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Semi quantitative chemical comparison of products obtained from rosehip pseudofruit and shell

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Abstract
Data from anti-inflammatory and antioxidant *in vitro* studies suggest that rosehip shell powder should be more effective than that of whole pseudo fruit powder. However, clinical data do not support the *in vitro* results. We used a photometric assay, ultra performance liquid chromatography and gas chromatography to quantify total phenols, total tannins, flavonoids, procyanidins and fatty acids for ten products from *Rosa canina* L. pseudo fruit and shell to compare their phytochemical compositon in order to get detailed information on their phytoequivalence. Our analyses showed that the content of important active compounds varies greatly between rose hip products and that the Folin-Ciocalteu's assay values were inappropriate to reflect the content of active compounds. UPLC® methods are required for the determination of flavonoids and procyanidins, and a gas chromatography method for the fatty acids, to obtain comparable information on the content of co-active ingredients in the rosehip products. The fermented aqueous rose hip shell extract contained more ellagic acid but lessof other co-active ingredients than the powders produced from rose hip shell or whole pseudofruit. The pseudofruit powder Litoflex ®is presently the only product with moderate evidence of effectiveness. Due to a lack of phytoequivalence, dose-determining studies are required for all other rose hipproducts before they should be considered in treatment schedules of osteoarthritis. Collagen hydro lysate and glucosamine mixtures with small quantities of aqueous rose hip shell extracts do not seem to be promising for osteoarthritic complaints as the evidence for efficacy is weak and our analysis found no traces of rose hip actives in these mixtures.

Keywords: Rose hip, shell, pseudofruit, aqueous extract, rose hip mixtures, osteoarthritis

Introduction
In vitro studies suggest that rose hip shell powder is clinically superior to whole pseudofruit powder in terms of anti-inflammatory and anti-oxidative [1] as well as chondroprotective activity [2]. These correlate well with a higher content of known anti-inflammatory and chondroprotective compounds in the shell compared to the seed [1-3]. However, four exploratory clinical studies have so far been unable to demonstrate any superiority of the shell powder versus whole pseudofruit powder in the treatment of osteoarthritic complaints. Three of the studies included patients suffering from osteoarthritis or chronic low back pain. The studies compared daily doses of (i) 2.25 g and 4.5 g shell powder versus 4.5 g pseudofruit powder (50% seed) [4], (ii) 2.2 g powder (10% seed) versus placebo [5] and (iii) 20 g shell powder (the control group consumed 5-10 g pseudofruit powder per day[6]. The remaining study included participants with self-reported knee complaints of unknown pathological origin. Compared to placebo, daily consumption of 2.25 gover 12 weeks did not alleviate pain or knee function nor the quality of life [7]. The primary outcome measure of this study was "biomechanical knee joint function during walking". Whether the observed increase in joint dynamics of the shell powder over placebois of clinical relevance still needs to be demonstrated. A dose of 2.25 g shell powder per day was ineffective in alleviating painful conditions and a dose of 20 g per day was not superior to 5-10 g pseudofruit powder per day. The aim of this study was to compare the phyto chemical compositon of various rose hip products in order to getdetailed information about their phyto equivalence.

Methods
Materials: (+)-Catechin hydrate ≥ 98%, 5,7-dimethoxycoumarin 98%, boron trifluoride-methanol solution 10%, acetic acid, F.A.M.E. Mix C_8-C_{22}, methyl oleate 99%, methyl linolenate ≥ 99% and methyl nonadecanoate ≥98% (GC) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Folin Ciocalteu's reagent, caffeic acid Reag. DAB, 2,6-di-tert-butyl-p-kresol for synthesis, hyperoside Reag.

Ph. Eur., methanol and acetonitrile were obtained from Merck Chemicals GmbH (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) anh., ellagic acid 95%, hesperidin ≈CHR and formic acid were from Carl Roth GmbH & Co. KG (Karlsruhe, Germany); quercetin dihydrate 97%was from Alfa Aesar (Thermo Fisher GmbH, Karlsruhe, Germany); n-hexane with less than 0,01% of water was from VWR International GmbH (Darmstadt, Germany); taxifolin 99%, quercitrin 90% and tiliroside 96% were from Phyto Lab GmbH & Co. KG (Vestenbergsgreuth, Germany); Freiberg hide powder was from the Research Institute for Leather and Plastic Sheeting GmbH (Freiberg, Germany). The water for analysis was generated by an Astacus Reagent bench (Membra Pure GmbH, Henningsdorf/Berlin, Germany).

Study Medications: The characteristics of the eleven products investigated are summarized in Table 1 (3aqueousextracts, 7powders).

Sample preparation for the Ultra Performance Liquid Chromatography (UPLC®) analysis: Samples were weighed (45.1 mg - 49.8 mg) and extracted in 1.00 mL methanol containing 5,7 dimethoxycoumarin (0.2 mg/mL) as internal standard in a supersonic bath at room temperature for 30 min and then centrifuged and filtrated. Extraction was performed in triplicates to ensure the reproducibility of this procedure.

Quantification of flavonoids and caffeic acid derivatives: An Acquity UPLC® equipped with an Acquity H-Class quaternary solvent manager, an Acquity FTN auto sampler, an Acquity eλ PDA detector, an Acquity FLR detector (Waters GmbH, Eschborn, Germany) and a Luna® omega 1.6 µm C18 100 Å 100 x 2.1 mm (Phenomenex, Aschaffenburg, Germany) was used. Column oven temperature was adjusted to 50 °C and the eluent passed a preheater with the same temperature. Injection volume was1 µL. Taxifolin, ellagicacid, hyperoside, quercitrin, hesperidin, quercetin and tiliroside were identified via reference substances. The other peaks with UV maxima from 308-335 nm were quantified as caffeicacid, peaks with UV maxima from 335-375 nm and the identified flavonoids as quercetin, while ellagicacid was quantified for its own, respectively. The quantification was achieved by 5,7-dimethoxycumarin as internal standard (5,7-dimethoxycoumarin: y = 954.1x +2273.8 (R^2 = 0.9995), 1.9-1975.8 µM, concentration of internal standard = 0,2 mg/mL; caffeic acid: y = 753.9x +1282.9 (R^2 = 0.9996), 2.8-2879.7 µM, correction factor = 1.29; quercetin: 1626.2x - 13980.0 (R^2 = 0.9995), 1.7-1691.4 µM, correction factor = 0.60; ellagic acid: y =961.0x – 3411.5 (R^2 = 0.9993), 1.4-1415.0 µM, correction factor = 1.02 ; calibration experiments were performed three times in duplicates). Concentrations were calculated as µmol per 1 g product [µmol/g]. A binary gradient with water (eluent A) and acetonitrile (eluent B), both containing 1% formic acid, and a flow of 0.5 mL/min was used as followed: 0.0-0.5 min 5% B isocratic; 0.5-9.0 min 5-30% B; 9.0-10.5 min 30-50% B; 10.5-11.5 min 50-100% B; 11.5-14.0 min 100% B isocratic; 14.0-15.0 min 100-5% B; 15.0-17.0 min 5% B isocratic. Quantification was achieved at a wavelength of 350 nm. Each experiment was performed in triplicates.

Quantification of proanthocyanidin clusters: The same Acquity UPLC® and an Acquity UPLC® Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 150 mm column (Waters GmbH) was used with a method developed by Hellenbrand *et al.* [8]. Quantification was achieved via the fluorescens detector (λ_{ex} = 280 nm, λ_{em} = 316 nm). All procyanidin clusters were quantified as catechin via external standard and related to the sample weights (y = 999174x – $2*10^6$ (R^2 = 0.9991), 0.03-156.3 µg/ml, calibration experiments were performed three times in duplicates). The peaks were grouped due to their degrees of polymerization. The same samples for UPLC® analysis were used as described above. The non-response of 5, 7-dimethoxycoumarin to this method was ensured before. Each experiment was performed in triplicates.

Determination of the total phenols and tannins: Of each UPLC®-sample 100 µL were vacuum dried in an exsiccator. The total phenols solutions (TPS) were gained by extracting the residues in 1.00 mL water in a supersonic bath for 30 min. After centrifugation (14000 rpm, 3 min), the procedure was continued as described in detail in Wiesneth and Jürgenliemk [9]. Tostay in the quantification range the solutions of the Natura Trend capsules had to be diluted with water (1 + 3, V/V). In short, hide powder was added to an aliquot of the TPS to adsorb the tannins to yield the solution containing the phenols not adsorbed by hide powder (NAPS). Phenols were quantified via a redox-reaction with the Folin-Ciocalteu's reagent as catechin. The tannins concentration wasgained by subtracting the NAPS-value from the TPS-value. The absence of the internal standard response to this assay was ensured in hexaplicates, where no difference to a water control could be shown. Eachexperiment concerning the samples was performed in triplicates.

Determination of fattyacids: Samples of 500.0 mg and 2 mLborontrifluoride-methanol solution 10% containing 2,6-di-tert-butyl-p-kresol (1 mg/mL) were mixed and boiled in a water bath for 2 h. Finally, the fattyacidsmethyl esters wereextracted in n-hexane. The n-hexane phases were reduced to dryness under a N_2-stream and dissolved in 1 mL of new n-hexane. These preparations were mixed 1+1 (V/V) with a methyl nonadecanoate solution in n-hexane (5.0 mg/mL). A 7890A GC with internal FID and a 220 ion trap equipped with a VF-5ms 30 x 0.25 (0.25) column (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) was used for analyzing the fatty acid methyl esters. Temperature program: 80 °C, hold time 0.5 min → ramp of 5 °C/min to 300 °C, hold time 10 min. Injection volume was 1 µL, using a split of 50:1, and the inlet temperature set to 300 °C. Flow was 1 mL/min using He_2 4.6 (Linde AG, Pullach, Germany) as carrier gas. Ionisation mode was set to EI-auto, scanning the ions from *m/z* 40-650. Methyl oleate was qualitatively identified via MS using the specific fragment *m/z* 264.2, since its peak was overlaying to the methyl linolenate one. Peak identification was achieved by a F.A.M.E. mix. Fatty acids' methyl esters were quantified as methyl nonadecanoate via an internal standard (methyl nonadecanoate: y = 121813.0x -4886.8 (R^2 = 0.9964), 0.09-14.87 mg/mL, concentration of internal standard= 2.5 mg/mL, calibration was performed in duplicate).The peaks for methyl heicosanoate and methyl tetracosanoate were only identified via EI spectra compared to the NIST database, since they were not contained in the F.A.M.E. mix. Each experiment concerning the samples was performed in triplicates.

Our strategy was to investigate phytoequivalence by a semi-quantitative approach to gain a comparable dataset concerning the phenolics and fatty acids. Every sample was prepared and analyzed in the same way in triplicates to ensure the reproducibility of the used methods. Thus, this study design enables a comparison of the investigated products due to their phytoequivalence.

Results

The content of flavonoids, ellagic and caffeic acid derivatives evaluated by UPLC® are presented in Fig. 1, the content of phenols and tannins assessed with the Folin-Ciocalteu assay are summarized in Fig.2 andthat of the procyanidin clusters in Fig. 3. The content of fatty acids is summarized in Table 2.

Discussion

Phyto equivalence is necessary to exchange herbal medicines in clinical treatment. Factors determining phyto equivalence include the quality of the drug, the extraction solvent and procedure and, the drug to extract ratio. The native extracts should be essentially similar in terms of co-active compounds used for standardization and those for pharmaceutical relevance as well as in their daily dosages [10]. Already Table 1 indicates that the preparations differ considerably. The use of photometry alone to quantify active ingredients is insufficient and can be misleading, so HPLC assessments are required to quantify the amount of co-active constituents in herbal medicinal products [11-13]. For rose hip products the discrepancyi seven more complex, as shown by our data and by others [14]. Figure 1revealsthatthe fermented aqueous extract of the shell contain e daconsider able amount of flavonoids in addition to ellagic acid and caffeic acid derivatives which would be unexpected from the photometric assessment (Fig. 2).In the commercial products of the fermented aqueous extract (one part mixed with 9 parts of collagen hydrolysate) practically no flavonoids were detected.

Our results confirm that pure shell powder contained more flavonoids and less unsaturated fatty acids than pseudo fruit powder (Fig. 1 and 3) [1, 2,14]. We also found that the content of flavonoids in powders varies considerably (Fig. 1) [15]. Flavonoids contribute to the anti-inflammatory activity mediated by the p38, LOX and COX-1 and 2 signaling pathways, to the antioxidative activity [16,17] and to the inhibition of cartilage-damaging collagenase [18] and hyluronidase [16]. The flavonoid tilirosideis only contained in the seed which also contains more hesperidin than the shell [3]. In our study, the rose hip shell products were free of tiliroside and hesperidin except VP Powder II. This powder contained double the amount of tiliroside and six times the amount of hesperidin than the powder with 50% seed (Litoflex®). We suggest therefore, that the VP Powder II is a mixture of seed and shell powder (ratio not declared) rather than a pure shell powder.

The high temperature used to obtain the water extract from fermented rose hip shell increases the solubility of flavonoids, in particular that of quercetin [19], which is a potent antioxidant [20]. A good correlation between the antioxidant activity evaluated by the FRAP assay and the content of phenolics in rose hippseudofruit was previously found by Jablonska-Rys *et al*. [21]. Due to the higher content of total phenolics, the radical scavenging activity of pure shell powder was higher than that of pseudofruit powder [1]. In the DPPH assay, polar extracts (methanol, water) proved to be the most effective [19]. The radical scavenging effect in this assay also correlated well with the content of phenolics and was not mediated by vitamin C [1, 22]. Recent investigations indicate that the rose hip antioxidative activity is composed of additive and synergisitic effects of phenolics, flavonoids, vitamin C and putative other bioactive compounds [14]. These non-phenolic antioxidative compounds, which are also contained in the aqueous fermented extract free of vitamin C [19], need to beidentified. It maywellbethatα-tocopherolor carotenoids found in higher quantities in the shell compared to the seed [2, 23] also contribute to the total antioxidative capacity [24, 25].

Schwager *et al*. [2] found higher contents of galactolipid, diglyceride derivatives and triterpenois (ursolic acid, oleanolic acid betulinic acid) in shell powder compared to pseudofruit powder. These lipophilic compounds also contribute to the inhibition of COX-1 and 2, LOX [1, 26] and the expression of cytokines [25, 27]. They are also dissolved in the aqueous extract from fermented rose hip shell due to the high temperature [19]. Our analyses confirm that the pseudo fruit powder Litoflex® contains more fatty acids than the shell powder Litozin® (Fig. 3) [2]. The main fatty acids, linolenic acid and α-linoleic acid [28] are active in the COX-1 and 2 assays, although more pronounced in the COX-2 assay [14, 29]. Both also inhibited weakly leukotrien formation discussed as the only target of oleic acid [1, 14]. The high content of unsaturated fatty acids in the VP powder II again indicates that this powder is not a pure shell powder. The content of unsaturated fatty acids in the NT pseudofruit products was remarkably low except that for palmitic and stearic acid in the capsules. It seems likely, that these fatty acids are auxiliary substances for the manufacturing process as well as those found in the Glucosamin Plus® tablets.

Procyanidins are contained in the pseudo fruit and shell in doses around 0.1% [30]. Fig. 4 shows that pure powders may contain more procyanidins than the fermented aqueous extract from the shell. Whether the small amounts of procyanidins in the mixture of glucosamine with aqueous (unfermented) extract derived from the rose hip pseudofruit or the acerola extract component, remains to be investigated. Nonetheless, the overall procyanidin amounts were very low compared to the amounts contained in pomegranate products used for the treatment of cancer [13] or for the prevention and treatment of cardiovascular diseases [31].

Due to a lack of available reference substances, we were unable to identify all of the 45 rose hip flavonoidspreviouslydescribed [14]. Not allflavonoids are considered important for the clinical activity. However, methyl gallate, isoquercitrin, catechin, epicatechin, eridictyol, apigenin and phloridzin are certainly of importance [32]. Since the detailed active principle of rose hip has not yet been identified, we cannot use our data to calculate equivalent doses of different rose hipproducts. But it is obvious that phyto equilanceis not given with in the investigated sample set.

The evidence of effectiveness for the pseudo fruit powder Litoflex® is moderate based on a number of studies with exploratory study designs [33, 34]. Although the clinical efficacy of the pseudo fruit powder Litoflex® has not been proven beyond doubt, the pain-relieving effect of the powder seems plausible due to the rational mechanism of action. The equivalent doses for all other rose hipproducts need to beassessed in clinical dose-finding studies, since they are showing defenetively another qualitative and quantitative composition of ingredients.

Collagen hydrolysate and/or glucosamine are combined with rose hip preparations in some medicinal products. A recent, independently performed, review did not find superiority of collagen hydrolysate products over placebo in patients suffering from kneeosteo arthritis [35]. In a later confirmatory study [36], the primary outcome measure was questionable [37]: The evidence of effectiveness of collagen hydrolysate is therefore presently "poor", and because important active rose hip ingredients could not be detected in the mixture of aqueous fermented rose hip shell extract and porcine collagen hydrolysate, we question the usefulness of the mixtures in the treatment of osteoarthritic complaints.

The clinical benefit of oral glucosamine has also been

questioned in patients suffering from osteoarthritis [38]. The mixture with about 6% aqueous rose hip pseudofruit extract (drug extract ratio not obtainable by the company, maltodextrine 2%) contained 1500 mg glucosamine in the daily dose and 4% acerola extract (with 2% maltodextrine). Important active rose hip ingredients could not be detected (Fig.1 and Table 2). Unless this product has not demonstrated effectiveness in clinical studies with a confirmatory study design in patients with painful osteoarthritis, other safe and approved options should be employed prior to a mixture with the risk of putative glucoseamin adverse events [39-41].

If an alleviation of osteoarthritis complaints can be achieved with particularrose hip products at least similar to that of Litoflex®, overall treatment costs could be reduced. These costs include those required for the treatment of adverse events and complications associated with nonsteoroidal anti-inflammatory drugs, the gold standard for the treatment of osteoarthritis [42].

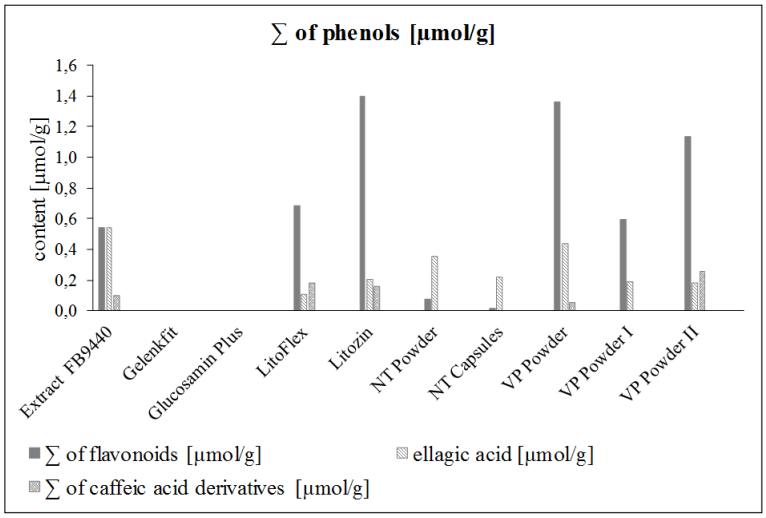

Fig 1: Summed contents of flavonoids, ellagic acid and caffeic acid derivatives [µmol/g]. NT Natura Trend, VP Vitapower.

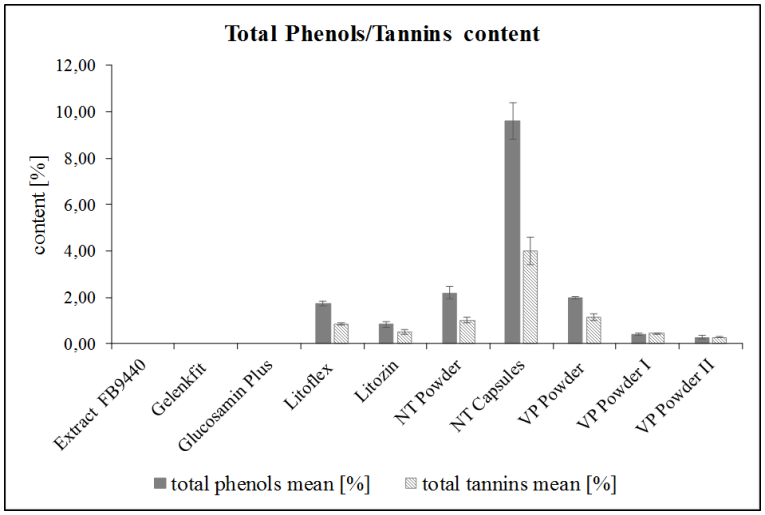

Fig. 2: Contents of total phenols / tannins [%]. NT Natura Trend, VP Vitapower.

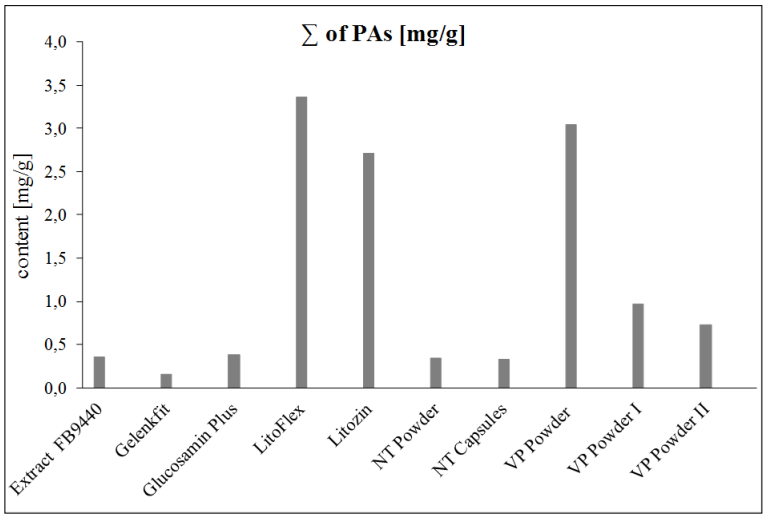

Fig. 3: Summed contents of procyanidin clusters [mg/g]. NT Natura Trend, VP Vitapower.

Table 1: Characteristics of the rose hip products analysed. *fermented plus vitamin C: #from Aspergillus niger plus acerola powder 3.6% (1.8% maltodextrin) plus vitamin C 12 mg in the daily dose.

Name	Producer	Plant Part	Characteristics	Batch	Exspire Date
FB9440	Finzelberg GmbH	Shell	Extract* (water, DER 4:1)	16004998	March 18
Gelenkfit	Sanct Bernhard	Shell	Extract* (water, DER 4:1); 1:10 (collagen hydrolysate)		
Glucosamin plus	Bioforce AG	Pseudofruit	Extract 5.6% (water, DER not stated), 42.7% glucosamine HCl#	2014002789 A	May 16
Litozin	Takeda	Shell	Powder	432999	Febuary 16
Litoflex	ContarixHealth AG	Pseudofruit (50% Seed)	Powder	5062081	July 17
Naturatrend	Dolsan AG	Pseudofruit	Powder in Capsules	070-001	
Naturatrend	Dolsan AG	Pseudofruit	Powder	401-001	
Hagebuttenpulver	Vitapower AG	Pseudofruit (wild), 30% Seed	Powder	17GR0223/D	
Hagebuttenpulver I	Vitapower AG	Pseudofruit, 20% Seed	Powder	15OA1125/S	
Hagebuttenpulver II	Vitapower AG	Shell (wild)	Powder	16GR0715/D	

Table 2: Contents of Fatty Acids (FA, mg/g ± standard deviation), calculated as methyl nonadecanoate. RT Retention time, NT Natura Trend, VP Vitapower, *additional presence of methyl oleate, identified via *m/z* 264.2.

fattyacid'smethylester	Corresponding fattyacid	RT [min]	FB9440	Gelenkfit	Glucosamin Plus	LitoFlex	Litozin	NT Powder	NT Capsules	VP Powder	VP Powder I	VP Powder II
Methyl laurate	lauricacid	18,6				0,1 ± 0,004	0,1 ± 0,002			0,1 ± 0,004		
Methyl tetradecanoate	myristicacid	23,0			0,1 ± 0,01	0,04 ± 0,004	0,1 ± 0,003		0,2 ± 0,004	0,04 ± 0,01		
Methyl palmitate	palmiticacid	27,1		0,04 ± 0,01	11,9 ± 0,9	1,0 ± 0,04	0,6 ± 0,03	0,1 ± 0,01	7,6 ± 0,1	0,7 ± 0,02	0,7 ± 0,1	1,5 ± 0,3
Methyl heptadecanoate	margaricacid	29,0	0,1 ± 0,001	0,1 ± 0,003	0,1 ± 0,01	0,1 ± 0,004	0,1 ± 0,002	0,1 ± 0,002	0,1 ± 0,004	0,1 ± 0,002	0,1 ± 0,005	0,1 ± 0,01
Methylinoleate	linoleicacid	30,3		0,05 ± 0,001		9,3 ± 0,2	0,4 ± 0,01	0,04 ± 0,001		1,1 ± 0,01	4,2 ± 0,8	13,9 ± 2,8
Methyl linolenate	linolenicacid	30,4			0,1 ± 0,004	7,0* ± 0,2	1,2 ± 0,03	0,04 ± 0,002		2,0* ± 0,05	3,0* ± 0,5	10,7* ± 2,2
trans-9-elaidic acid methyl ester	trans-9-elaidic acid	30,5				0,04 ± 0,001						0,1 ± 0,003
Methyl octadecanoate	stearicacid	30,8			13,9 ± 1,1	0,5 ± 0,04	0,2 ± 0,04		6,1 ± 0,1	0,2 ± 0,01	0,3 ± 0,05	0,9 ± 0,2
Methyl cis-11-eicosenoate	cis-11-eicosenoic acid	33,8				0,1 ± 0,01						0,1 ± 0,02
Methyl arachidate	arachidicacid	34,3			0,2 ± 0,01	0,2 ± 0,001	0,05 ± 0,002		0,1 ± 0,001	0,05 ± 0,004	0,1 ± 0,02	0,3 ± 0,1
Methyl heicosanoate	heneicosanoicacid	35,8				0,1 ± 0,01	0,04 ± 0,003			0,05 ± 0,005	0,1 ± 0,01	0,1 ± 0,01
Methyl docosanoate	behenicacid	37,4				0,05 ± 0,001						0,1 ± 0,02
Methyl tetracosanoate	lignocericacid	40,3				0,04 ± 0,001	0,04 ± 0,001					0,05 ± 0,01
∑ [mg/g]			0,1	0,2	26,2	11,5	2,7	0,2	14,1	2,2	5,4	17,0

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper

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