constant flow of 1.5 mL/min. The oven temperature program was as follows: initial temperature, 40 °C (2 min); programmed at 100 °C/min to 100 °C, then at 15 °C/min to 310 °C (2 min), and at 1.5 °C/min to 340 °C (3 min). The detector temperature was set to 360 °C and nitrogen was used as makeup gas (25 mL/min). Data acquisition was performed by ChemStation.

Quantitation by Online LC-GC-FID. Free sterols/stanols were quantitated as their trimethylsilyl (TMS)-derivatives using 5α-cholestan-3β-ol as internal standard (IS); the employed response factor (Rf) of 1.0 was experimentally determined using stigmasterol and sitostanol as representatives.

The responses of individual steryl/stanyl esters were determined within the calibration range using five-point calibration functions and showed good linearity (coefficient of determination (R^2) ≥0.995). On this basis, steryl/stanyl fatty acid esters were subsequently quantitated by generating three-point calibration functions either of the synthesized or of the commercially obtained steryl ester mixture (range: 0.8−14.2 or 0.8−20.8 ng total steryl/stanyl esters per μL of injection volume, respectively) in the presence of 5 ng/μL cholesteryl palmitate. Each calibration point was done in triplicate and linear regression was confirmed in ratio of areas (area steryl or stanyl ester/area IS) and amounts (amount steryl or stanyl ester/amount IS). Steryl/stanyl esters that were not included in the mixtures were quantitated with a Rf of 1.0 in relation to the IS cholesteryl palmitate.

Tocopherols and squalene were calculated via 5α-cholestan-3β-ol as IS with a Rf of 1.2 and 0.92, respectively.

Identification by Online LC-GC-MS. Identification was carried out on an online LC-GC-MS system. The GC part was coupled via a transfer line to an inert 5975C mass spectrometer with triple axis detector (Agilent Technologies, Waldbronn, Germany). Mass spectra were obtained by positive electron impact ionization at 70 eV in the scan mode at unit resolution from 40 to 700 Da. The interface was heated to 280 °C, the ion source to 250 °C, and the quadrupole to 150 °C. GC-separations were performed on a 30 m × 0.25 mm i.d., 0.1 μm film, Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). The remaining GC-conditions were as described for online

Table 2. Contents of Lipids and Dry Matter as well as Contents and Compositions of Free Sterols/Stanols and Steryl/Stanyl Fatty Acid Esters in Nuts

	almonds	Brazil nuts	cashews	hazelnuts	macadamias	peanuts	pecan nuts	pine nuts	pistachios	walnuts
lipid content [%][a]	45.3 ± 7.1	63.0 ± 3.0	44.9 ± 1.1	52.0 ± 4.8	57.9 ± 3.1	46.7 ± 0.5	67.4 ± 0.8	42.4 ± 4.0	44.3 ± 1.2	58.5 ± 2.0
dry matter [%][b]	97.2 ± 0.2	97.5 ± 0.1	95.8 ± 0.1	96.7 ± 0.2	97.8 ± 0.2	97.8 ± 0.5	95.6 ± 2.6	95.4 ± 1.1	96.6 ± 0.8	97.2 ± 0.2
∑ free sterols/stanols [mg/g nut][a]	1.09 ± 0.17	0.89 ± 0.08	0.65 ± 0.03	0.62 ± 0.13	1.18 ± 0.07	0.84 ± 0.11	1.12 ± 0.12	1.57 ± 0.10	1.61 ± 0.22	0.95 ± 0.19
∑ free sterols/stanols [μg/100 mg oil][a]	241.7 ± 10.5	141.3 ± 10.4	146.1 ± 8.8	118.7 ± 14.6	204.7 ± 11.9	180.0 ± 25.7	165.9 ± 18.4	369.7 ± 13.7	362.6 ± 48.1	162.4 ± 34.3
cholesterol[%][c]	–[f]	–	<LOD-0.4	–	–	–	–	–	–	–
campesterol [%][c]	2.7 ± 0.4	1.6 ± 0.4	6.3 ± 0.4	4.9 ± 0.9	7.4 ± 0.7	11.2 ± 1.0	3.4 ± 0.2	15.6 ± 0.4	4.5 ± 0.3	3.8 ± 0.2
stigmasterol [%][c]	1.1 ± 0.3	4.8 ± 0.3	0.4 ± 0.2	1.3 ± 0.2	–	11.0 ± 3.4	1.3 ± 0.2	<LOQ-0.2	0.6 ± 0.2	0.3 ± 0.1
campestanol [%][c]	–	–	–	–	–	–	–	<LOQ-0.2	–	–
clerosterol [%][d]	1.2 ± 0.0	1.0 ± 0.1	1.7 ± 0.0	1.4 ± 0.1	0.9 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.7 ± 0.2	1.4 ± 0.2	0.6 ± 0.0
sitosterol [%][c]	86.0 ± 2.2	60.9 ± 2.6	75.6 ± 3.4	82.2 ± 0.5	84.7 ± 2.5	67.6 ± 2.2	75.2 ± 3.6	76.2 ± 0.9	80.7 ± 1.3	69.4 ± 0.6
Δ^5-avenasterol [%][d]	0.7 ± 0.0	1.2 ± 0.3	0.6 ± 0.2	0.8 ± 0.1	0.3 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	5.8 ± 0.4	2.0 ± 0.1	1.6 ± 0.3
sitostanol [%][c]	2.5 ± 0.3	3.9 ± 0.1	1.1 ± 0.2	2.8 ± 0.2	0.8 ± 0.2	1.2 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.4 ± 0.0	0.8 ± 0.0
Δ^7-sitosterol [%][d]	2.2 ± 0.6	3.0 ± 0.3	–	1.8 ± 1.2	–	–	1.1 ± 0.1	–	1.1 ± 0.2	–
α-amyrin [%][d]	–	17.0 ± 2.3	–	–	–	–	–	–	0.7 ± 0.1	–
β-amyrin [%][d]	–	4.7 ± 1.0	–	–	–	1.5 ± 0.2	1.2 ± 0.8	–	0.4 ± 0.1	–
cycloartenol [%][c]	<LOQ[g]-1.6	0.8 ± 0.2	6.8 ± 1.8	–	–	1.9 ± 1.4	8.5 ± 2.4	–	1.5 ± 0.4	20.3 ± 0.5
cycloartanol[%][c]	–	–	1.9 ± 0.4	–	–	–	–	–	–	–
24-methylene cycloartanol[c] + citrostadienol[d][%]	3.5 ± 1.3	1.2 ± 0.2	5.4 ± 1.4	4.7 ± 0.7	5.8 ± 1.9	3.9 ± 1.2	6.8 ± 0.6	0.5 ± 0.3	6.1 ± 0.9	3.2 ± 0.5
∑ steryl fatty acid esters [mg/g nut][a]	0.33 ± 0.12	0.11 ± 0.05	0.24 ± 0.07	0.24 ± 0.03	0.17 ± 0.02	0.28 ± 0.17	0.28 ± 0.12	1.07 ± 0.18	1.26 ± 0.67	0.30 ± 0.06
∑ steryl fatty acid esters [μg/100 mg oil][a]	74.8 ± 34.8	17.7 ± 7.2	52.5 ± 15.1	46.8 ± 6.8	29.3 ± 4.9	60.2 ± 36.8	41.3 ± 18.5	253.8 ± 48.6	282.4 ± 142.9	51.3 ± 11.4
campesteryl-16:0/16:1 [%][e]	<LOQ	<LOQ-1.5	0.7 ± 0.5	0.5 ± 0.4	1.3 ± 0.3	1.5 ± 0.6	<LOQ-0.5	0.3 ± 0.1	0.3 ± 0.2	<LOQ-0.3
sitosteryl-16:0/16:1 [%][e]	1.8 ± 1.3	12.6 ± 3.8	6.6 ± 1.8	7.9 ± 0.9	13.2 ± 2.4	4.5 ± 0.4	4.6 ± 2.5	1.1 ± 0.0	2.2 ± 1.1	5.1 ± 2.1
cycloartenyl-16:0/16:1 [%][e]	–	–	1.4 ± 0.4	–	–	–	2.1 ± 1.1	–	–	1.2 ± 0.2
campesteryl-18:0/18:1 [%][e]	1.1 ± 0.7	<LOQ-3.0	3.0 ± 1.0	3.5 ± 1.1	5.3 ± 0.8	4.4 ± 2.8	1.4 ± 1.1	1.8 ± 0.2	0.9 ± 0.5	1.0 ± 0.3
campesteryl-18:2 [%][e]	5.7 ± 1.2	<LOQ-8.4	6.7 ± 2.2	2.3 ± 1.2	2.0 ± 1.3	14.1 ± 2.8	2.5 ± 0.5	13.9 ± 0.5	4.8 ± 1.0	2.4 ± 0.3
campesteryl-18:3 [%][e]	–	–	–	–	–	–	–	–	–	1.5 ± 0.2
stigmasteryl-18:2 [%][e]	<LOQ-2.9	<LOD-2.4	–	–	–	1.4 ± 0.7	–	–	–	–
sitosteryl-18:0/18:1 [%][e]	24.5 ± 11.9	30.7 ± 5.4	31.8 ± 3.0	54.6 ± 4.7	56.7 ± 4.7	19.0 ± 9.8	26.5 ± 8.3	7.9 ± 0.5	13.0 ± 4.6	11.9 ± 1.6
sitosteryl-18:2 [%][e]	65.9 ± 11.1	48.2 ± 6.1	41.3 ± 9.2	30.5 ± 2.3	21.5 ± 3.9	54.4 ± 14.1	50.1 ± 14.1	71.9 ± 1.9	73.0 ± 4.9	49.2 ± 3.7
sitosteryl-18:3 [%][e]	–	–	–	–	–	<LOQ-2.1	–	–	–	20.8 ± 1.0
cycloartenyl-18:0/18:1 [%][e]	–	–	<LOQ-4.6	–	–	–	3.9 ± 1.6	–	–	<LOQ
cycloartenyl-18:2 [%][e]	–	–	3.3 ± 0.4	–	–	–	3.6 ± 1.5	–	2.7 ± 1.5	4.9 ± 1.0
24-methylene cycloartanyl-18:0/18:1 [%][e]	–	–	1.4 ± 0.5	–	–	–	–	–	–	–
24-methylene cycloartanyl-18:2 [%][e]	–	–	1.0 ± 0.3	–	–	–	–	<LOQ-0.9	3.1 ± 1.4	1.8 ± 0.8
others	–	–	–	<LOQ-2.4	–	–	5.2 ± 0.9	2.6 ± 0.3	–	–

[a]Means ± standard deviations of three samples, analyzed in triplicate. [b]Means ± standard deviation of three samples, analyzed in duplicate. [c]Identified on the basis of commercially obtained reference compounds. [d]Tentatively identified according to mass spectrometric data from literature.[29−31] [e]Identified on the basis of synthesized reference compounds. [f](–) Below limit of detection (LOD): 5 ng/mL of injection volume (i.v.) for free sterols/stanols and 14 ng/mL i.v. for steryl fatty acid esters (determined on the basis of 20 μL i.v.). [g]Below limit of quantitation (LOQ): 15 ng/mL i.v. for free sterols/stanols and 40 ng/mL i.v. for steryl fatty acid esters (determined on the basis of 20 μL i.v.).

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LC-GC-FID analysis. Data acquisition was performed by MSD Productivity ChemStation.

Minor sterols, which were not commercially available, were tentatively identified by comparison of their mass spectral data to those reported in the literature.[29−31] The following characteristic mass fragments (m/z (%)) were used: clerosterol {484 (6), 469 (2), 394 (8), 379 (4), 355 (7), 255 (4), 129 (58), 55 (100)}; Δ^5-avenasterol {484 (3), 394 (5), 379 (2), 255 (5), 213 (10), 129 (24), 55 (100)}; β-amyrin {498 (1), 483 (1), 408 (2), 393 (2), 255 (1), 213 (1), 218 (100), 129 (20), 55 (29)}; Δ^7-sitosterol {486 (22), 471 (7), 396 (5), 381 (6), 255 (47), 213 (21), 129 (26), 55 (100)}; α-amyrin {498 (1), 483 (1), 408 (1), 393 (2), 255 (2), 213 (1), 218 (100), 129 (20), 55 (35)}; citrostadienol {498 (2), 483 (4), 400 (16), 393 (4), 357 (41), 255 (2), 213 (7), 129 (28), 55 (96)}.

Recovery and Repeatability. Recoveries were determined by spiking 2 g of ground walnut, hazelnut, and peanut with defined amounts of selected reference compounds: 100 μg stigmasterol and 700 μg sitosterol as reference compounds for free sterols/stanols; 100 μg of stigmasteryl palmitate as reference compound for steryl/stanyl fatty acid esters; 50 μg of α-tocopherol and δ-tocopherol, respectively, as reference compounds for tocopherols. The recovery experiments were performed in triplicate for each nut type; the means and standard deviations were calculated on the basis of all individual values obtained for the respective substance class. The repeatability of the approach, comprising lipid extraction and online LC-GC analysis, was confirmed by working up a control sample (ground walnut) once on each day of analysis (in total, ten replicates). The limits of detection (LOD) and the limits of quantitation (LOQ) were determined via the linear regression methodology according to German standard procedures and criteria,[32] and were calculated on the basis of the respective injection volume (20 μL). Each regression analysis was performed in triplicate.

RESULTS AND DISCUSSION

Online LC-GC Analysis. The simultaneous analysis of free sterols/stanols and intact steryl/stanyl fatty esters as well as of tocopherols and squalene in ten different types of nuts was performed via online LC-GC. The applied online LC-GC system was equipped with a programmable temperature vaporizer interface and has recently been successfully established for the analysis of free sterols/stanols and intact steryl/stanyl esters in cereal grains.[26] Due to silylation of the nut lipids, steryl/stanyl fatty acid esters and squalene as well as the TMS-derivatives of free sterols/stanols and tocopherols eluted at the same time under the employed LC-conditions and could thus be transferred together to the GC. Hence, all compounds of interest could be analyzed within one single GC-run. The online LC-GC analysis is exemplarily shown for a walnut sample in Figure 2.

Additionally, all samples were screened for the presence of *trans*-steryl/stanyl ferulic acid esters, which can be transferred in a separate LC-fraction to the GC, as shown previously for corn.[26] However, no esters of that type could be detected in the investigated tree nut and peanut samples.

Recoveries were determined by spiking walnut, peanut, and hazelnut meal with known amounts of selected reference compounds and were on average 98.6 ± 3.6% for free sterols/stanols, 96.3 ± 5.4% for steryl/stanyl esters, and 104.9 ± 3.7% for tocopherols. To confirm the repeatability of the approach, a ground walnut sample was analyzed as control sample once on each day of analysis. The low relative standard deviations of the total contents of free sterols/stanols (3.0%), steryl/stanyl esters (8.7%), and tocopherols (3.1%) of ten replicate analyses indicated a good repeatability of the approach.

Total Lipids. The average lipid contents determined in the present study ranged from 42% in pine nuts to 67% in pecan nuts (Table 2); they were comparable to those previously described.[20,33−37] For some nut types, large variations in lipid contents have been reported; such differences may be explained by the influence of cultivar, geographical location, and growing conditions as well as by the employed extraction procedures.[13,17,19,38]

Free Sterols/Stanols. *Compositions.* Five sterols as well as one stanol (sitosterol, campesterol, 24-methylene cycloartanol (coeluted with citrostadienol), Δ^5-avenasterol, clerosterol, and sitostanol) could be identified and quantitated in the investigated nuts (Table 2). Sitosterol was consistently predominant, accounting for >60% of total free sterols/stanols. This is in agreement with previous findings, where sitosterol was also identified as the main free sterol in almonds, hazelnuts, peanuts, and walnuts as well as in refined peanut and walnut oils.[23−25,39] Campesterol was generally the second most abundant sterol in macadamias, hazelnuts, peanuts, and pine nuts. Cycloartenol, which could be detected in 19 of the 30 investigated nut samples, was the second most abundant sterol in walnuts, accounting for approximately 20% of total free sterols/stanols. This is in agreement with the few studies, in which total sterols/stanols in walnuts have been analyzed;[20,40] other studies, in turn, did not mention cycloartenol.[12,15,19,23,25] Even though Δ^5-avenasterol was present in all samples, the amounts were lower than 2%, with the exception of pine nuts where it made up on average 5.8%. Several studies examining total sterols/stanols in pine nuts also detected high amounts of Δ^5-avenasterol.[16,18,20] Relatively high levels of α- and β-amyrin were characteristic for Brazil nuts. High contents of α-amyrin have also been observed during analysis of total sterols/stanols in Brazil nuts; β-amyrin, in turn, has not been mentioned.[20] Sitostanol was a minor component accounting for 0.8−3.9% of total free sterols/stanols.

Contents. On the basis of fresh nuts, pine nuts and pistachios revealed the highest total levels of free sterols/stanols, averaging 1.57 and 1.61 mg/g, respectively (Figure 3). The average total

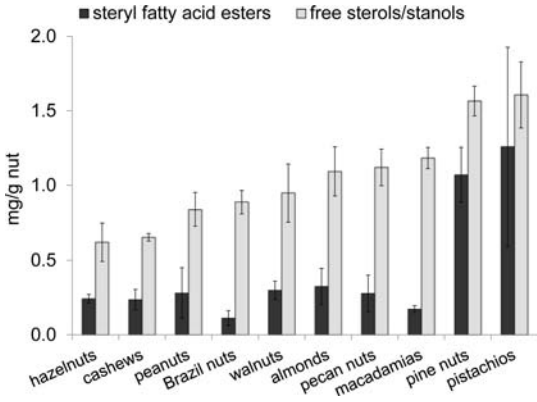

Figure 3. Mean total contents of steryl fatty acid esters and free sterols in different types of nuts.

contents of the other nut types ranged from 0.62 to 1.18 mg/g, being lowest in hazelnuts and cashew kernels. Despite the variations between the different nuts, the profiles and total contents of free sterols/stanols within a certain type of nut were almost comparable (Table 2). This phenomenon was observed for all nuts; as examples, the total contents of the three investigated samples of almonds and pistachios are shown in Table 3. The average total contents of free sterols/stanols determined in the extracted nut oils were between 0.15 and

Table 3. Total Contents of Free Sterols/Stanols and Steryl Esters Determined in the Three Samples of Almonds and Pistachios

	free sterols/stanols [mg/g nut]	steryl fatty acid esters [mg/g nut]
almond no. 1[a]	1.22 ± 0.01[b]	0.40 ± 0.02
almond no. 2	0.91 ± 0.02	0.39 ± 0.01
almond no. 3	1.16 ± 0.02	0.18 ± 0.00
pistachio no. 1	1.85 ± 0.02	0.75 ± 0.02
pistachio no. 2	1.43 ± 0.05	1.01 ± 0.05
pistachio no. 2	1.54 ± 0.03	2.02 ± 0.14

[a]The sample numbers correspond to those in Table 1. [b]Means ± standard deviations, triplicate analysis.

0.37%, being highest in pine nuts and pistachios and lowest in cashew nuts (Table 2). The contents of free sterols/stanols of 0.3% in hazelnut oil, 0.6% in almond oil, and 0.7% in walnut oil, as determined by Momchilova and Nikolova-Damyanova, are above these levels.[23] The total content of free sterols/stanols, which has been reported for a single almond sample, was comparable to the amounts determined in the present study; those for Brazil nuts, hazelnuts, peanuts, pecan nuts, and walnuts were somewhat higher, but in the same order of magnitude.[22,24] The levels quantitated for a single pine nut and pistachio sample were, in turn, 1.9- or 2.8-fold lower.[22]

Steryl/Stanyl Fatty Acid Esters. *Compositions.* Up to 14 individual steryl fatty acid esters were identified (Table 2). Stanols, which were found in small amounts in free form, could, however, not be detected as fatty acid esters. Six steryl esters, namely sitosteryl-18:2, sitosteryl-18:0/18:1, campesteryl-18:2, campesteryl-18:0/18:1, sitosteryl-16:0/16:1, and campesteryl-16:0/16:1, were present in all samples. The main part of the fatty acids occurred as sitosteryl esters of at least 74% (cashew no. 2), most often followed by esters of campesterol. 4,4-Dimethylsteryl fatty acid esters were detected in cashew nuts, pecan nuts, pine nuts, pistachios, and walnuts, where they represented 0.9−13.9% of total steryl fatty acid esters. Considering the fatty acid part, the majority of steryl esters was made up by C18-fatty acids, accounting for 83.0−99.6%. In detail, sitosteryl-18:2 was the predominant ester in almonds, Brazil nuts, cashew kernels, peanuts, pecan nuts, pine nuts, pistachios, and walnuts. Amounts of C16-fatty acid esters were remarkably high in macadamias (12.1−17.0%) and Brazil nuts (10.1−16.9%). In contrast to all other investigated nuts, the majority of sterols in hazelnuts and macadamias occurred as esters of oleic/stearic acid. Walnuts showed a distinct profile with esters of linolenic acid accounting for approximately 22% of total steryl fatty acid esters. A previously published study reported that sitosterol was the only sterol present within the fractions of steryl esters isolated from hazelnuts and walnuts.[23] Furthermore, the described fatty acid compositions of the steryl esters, particularly regarding walnuts, differed from that determined in the present study. Other studies reported sitosterol, campesterol, stigmasterol, and Δ^5-avenasterol as main esterified sterols in walnut and peanut oils.[24,39]

Under the employed GC-conditions, the resolution of the individual steryl esters was hampered for steryl esters of palmitic/palmitoleic acid and stearic/oleic acid. The exact distributions of these acids were determined by GC-FID as fatty acid methyl esters after methanolysis of the respective steryl fatty acid ester fraction isolated via SPE for one sample of each kind of nut.[41] The ratios of palmitic/palmitoleic acid in hazelnuts, pecan nuts, and Brazil nuts were 73:1, 94:1, and 118:1, respectively. In almonds, cashew nuts, peanuts, pine nuts, and pistachios no palmitoleic acid was detected. The steryl esters of macadamia nuts, in turn, revealed a remarkably high amount of palmitoleic acid (ratio palmitic/palmitoleic: 1:1). A predominance of oleic acid was observed in all nut samples. The amounts of oleic acid were 2- to 19-fold above the amounts of stearic acid, being lowest in Brazil nuts and highest in pistachios.

Contents. In agreement with the results obtained for free sterols/stanols, pine nuts and pistachios also revealed the highest levels of steryl fatty acid esters, averaging 1.07 and 1.26 mg/g (Figure 3). Six nut types showed levels in the range of 0.24 and 0.33 mg/g. The lowest total contents were determined in Brazil nuts and macadamias (0.11 and 0.17 mg/g, respectively). Large variations were observed in the total contents not only between the different nut types, but also within a single kind of nut. Particularly, the three individual samples of almonds, Brazil nuts, peanuts, pecan nuts, and pistachios exhibited considerable differences in their steryl/stanyl fatty acid esters levels. This effect is exemplarily shown for almonds and pistachios, whose amounts ranged from 0.18−0.40 mg/g and from 0.75−2.02 mg/g, respectively (Table 3). The profiles of the individual esters were, in turn, almost comparable. Due to the lack of information on horticultural characteristics, e.g., growing conditions or cultivar, the variation in total contents cannot be explained at the moment. The amounts of steryl fatty acid esters, which have previously been described for almonds, hazelnuts, peanuts, and pecan nuts, are in good agreement with the present data.[22,24] The levels reported for Brazil nuts and walnuts were slightly higher; those of pine nuts and pistachios lower.[22] Momchilova and Nikolova-Damyanova[23] reported contradictory results as on the one hand they could not detect steryl esters in almonds, and on the other hand the contents determined in hazelnuts and walnuts were 10- and 31-fold above the levels calculated in the present study.

Proportions of Free Sterols/Stanols and Steryl Fatty Acid Esters. All tree nut and peanut samples exhibited higher amounts of free sterols/stanols than of steryl fatty acid esters, with the exception of a single pistachio sample, which revealed a reversed distribution. The dominance of free sterols/stanols in nuts has already been reported for almonds, Brazil nuts, hazelnuts, peanuts, pecan nuts, pine nuts, pistachios, and walnuts.[22,24,42] Only one study described higher amounts of steryl esters than of free sterols in hazelnuts and walnuts.[23]

Distribution Patterns of Total Fatty Acids and of Fatty Acids Esterified with Sterols. The total fatty acid compositions of the nuts determined in the present study (data not shown) are in accordance with earlier reported results.[11,12,14,15,17,20,36−38,43,44] However, the distributions of fatty acids esterified to sterols, calculated on the basis of intact esters, differed from those of the total lipids. The steryl fatty acid esters of all nuts contained higher proportions of linoleic acid and lower proportions of oleic/stearic acid than the corresponding total lipids. Moreover, palmitic/palmitoleic acid were found to be esterified to a lower degree with sterols than in the whole oil, except for Brazil nuts, cashew kernels, hazelnuts, and pecan nuts, where the proportions were almost equal. Linolenic acid, which was detected as ester with sitosterol in walnuts and one peanut sample, was found to a lower proportion in total lipids. The described observations are exemplarily shown for the mean fatty acid compositions of walnuts and pine nuts in Figure 4.

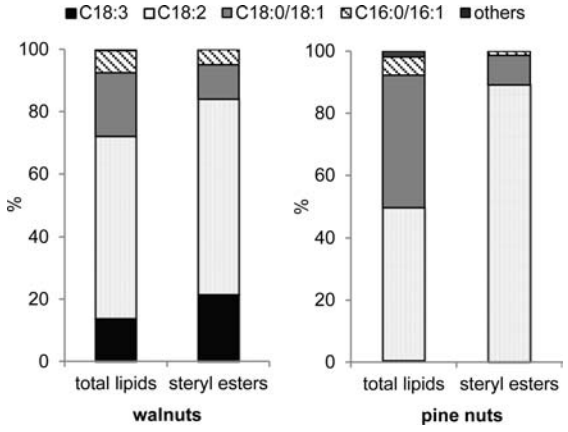

Figure 4. Mean total fatty acid compositions in total lipids and in steryl/Stanyl esters of walnuts and pine nuts.

Comparable results have previously been reported for the fatty acid distributions of two peanut cultivars.[24]

Compositions and Contents of Tocopherols. The compositions and contents of tocopherols determined in the different nut types are given in Table 4. γ-Tocopherol was the predominant form in Brazil nuts, cashew kernels, pecan nuts, pine nuts, pistachios, and walnuts, whereas α-tocopherol was most abundant in almonds and hazelnuts. δ-Tocopherol was present in all samples, except for hazelnuts and macadamias. The results obtained for the distributions of tocopherols in almonds, Brazil nuts, cashew kernels, hazelnuts, pecan nuts, pine nuts, pistachios, and walnuts are comparable to those described in literature and total contents, which ranged from 0.07 ± 0.00 mg/g in cashew kernels to 0.33 ± 0.07 mg/g in pine nuts were in the same order of magnitude.[14,15,19,20,22,33−35,45−48] In contrast to all other tree nuts, tocopherols could not be detected in macadamia nuts. This is in agreement with data reported by Kornsteiner et al.[34] Robbins et al.[20] reported γ-tocopherol contents of 0.1 ± 0.2 μg/g for three independent macadamia nuts, but they could not detect α-, β-, and δ-tocopherol. Kaijser et al.[11] identified α- and δ-tocopherol in macadamias, but total amounts were lower than 5.6 μg/g oil; Maguire et al. [33] reported, in turn, levels of about 185 μg/g oil. The three investigated peanut samples revealed large differences in their tocopherol contents and compositions. A 36-fold margin was observed between the highest and lowest tocopherol level. Whereas γ-tocopherol was predominant in two peanut samples with percentage amounts of 84% and 65%, respectively, the tocopherols of the third sample consisted mainly of δ-tocopherol (40%); α- and γ-tocopherol both made up 30%, respectively. Considering the fact that the peanuts investigated in the present study were roasted, comparison to literature is difficult as roasting conditions as well as storage of roasted peanuts have been shown to influence tocopherol contents and compositions.[49,50] Literature data concerning tocopherols in raw peanuts are also inconsistent, whereas α- and γ-tocopherol each made up approximately 48% in a total of 151 raw peanut samples,[51] other studies identified α-tocopherol as the predominant form in peanuts.[52,53]

Contents of Squalene. The contents of squalene showed large variations between the different kinds of nuts (Table 4). The lowest values were determined in walnuts and pine nuts, averaging 0.01 and 0.02 mg/g, respectively. The other nut types exhibited squalene contents between 0.06 and 0.18 mg/g, with the exceptions of Brazil nuts, which contained by far the highest

Table 4. Contents and Compositions of Tocopherols and Squalene in Nuts

	almonds	Brazil nuts	cashews	hazelnuts	macadamias	peanuts	pecan nuts	pine nuts	pistachios	walnuts
$\sum$ tocopherols [mg/g nut][a]	0.21 ± 0.10	0.16 ± 0.02	0.07 ± 0.00	0.17 ± 0.10	–	0.05 ± 0.04	0.24 ± 0.04	0.33 ± 0.07	0.25 ± 0.02	0.26 ± 0.04
$\sum$ tocopherols [μg/100 mg oil][a]	49.6 ± 30.4	26.1 ± 3.7	15.5 ± 1.0	30.8 ± 17.2	–	12.0 ± 12.7	35.2 ± 6.0	77.0 ± 10.8	56.9 ± 6.3	45.0 ± 5.8
α-tocopherol [%][b]	94.7 ± 1.3	28.8 ± 5.7	6.1 ± 1.2	93.1 ± 2.3	–	21.0 ± 15.0	6.1 ± 4.3	9.9 ± 1.8	2.4 ± 1.9	3.2 ± 0.4
γ-tocopherol [%][b]	4.6 ± 0.2	69.9 ± 5.5	88.6 ± 0.4	6.9 ± 2.3	–	59.3 ± 27.4	93.4 ± 4.4	89.3 ± 1.9	95.2 ± 1.8	87.8 ± 2.2
δ-tocopherol [%][b]	<LOQ[c]-2.0	1.3 ± 0.2	5.3 ± 0.9	–[d]	–	19.7 ± 17.7	0.6 ± 0.1	0.8 ± 0.2	2.4 ± 0.1	9.0 ± 2.5
squalene [mg/g nut][a,b]	0.06 ± 0.03	1.11 ± 0.05	0.06 ± 0.04	0.13 ± 0.02	0.16 ± 0.05	0.11 ± 0.04	0.18 ± 0.05	0.02 ± 0.00	0.07 ± 0.01	0.01 ± 0.00
squalene [μg/100 mg oil][a,b]	13.4 ± 6.2	177.3 ± 16.0	12.7 ± 8.3	24.9 ± 1.9	27.4 ± 9.0	30.0 ± 13.1	26.1 ± 7.2	3.9 ± 1.0	15.5 ± 2.4	1.7 ± 0.6

[a]Means ± standard deviations of three samples, analyzed in triplicate. [b]Identified on the basis of commercially obtained reference compounds. [c]Below limit of quantitation (LOQ): 16 ng/mL i.v. (determined on the basis of 20 μL i.v.). [d](–) Below limit of detection (LOD): 6 ng/mL i.v. (determined on the basis of 20 μL i.v.).

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amounts, with 1.11 mg/g on average. The observed values are in agreement with those reported elsewhere.[13,33,35]

In conclusion, the present study extends the current knowledge regarding minor lipids in nuts by the spectrum of free sterols/stanols and individual steryl fatty acid esters. The data provide new and detailed information on the contents and compositions of these lipid constituents in ten commercially important nut types. The analysis was enabled by the application of a comprehensive online LC-GC-based approach. This methodology further allowed the simultaneous analysis of tocopherols and squalene in addition to the steryl conjugates. Among the studied nuts, pistachios and pine nuts revealed the highest contents of free sterols/stanols and steryl/stanyl fatty acid esters; tocopherols were highest in pine nuts and squalene highest in Brazil nuts. Considerable differences were observed between the various nut types not only in content, but also in the distribution patterns of free sterols/stanols, steryl/stanyl fatty acid esters, and tocopherols. As information on cultivar, harvest year, stage of maturity or growing conditions was not available for most of the examined nuts, further studies should be designed to investigate the influence of these parameters on the contents and profiles of the minor lipid constituents.

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Notes

The authors declare no competing financial interest.

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