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NMR metabolomics of ripened and developing oilseed rape (*Brassica napus*) and turnip rape (*Brassica rapa*)

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ABSTRACT

The oilseeds of the commercially important oilseed rape (*Brassica napus*) and turnip rape (*Brassica rapa*) were investigated with ^{1}H NMR metabolomics. The compositions of ripened (cultivated in field trials) and developing seeds (cultivated in controlled conditions) were compared in multivariate models using principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). Differences in the major lipids and the minor metabolites between the two species were found. A higher content of polyunsaturated fatty acids and sucrose were observed in turnip rape, while the overall oil content and sinapine levels were higher in oilseed rape. The genotype traits were negligible compared to the effect of the growing site and concomitant conditions on the oilseed metabolome. This study demonstrates the applicability of NMR-based analysis in determining the species, geographical origin, developmental stage, and quality of oilseed *Brassicas*.

1. Introduction

As a raw material for food oils, animal feed, and biofuels, the oleiferous plants of genus *Brassica* are versatile crops. Oilseed rape (*Brassica napus* L. subsp. *oleifera*) is one of the most cultivated oil crops in the world, while turnip rape (*Brassica rapa* L. subsp. *oleifera*) is grown only in limited areas in Northern Europe, and more precisely in Finland, where the summer is too short for oilseed rape. The oils extracted from their seeds are naturally rich in α-linolenic acid, resulting in an ideally low ratio of $n-6/n-3$ fatty acids for human diet (Simopoulos, 2002). However, the abundance of unsaturated bonds can lead to the production of oxidised species during storage, cooking, and digestion, which may cause adverse health effects through oxidative stress and inflammation (Awada et al., 2012; Tarvainen, Phuphusit, Suomela, Kuksis, & Kallio, 2012). The oil content and quality of the raw material used for oil is determined by the genome of the oil plant (mainly through maternal factors) and interactions with the environment (weather, soil, cultivation techniques, biotic and abiotic stresses) (Weselake et al., 2009). The oil content of *Brassicas* is also associated with protein and fibre content and seed colour (Abbadi & Leckband, 2011; Snowdon, Lühs, & Friedt, 2007). Crushed seeds and seed extrudates are important sources of plant protein, but antinutrients, such as glucosinolates, have shadowed their use as feedstuffs. The breeding of *Brassica* has managed to produce seeds with high yield and oil content, free of erucic acid, glucosinolates, sinapic acid esters, and chlorophyll (Abbadi & Leckband, 2011). Glucosinolates are the main group of secondary metabolites, along with phenolic choline esters. The differences in the composition and concentrations of triacylglycerols between *B. napus* and *B. rapa* are not usually significant (Vuorinen et al., 2014). Therefore, the examination of minor components of the oilseeds may be of great importance, since they are more susceptible to exogenous and endogenous variation (Spyros & Dais, 2013).

Many of the nuclear magnetic resonance (NMR) spectroscopy studies on vegetable oils have focused on classification, authentication, and determining of the geographical origin of olive oils with proton NMR (Alonso-Salces et al., 2010; Longobardi et al., 2012; Sacchi et al., 1998; Sacco et al., 2000). ^{13}C and ^{31}P NMR techniques have also been successful in oil analyses (Hatzakis, Koidis, Boskou, & Dais, 2008; Sacchi, Addeo, & Paolillo, 1997; Vigli, Philippidis, Spyros, & Dais, 2003). Apart from studies by Chen, Li, Lei, Zhu, and Zhang (2010), little attention has been paid to the NMR metabolomics of rapeseed oils *per se*, although ^{1}H NMR fingerprinting has been used to distinguish rapeseed (or canola) oil from other vegetable oils and detect its adulteration by animal fats (Fang, Goh, Tay, Lau, & Li, 2013). However, other analytical methods, such as pulse-NMR and near-infrared spectroscopy (NIR), have also been

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used in oilseed analyses (Barthet, 2013). The *B. rapa* leaf composition has been studied using 1D and 2D NMR techniques by Abdel-Farid, Kim, Choi, and Verpoorte (2007) with respect to the effect of cultivar and developmental stage.

The aim of the present work was to study the composition of oilseed rape and turnip rape seeds after a facile extraction procedure, by using NMR spectroscopy and chemometrics. The effects of species, developmental stage, geographical origin, and related growing conditions on the seed composition were specifically viewed.

2. Materials and methods

2.1. Sample material

Ripened seeds of low erucic acid spring rape (oilseed rape; *Brassica napus* L. subsp. *oleifera*) and spring turnip rape (*Brassica rapa* L. subsp. *oleifera*) of the 2011 crops were received *via* MTT Agrifood Research Finland from the official variety test trial sites in Hauho, Inkoo, Jokioinen, Maaninka, and Pernaja (Kangas et al., 2011). The trial sites situate in different cultivation zones according to their geographical location, Inkoo (60.08°N, 24.89°E) and Pernaja (60.45°N, 26.15°E) belonging to zone 1, Jokioinen (60.81°N, 23.50°E) and Hauho (61.15°N, 24.59°E) to zone 2, and Maaninka (63.14°N, 27.32°E) to zone 3, respectively (Supplementary Fig. S1). Classification of zones is based on the temperature sum accumulation of a region during growing season. The turnip rape genotypes chosen for this study were Aurea CL, Bor 05075, Bor 05100, Bor 07010, Cordelia, Juliet, SW Petita, and Viikki 11, while the oilseed rape genotypes were Belinda (hybrid), DLE 1006, DLE 1107, Highlight, Marie, Mirco CL (hybrid), Proximo, Majong (hybrid), SW Q2865, Trapper (hybrid), Tamarin, Brando (hybrid), Early Bird, and Lunedie. Weeds and pests were controlled according to the protocol of the test sites. Plots were harvested when fully matured and the yield obtained was dried after harvest to a moisture content of approximately 9%. Seeds from one or two block samples were randomly selected for extraction. The weather data for each experimental site was calculated based on the data collected by the Finnish Meteorological Institute (Helsinki, Finland) (Table 1).

The oilseed rape genotypes Marie and Bor 01000, and the turnip rape genotypes SW Petita and Bor 05075 were cultivated in controlled conditions as described by Vuorinen et al. (2014). Optimal growing conditions were created in a growth room and in a greenhouse at 22 °C with 16 h day length and at 15–20 °C with 16–19 h day length. Stress conditions were created with the reduced temperature (15 °C, 16 h) in a growth chamber. The siliques were harvested at different time points (2, 3, and 4 weeks after flowering, WAF, with ±3 days marginal per weekly time point) after the start of flowering and stored at −20 °C until further treatment.

2.2. Chemicals

Analytical grade cyclohexane was purchased from Lab-Scan (Dublin, Ireland). Chloroform-*d* ($CDCl_3$, 99.8 atom % D) was from Sigma–Aldrich (St. Louis, MO), and methanol-d_4 (CD_3OD, 99.8 atom % D) from Sigma–Aldrich and VWR International Oy (Espoo, Finland).

2.3. Sample preparation

Ripened seeds (100 mg) were homogenised and extracted with 1.5 ml cyclohexane using an ULTRA-TURRAX T 25 homogeniser (IKA Works, Wilmington, NC), equipped with an 8 G dispenser element. The cyclohexane extract, separated after centrifugation of

Table 1
Accompanying information on the growing sites. Average values of the weather parameters were calculated separately for *B. napus* (*BN*) and *B. rapa* (*BR*). The parameters were calculated (from sowing to ripening) based on the data provided by the Finnish Meteorological Institute (Helsinki, Finland).

Growing site				Weather station[a]		Sowing date	Growing time (days)		Number of hot days[b]		Average daily temperature (°C)		Temperature sum (°C)		Growing degree days (°Cd)[c]		Global radiation sum (10^4 kJ/m^2)		Average relative humidity (%)		Precipitation sum (mm)	
Site	Coordinates	Zone	Soil type	Coordinates	Altitude		*BN*	*BR*	*BN*	*BR*	*BN*	*BR*	*BN*	*BR*	*BN*	*BR*	*BN*	*BR*	*BN*	*BR*	*BN*	*BR*
Inkoo	60.08°N, 24.89°E	1	Sandy clay	60.21°N, 24.74°E	31	May 6th	110	101	23	23	17	17	1850	1690	1300	1190	220	207	72	71	166	143
Pernaja	60.45°N, 26.15°E	1	Sandy clay, silty clay	60.39°N, 25.61°E	22	May 9th	127	114	25	25	17	17	2130	1950	1490	1370	234	221	74	72	285	234
Jokioinen	60.81°N, 23.50°E	2	Sandy clay, silty clay	60.81°N, 23.50°E	104	May 22nd	116	94	26	25	16	17	1870	1570	1290	1100	185	166	81	79	342	281
Hauho[d]	61.15°N, 24.59°E	2	Coarse silt	61.05°N, 25.04°E	125	May 9th	116	94	26	24	16	16	1860	1520	1240	1020	185	166	75	72	201	175
Maaninka	63.14°N, 27.32°E	3	Fine sandy till, till	63.14°N, 27.31°E	90	May 24th	–	77	–	21	–	17	–	1310	–	923	–	147	–	71	–	228

[a] Weather stations with closest match to the growing sites were chosen. The radiation data for Inkoo and Pernaja, and the precipitation data for Inkoo were from a substituting weather station at coordinates 60.33°N, 24.96°E (altitude 51 m). The radiation data for Hauho was calculated using the data recorded at the station closest to Jokioinen.
[b] Daily maximum temperature above +25 °C.
[c] The effective temperature sum (the sum of the positive differences between average daily temperatures and +5 °C).
[d] Growing time for Hauho was estimated using the data from the nearest site in Jokioinen, because the ripening dates were not recorded in Hauho.

10 min with 1730 × *g*, was evaporated under a nitrogen flow and dissolved in 1.5 ml of $CDCl_3$. The seed material was re-extracted, first with 1.5 ml of $CDCl_3$, and then with 1.5 ml of CD_3OD, sonicating 30 min each time. All the extracts were filtered through a 0.45 μm PTFE syringe filter and stored at −20 °C prior to analyses (extracts stored longer than one week were kept at −80 °C).

The unripe, developing seeds were collected from the siliques and seeds from the same time point were combined into one sample. A total of 50 mg of seeds were extracted with 800 μl $CDCl_3$ in an Eppendorf tube using a stirring rod for pounding. The mixture was vortexed for 1 min and sonicated for 10 min. The extract was filtered and stored at −80 °C prior to analyses.

2.4. Data acquisition

The extracts (600 μl of each) were transferred into 5 mm NMR tubes for analysis. The ^{1}H NMR spectra of the extracts were recorded at 298 K with a 500 MHz Bruker Avance spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a broadband inverse (BBI-5mm-Zgrad-ATM) autotune probe. A standard *zg30* pulse program with 160 scans, collecting 64 k data points and covering a spectral range of 10,000 Hz, was used. The acquisition time was 3.28 s, the relaxation delay 7 s and the 90 degree pulse was 6.90 μs. When measuring the CD_3OD extracts, the receiver gain was set to 90, otherwise the gain was set automatically with the *rga* command for each experiment. Standard DQF-COSY (*cosygpmfqf*), HSQC (*hsqcetgpsi2*), CH_2-edited HSQC (*hsqcedetgpsisp2*), HMBC (*hmbcgplpndqf*), and 1D TOCSY (*selmlgp*) experiments were conducted with selected samples.

2.5. Data processing and spectral assignment

The spectra were baseline and phase corrected with the TopSpin 1.3 software (Bruker BioSpin GmbH, Rheinstetten, Germany). The spectra were aligned in respect to the chemical shift of the residual solvent peak, to be precise, 7.26 ppm and 3.31 ppm for chloroform and methanol, respectively. The spectra were binned then into equal widths of 0.04 ppm in the AMIX software (version 3.9.4, Bruker BioSpin GmbH). The sum of positive intensities was applied as the integration mode, while the spectral scaling was set to the total intensity. Spectral ranges were 5.75–0.65 ppm (excluding buckets 2.21, 2.17 and 1.93–1.09 ppm) for cyclohexane and $CDCl_3$ extracts and 7.75–0.70 ppm (excluding 5.17–4.45, 3.33–3.29, 2.17 and 1.81–1.25 ppm) for CD_3OD extracts. For the developing seeds, the spectral range was 5.50–0.66 ppm, excluding buckets 2.16 and 1.92–1.48 ppm.

The ^{1}H NMR peaks were assigned by consulting the literature, with Chenomx NMR Suite 7.5 software (Chenomx, Inc., Edmonton, Alberta, Canada), the HMDB database (Wishart et al., 2013), and experimental data from 1D and 2D measurements.

2.6. Multivariate data analysis

The SIMCA-P+12.0 software was used for the multivariate data analyses. Separate datasets were created for each set of extracts: the dataset dimensions (observations × variables) were 96 × 104, 98 × 104, and 134 × 140 for the cyclohexane, chloroform, and methanol extracts, respectively. The dataset for the developing seeds was 46 × 106. All the datasets were Pareto scaled with the scaling weight of $1/\sqrt{s_k}$. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA) were used as the multivariate methods. The unsupervised method PCA was used to observe general model overview and possible trends, while the supervised methods PLS-DA and OPLS-DA were used to highlight the group separation and increase model interpretability (Eriksson et al., 2006).

3. Results and discussion

3.1. NMR spectroscopic data

The cyclohexane, chloroform, and methanol extracts of the mature seeds gave different NMR profiles of the seed compositions (Fig. 1). Most of the major seed lipids were extracted with cyclohexane, while the chloroform extracts also contained choline. By using cyclohexane and chloroform we were able to wash out most

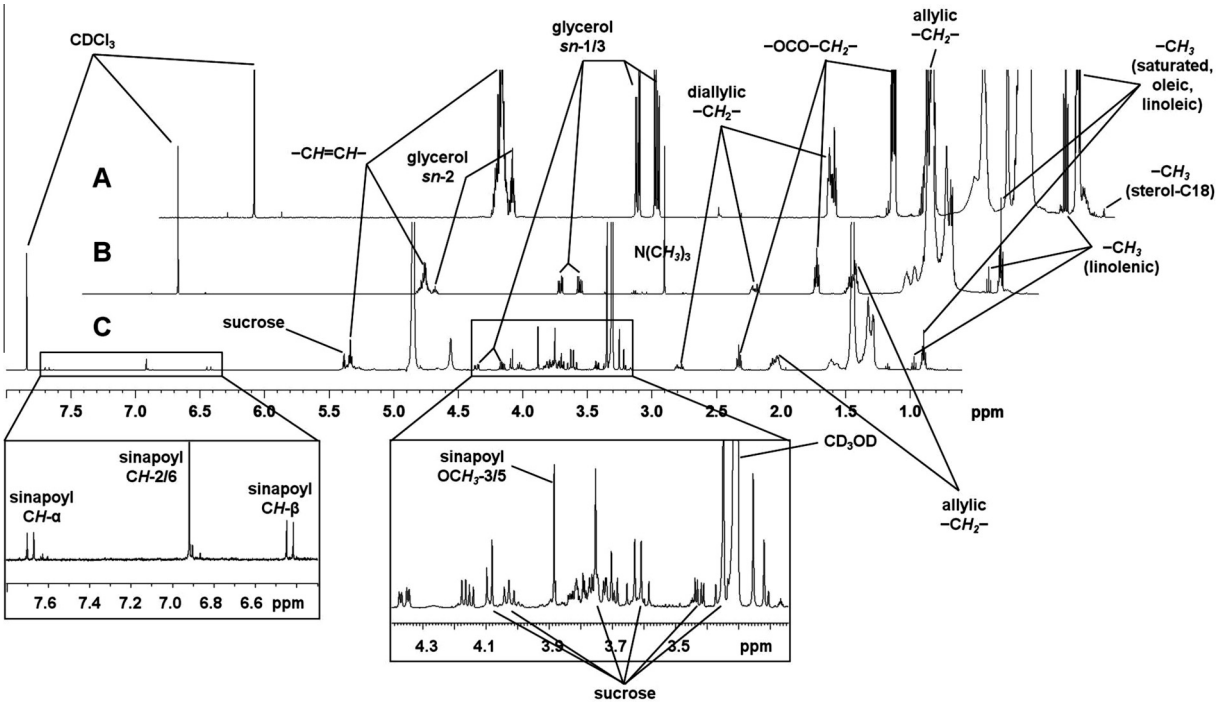

Fig. 1. ^{1}H NMR spectra of the seed extracts (example: turnip rape Bor 05100). (A) Cyclohexane extract in $CDCl_3$, (B) $CDCl_3$ extract, and (C) CD_3OD extract.

of the major lipids before the methanol extractions. The methanol fractions contained storage sugar and sinapic acid esters, while some triacylglycerols remained. The spectral profiles of the cyclohexane extracts resembled typical oil spectra (Fig. 1A), while a large singlet from an $N(CH_3)_3$ moiety was present at 3.49 ppm in the chloroform extracts (Fig. 1B). Two singlets at 0.68 and 0.69 ppm from the C18-steroid groups were observed in the spectra. The main sterols in rapeseed oils are β-sitosterol and campesterol (Schwartz, Ollilainen, Piironen, & Lampi, 2008). Other minor components of the seeds, many of which are lost during normal oil processing, were extracted in the methanolic fraction (Fig. 1C). In addition to the residual major lipophilic components, storage sugar and sinapic acid esters were present. The major sugar was sucrose, but a small anomeric signal at 5.41 ppm (J = 3.9 Hz) from an α-glucose moiety revealed the presence of minor sugar, raffinose or stachyose, characteristic to brassicaceous oilseeds. The Supplementary Table S1 shows the peak assignments. The ^{13}C shifts were obtained from the HSQC experiments. A singlet at 3.89 ppm had an HMBC-correlation to a carbon at 148.0 ppm, which confirmed that this methoxy group (OCH_3) is attached to an aromatic ring. Several cholinyl singlets were present in the methanol extracts.

The spectra from the developing seed extracts were corresponding to the $CDCl_3$ extracts of the ripened seeds, although the stage of maturity naturally impacted the profiles. A few of the major changes between the seeds harvested 2 weeks after flowering (2 WAF) compared to the seeds harvested at later stage (3–4 WAF) are shown in Fig. S2: the oil accumulation can be seen in the acyl resonances at δ 0.88, 0.97, 1.25, and 1.30 ppm, the second and fourth of which show the increase in polyunsaturated fatty acid levels. Meanwhile, a decrease in the δ 0.85 ppm methyl region is observed.

3.2. Cyclohexane extracts

Separate datasets were created for each set of extracts in order to see whether the most important discriminating factors were basic lipids or the minor components and how the composition is changed during seed development. The exclusion areas eliminated residual acetone (washing solvent for NMR tubes), cyclohexane and water resonances from the spectra. Some of the fatty acid signals were concurrently excluded, however, this was not considered a disadvantage as the relatively large methylene signals between 1.40 and 1.20 ppm would have distorted the multivariate models (the methylene signals were included in the spectra of developing seeds though).

In the PCA models ($R^2X_{(cum)}$ = 0.99, $Q^2_{(cum)}$ = 0.95) of the cyclohexane extracts, the first two components showed a partial separation of *B. napus* and *B. rapa* (Fig. 2A). The separation between the first two components, which covered 78% of the total variation, was greatly influenced by the variable buckets 0.81, 0.85, 2.01, and 2.05 ppm (Fig. 2B). Buckets at 2.01 and 2.05 ppm were from allylic methylene protons of saturated/monounsaturated and polyunsaturated fatty acids, respectively, therefore these adjacent variables were situated at opposite coordinates in the models. This suggested a higher content of unsaturated fatty acids in *B. rapa* samples, especially those cultivated in the northernmost site (Maaninka) (Fig. 2C). Bucket 0.81 contained unspecified methyl resonances, while the subsequent bucket 0.85 also contained part of the terminal methyl

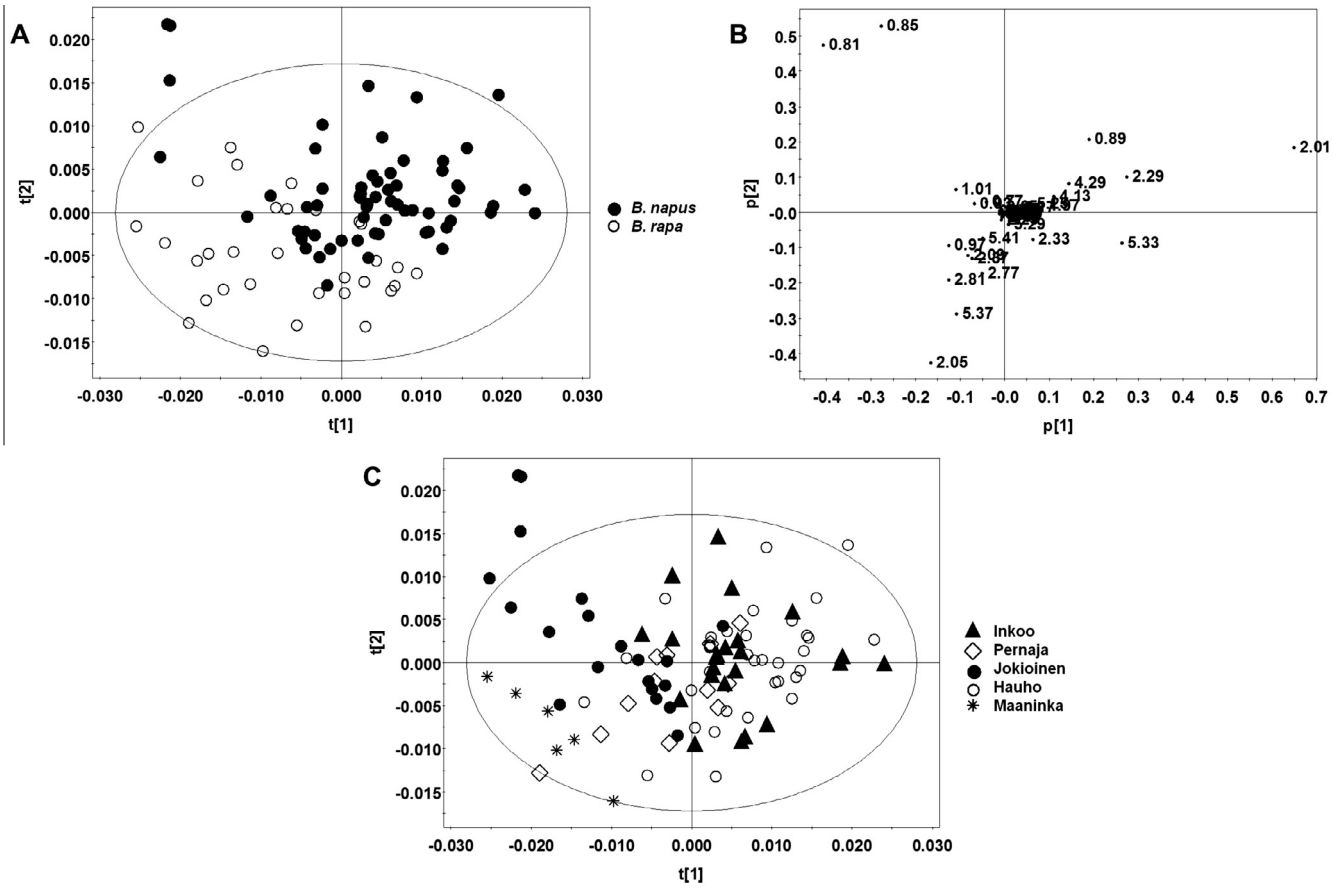

Fig. 2. PCA model of the cyclohexane extracts in $CDCl_3$ (96 × 104, $R^2X[1]$ = 0.57, $Q^2[1]$ = 0.43; $R^2X[2]$ = 0.21, $Q^2[2]$ = 0.39). (A) Scores plot $t[2]$ vs. $t[1]$ with observations coloured according to species. (B) Loadings plot $p[2]$ vs. $p[1]$. (C) Scores plot $t[2]$ vs. $t[1]$ with observations coloured according to growing place.

signal from fatty acids. Some clear grouping was observed amongst cultivation sites despite some overlap (Fig. 2C).

Since the separation of *B. napus* and *B. rapa* was not satisfactory, supervised methods PLS-DA and OPLS-DA were applied to emphasise the class discrimination between the species. The PLS-DA model (Fig. S3A) was validated by a permutation test (Fig. S3B), which gave the *Y*-intercept values of 0.01 for R^2 and −0.12 for Q^2, showing model validity (R^2Y-intercept < 0.3–0.4 and Q^2Y-intercept < 0.05; Eriksson et al., 2006). However, the goodness-of-fit and the predictive ability of the PLS-DA model were poor, being < 0.5. A list of the variable importance for the projection (VIP) values are shown in Table S2. The most influential *X*-variables were the allylic (2.01, 2.05), α-methylene (2.29, 2.37), olefinic (5.37, 5.33), diallylic (2.81, 2.77), *sn*-1/3 glycerol (4.29, 4.13), and methyl (0.89, 0.81) resonances. The use of OPLS-DA aimed for higher interpretability and enhanced variation between the groups compared to PLS-DA, but the discriminating power in the OPLS-DA model (Fig. S3C) was not adequate for a clear-cut separation of the two species either. The model composed of only one predictive component (related to *Y*), and one orthogonal component without relations (orthogonal) to *Y* (Eriksson et al., 2006). Chen et al. (2010) used chemometrics to discriminate rapeseed oil brands analysed by ^{1}H NMR and determined that all acyl chains, linonenoyl and linoleoyl chains, and triacylglycerols were responsible for the discrimination. They focused on methods which selectively reduced the number of variables. However, the use of a wide bucket size (0.04 ppm)

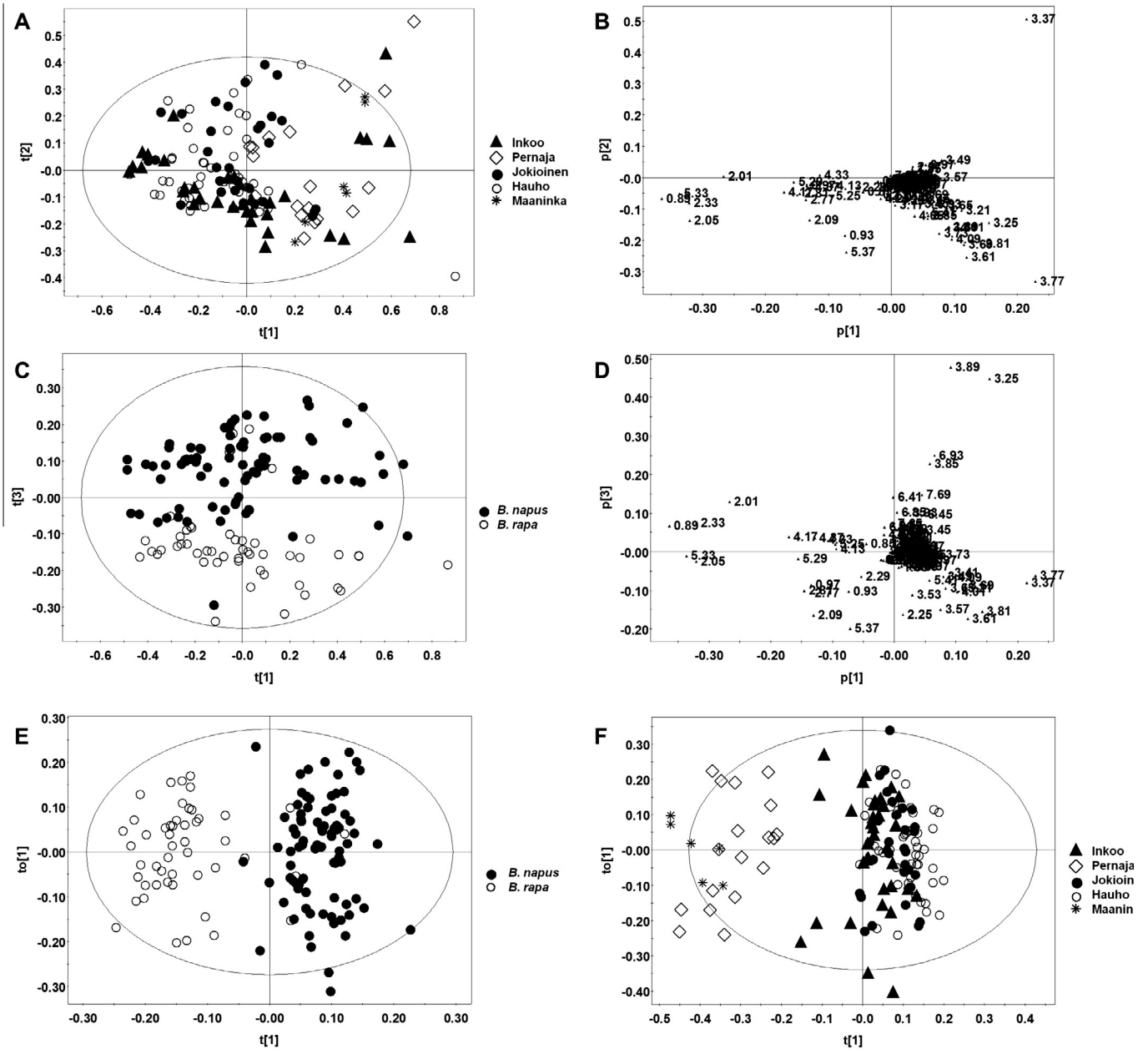

Fig. 3. Multivariate models of the CD_3OD extracts (134 × 140). (A) PCA scores plot *t*[2] vs. *t*[1] with observations coloured according to growing place (R^2X[1] = 0.42, Q^2[1] = 0.37; R^2X[2] = 0.16, Q^2[2] = 0.19). (B) PCA loadings plot *p*[2] vs. *p*[1]. (C) PCA scores plot *t*[3] vs. *t*[1] with observations coloured according to species (R^2X[3] = 0.12, Q^2[3] = 0.16). (D) PCA loadings plot *p*[3] vs. *p*[1]. (E) OPLS-DA scores plot *to*[1] vs. *t*[1] with observations classified by species ($R^2X_{(cum)}$ = 0.83, $R^2Y_{(cum)}$ = 0.73, $Q^2_{(cum)}$ = 0.60). (F) OPLS-DA scores plot *to*[1] vs. *t*[1] with observations classified by growing place ($R^2X_{(cum)}$ = 0.92, $R^2Y_{(cum)}$ = 0.64, $Q^2_{(cum)}$ = 0.48).

already reduces the number of variables, while the similar outcome is achieved, as shown herein.

In Finland, the oil from *B. napus* and *B. rapa* are sold as one product (rapeseed oil). The quality of the oilseed is essentially determined by the amount of oil. High α-linolenic acid content may also be a desirable quality for healthier oils for human consumption, but its susceptibility to oxidation may diminish possible health benefits. Several biotic and abiotic stresses may affect the oil composition of the oil plants grown under open field conditions (Quijada, Cao, Wang, Hirai, & Kole, 2007). Oilseed rape is more prone to develop higher levels of chlorophyll because it may not reach the same level of maturity as turnip rape within same time frame (Barthet, 2013). For turnip rape, lower chlorophyll content is related to higher yield plasticity (Peltonen-Sainio, Jauhiainen, & Sadras, 2011). Turnip rape is a freely cross-pollinating crop, while oilseed rape is mainly self-pollinating. However, in the test field sites, the oilseed rape is also susceptible to cross-pollination, thus likely to produce less pure genotypes. However, the pollen is not regarded as a genetically defining factor for the oil content of the seed when compared to the maternal genotype (Weselake et al., 2009).

3.3. Chloroform extracts

The PCA models ($R^2X_{(cum)} = 0.98$, $Q^2_{(cum)} = 0.91$) for the chloroform extracts are shown in Fig. S4. The first component did not have a great impact on the separation between the species (Fig. S4A), although the singlet at 3.49 ppm from cholinyl $N(CH_3)_3$ was a very dominating variable (Fig. S4B). The methyl resonances 0.81 and 0.85 had strong positive loadings in the second component. Otherwise the models followed the same pattern as with the cyclohexane models: variables 0.89, 2.01, 2.29, and 5.33 correlated with oilseed rape and 0.97, 2.05, 2.81, and 5.37 correlated with turnip rape (Fig. S4C–F).

3.4. Methanol extracts

Fig. 3 and Fig. S5 show the multivariate models for the methanol extracts. The first and the second component of the PCA model ($R^2X_{(cum)} = 0.96$, $Q^2_{(cum)} = 0.85$) failed to show any distinct grouping of the species (Fig. S5), but when the model was coloured according to growing place (Fig. 3A), some trends were clearer, *e.g.* the first component separated samples from Pernaja and Maaninka to the positive side and samples from Hauho and Jokioinen mainly to the negative side. The Inkoo samples had more variation, but the majority of them were situated on the negative side of the second component. In addition to the same influential variables as seen in the previous models, sucrose had a strong emphasis on the first component to the opposite direction (Fig. 3B). Endospermic sucrose is an important carbon source for the developing seed embryo (Hill, Morley-Smith, & Rawsthorne, 2003). The equilibrium of the concentrations of glucose, fructose, and sucrose may also have some role in controlling the gene expression of the embryo (Hill et al., 2003).

A partial grouping of *B. napus* and *B. rapa* was seen along the third component in Fig. 3C. *B. napus* situated on the positive and *B. rapa* on the negative side of the component. The sinapine signals influenced the third component positively and were more characteristic to *B. napus*, whereas the sucrose signals influenced negatively, and were more characteristic to *B. rapa*, respectively (Fig. 3D). Low levels of phenolic choline esters, along with glucosinolates, are desirable due to their anti-nutritive properties when the

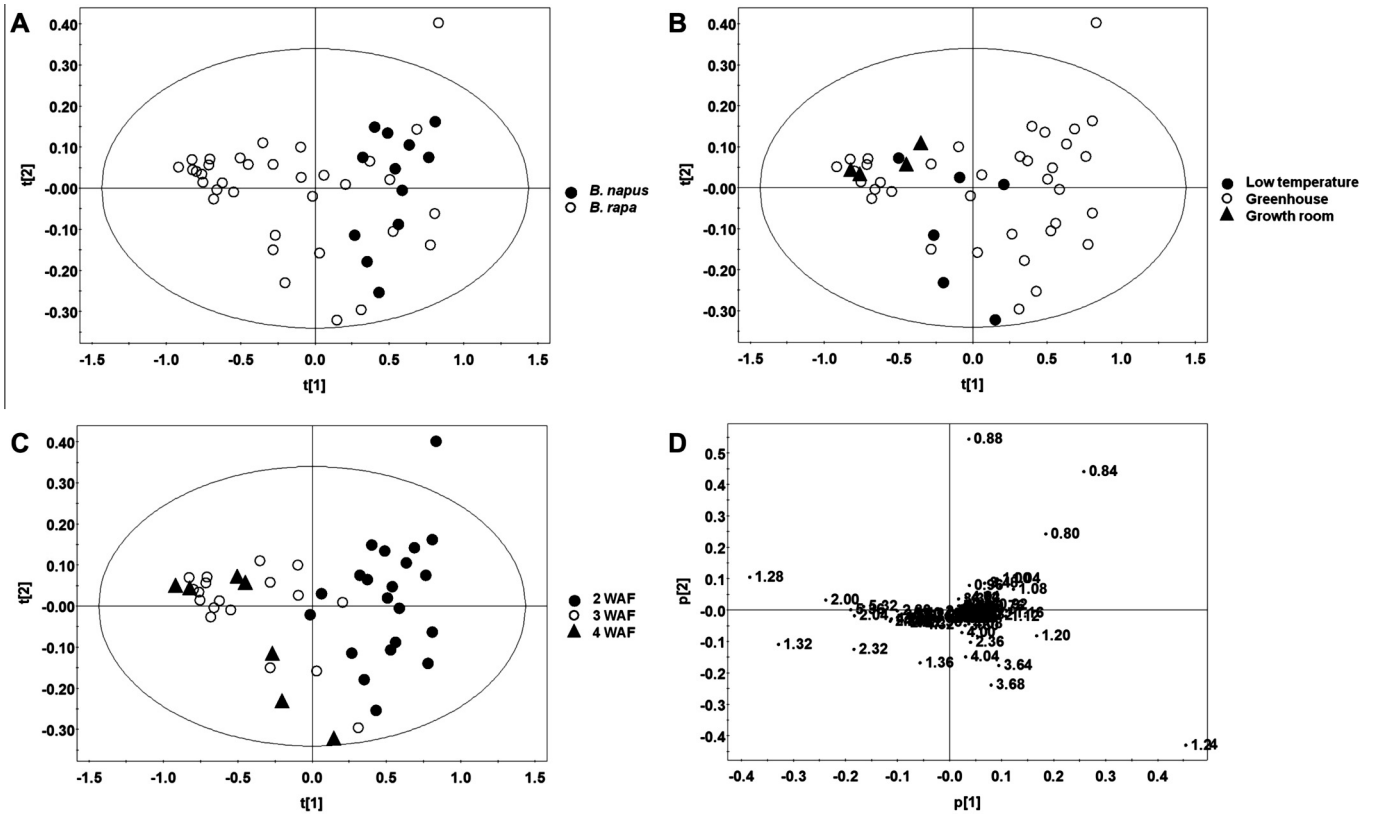

Fig. 4. PCA models of the developing seed extracts (46 × 106; $R^2X[1] = 0.88$, $Q^2[1] = 0.86$; $R^2X[2] = 0.05$, $Q^2[2] = 0.16$). (A) Scores plot $t[2]$ vs. $t[1]$ with observations coloured according to species. (B) Scores plot $t[2]$ vs. $t[1]$ with observations coloured according to growing conditions. (C) Scores plot $t[2]$ vs. $t[1]$ with observations coloured according to stage of ripeness (WAF = weeks after flowering). (D) Loadings plot $p[2]$ vs. $p[1]$.

seed meal is used as an animal feed (Wang & Ellis, 1998). As the seed develops, the sinapate is turned into 1-*O*-sinapoyl-β-D-glucose and sinapoylcholine, catalysed by UDP-glucose:sinapate glucosyltransferase (SGT) and sinapoylglucose:choline sinapoyltransferase (SCT), respectively. Sinapoylglucose is an important substrate both for the formation of sinapine and for an array of other sinapate esters in the seed (Baumert et al., 2005; Wang & Ellis, 1998). During germination, the accumulated sinapine is hydrolysed and sinapoylmalate is formed in the leaves of the plant (Milkowski & Strack, 2010; Wang & Ellis, 1998). Sinapine may have antioxidant effects and it serves as a storage metabolite for choline (Snyder & Weselake, 2013).

With this dataset, the OPLS-DA was also tested to see if this multivariate method would improve the discrimination between species and growing place classes (Fig. 3E). The model parameters ($R^2Y_{(cum)}$ = 0.73, $Q^2_{(cum)}$ = 0.60) were now improved compared to the OPLS-DA models of the cyclohexane extracts discriminating *B. napus* and *B. rapa* (Fig. S3C). This model had one predictive component and five orthogonal components. In the OPLS-DA model with four predictive and six orthogonal components, the samples from Maaninka and Pernaja were clearly separated from the rest (Fig. 3F), due to the influence of sucrose and cholinyl resonances. The conditions at the northernmost site, Maaninka in zone 3 (63.14°N, 27.32°E), are able to sustain only turnip rape. The growing season is several days shorter than in zones 1 and 2 (Table 1). Surprisingly, the samples from Maaninka had similarities with the Pernaja samples, although Pernaja was the second southernmost location and had the highest effective temperature (growing degree days) and global radiation sum. The highest average relative humidity and precipitation were recorded in Jokioinen.

For geographical classification of olive oils, minor components in the unsaponifiable (Alonso-Salces et al., 2010) and phenolic (Sacco et al., 2000) fractions have also been studied. These subfractions described the geographical origin of the oils better than the fatty acid composition data alone. This might also be the case with the rapeseed methanol extracts, since they contain more metabolites compared to the lipid extracts.

3.5. Developing seeds

Multivariate models were also created for the dataset of developing seeds. The PCA plots ($R^2X_{(cum)}$ = 0.99, $Q^2_{(cum)}$ = 0.95) in Fig. 4A–C show the distribution of observations coloured according to species, growing conditions, and developmental stage, respectively. All the oilseed rape samples were collected at the 2 WAF stage, which might explain the separation between *B. napus* and *B. rapa* by the first component in Fig. 4A. The first component in Fig. 4C separated the 2 WAF samples to the positive side, while 3 and 4 WAF were on the negative side, which correlated with the methylene signals from unsaturated fatty acids (δ 1.28, 1.32) (Fig. 4D). This showed that the level of unsaturated fatty acids increased compared to the level of monounsaturated and saturated fatty acids after the 2 WAF time point in the developing seeds, which was also seen in Fig. S2. The primary biosynthesis of saturated fatty acids and oleic acid takes place in the plastids, after which they are further desaturated by Δ12 and Δ15 desaturases (FAD2 and FAD3) in the endoplasmic reticulum (Snyder & Weselake, 2013). In our earlier study, the co-expression of several Kennedy pathway genes involved in triacylglycerol assembly was observed during the seed development in *B. napus* and *B. rapa* (Vuorinen et al., 2014). The low temperature treatment increased the level of α-linolenic acid in turnip rape, but any major increases in the *FAD3* expression were not measured. A higher α-linolenic acid content was also seen with the field grown samples from Maaninka, where the temperature sums remain low due to the shorter season.

4. Conclusions

A clear trend in the distribution of *B. napus* and *B. rapa* was observed in several models, although the discrimination of the species was not absolute even after the use of supervised multivariate methods. Higher levels of polyunsaturated fatty acids, especially α-linolenic acid, and sucrose were more characteristic to turnip rape than oilseed rape, while sinapine levels were higher in the latter. Some trends were also seen amongst the growing places, particularly samples from Maaninka and Pernaja, as well as the samples from Jokioinen and Hauho shared some similar characteristic, respectively. Complimentary information was gained by using different extraction solvents.

The knowledge on crop quality and differences between species, cultivars, and growing sites in order to find the optimum conditions for producing profitable crops may rise to even greater importance in the near future, considering *e.g.* climatic changes and the current debate on the commonly used pesticides (neonicotinoids) in the protective treatment of the oilseeds. The methodology used here could potentially be used for detecting genetically modified oilseeds with *e.g.* altered fatty acid composition before their production into food oils. Sample throughput could be increased by focusing on single-solvent extraction (depending on the metabolites of interest) while saving time on sample handling. By including samples from several crop years the multivariate models could be further improved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.040.

References

Abbadi, A., & Leckband, G. (2011). Rapeseed breeding for oil content, quality, and sustainability. *European Journal of Lipid Science and Technology, 113*, 1198–1206.

Abdel-Farid, I. B., Kim, H. K., Choi, Y. H., & Verpoorte, R. (2007). Metabolic characterization of *Brassica rapa* leaves by NMR spectroscopy. *Journal of Agricultural and Food Chemistry, 55*, 7936–7943.

Alonso-Salces, R. M., Héberger, K., Holland, M. V., Moreno-Rojas, J. M., Mariani, C., Bellan, G., Reniero, F., & Guillou, C. (2010). Multivariate analysis of NMR fingerprint of the unsaponifiable fraction of virgin olive oils for authentication purposes. *Food Chemistry, 118*, 956–965.

Awada, M., Soulage, C. O., Meynier, A., Debard, C., Plaisancié, P., Benoit, B., et al. (2012). Dietary oxidized *n*-3 PUFA induce oxidative stress and inflammation: Role of intestinal absorption of 4-HHE and reactivity in intestinal cells. *Journal of Lipid Research, 53*, 2069–2080.

Barthet, V. J. (2013). Measurement of oil content by rapid analytical techniques. In U. Thiyam-Holländer, N. A. M. Eskin, & B. Matthäus (Eds.), *Canola and rapeseed: Production, processing, food quality, and nutrition* (pp. 125–144). Boca Raton: CRC Press.

Baumert, A., Milkowski, C., Schmidt, J., Nimtz, M., Wray, V., & Strack, D. (2005). Formation of a complex pattern of sinapate esters in *Brassica napus* seeds, catalyzed by enzymes of a serine carboxypeptidase-like acyltransferase family? *Phytochemistry, 66*, 1334–1345.

Chen, X., Li, H., Lei, X., Zhu, X., & Zhang, A. (2010). Application of a hybrid variable selection method for the classification of rapeseed oils based on ^{1}H NMR spectral analysis. *European Food Research and Technology, 230*, 981–988.

Eriksson, L., Johansson, E., Kettaneh-Wold, N., Wold, S., Trygg, J., & Wikström, C. (2006). *Multi- and megavariate data analysis part I: Basic principles and applications* (2nd ed.). Umeå: Umetrics AB.

Fang, G., Goh, J. Y., Tay, M., Lau, H. F., & Li, S. F. Y. (2013). Characterization of oils and fats by ^{1}H NMR and GC/MS fingerprinting: Classification, prediction and detection of adulteration. *Food Chemistry, 138*, 1461–1469.

Hatzakis, E., Koidis, A., Boskou, D., & Dais, P. (2008). Determination of phospholipids in olive oil by ^{31}P NMR spectroscopy. *Journal of Agricultural and Food Chemistry, 56*, 6232–6240.

Hill, L. M., Morley-Smith, E. R., & Rawsthorne, S. (2003). Metabolism of sugars in the endosperm of developing seeds of oilseed rape. *Plant Physiology, 131*, 228–236.

Kangas, A., Laine, A., Niskanen, M., Salo, Y., Vuorinen, M., Jauhiainen, L., et al. (2011). *MTT Kasvu 18: Virallisten lajikekokeiden tulokset 2004–2011 (Results of official variety trials 2004–2011)* (2nd ed.). Jokioinen: MTT.

Longobardi, F., Ventrella, A., Napoli, C., Humpfer, E., Schütz, B., Schäfer, H., et al. (2012). Classification of olive oils according to geographical origin by using ^{1}H NMR fingerprinting combined with multivariate analysis. *Food Chemistry, 130*, 177–183.

Milkowski, C., & Strack, D. (2010). Sinapate esters in brassicaceous plants: Biochemistry, molecular biology, evolution and metabolic engineering. *Planta, 232*, 19–35.

Peltonen-Sainio, P., Jauhiainen, L., & Sadras, V. O. (2011). Phenotypic plasticity of yield and agronomic traits in cereals and rapeseed at high latitudes. *Field Crops Research, 124*, 261–269.

Quijada, P., Cao, J., Wang, X., Hirai, M., & Kole, C. (2007). *Brassica rapa*. In C. Kole (Ed.), *Genome mapping and molecular breeding in plants. Oilseeds* (Vol. 2, pp. 211–263). Berlin Heidelberg: Springer-Verlag.

Sacchi, R., Addeo, F., & Paolillo, L. (1997). ^{1}H and ^{13}C NMR of virgin olive oil. An overview. *Magnetic Resonance in Chemistry, 35*, S133–S145.

Sacchi, R., Mannina, L., Fiordiponti, P., Barone, P., Paolillo, L., Patumi, M., et al. (1998). Characterization of Italian extra virgin olive oils using ^{1}H-NMR spectroscopy. *Journal of Agricultural and Food Chemistry, 46*, 3947–3951.

Sacco, A., Brescia, M., Liuzzi, V., Reniero, F., Guillou, G., Ghelli, S., et al. (2000). Characterization of Italian olive oils based on analytical and nuclear magnetic resonance determinations. *Journal of the American Oil Chemists' Society, 77*, 619–625.

Schwartz, H., Ollilainen, V., Piironen, V., & Lampi, A. (2008). Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. *Journal of Food Composition and Analysis, 21*, 152–161.

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & Pharmacotherapy, 56*, 365–379.

Snowdon, R., Lühs, W., & Friedt, W. (2007). *Brassica napus*. In C. Kole (Ed.), *Genome mapping and molecular breeding in plants. Oilseeds* (Vol. 2, pp. 55–114). Berlin Heidelberg: Springer-Verlag.

Snyder, C. L., & Weselake, R. J. (2013). Modification of seed oil formation in *Brassica* oilseed species. In U. Thiyam-Holländer, N. A. M. Eskin, & B. Matthäus (Eds.), *Canola and rapeseed: Production, processing, food quality, and nutrition* (pp. 101–123). Boca Raton: CRC Press.

Spyros, A., & Dais, P. (2013). Fats and oils. In P. S. Belton (Ed.), *NMR spectroscopy in food analysis* (pp. 149–201). Cambridge: Royal Society of Chemistry.

Tarvainen, M., Phuphusit, A., Suomela, J., Kuksis, A., & Kallio, H. (2012). Effects of antioxidants on rapeseed oil oxidation in an artificial digestion model analyzed by UHPLC–ESI–MS. *Journal of Agricultural and Food Chemistry, 60*, 3564–3579.

Vigli, G., Philippidis, A., Spyros, A., & Dais, P. (2003). Classification of edible oils by employing ^{31}P and ^{1}H NMR spectroscopy in combination with multivariate statistical analysis. A proposal for the detection of seed oil adulteration in virgin olive oils. *Journal of Agricultural and Food Chemistry, 51*, 5715–5722.

Vuorinen, A. L., Kalpio, M., Linderborg, K. M., Kortesniemi, M., Lehto, K., Niemi, J., et al. (2014). Coordinate changes in gene expression and triacylglycerol composition in the developing seeds of oilseed rape (*Brassica napus*) and turnip rape (*Brassica rapa*). *Food Chemistry, 145*, 664–673.

Wang, S. X., & Ellis, B. E. (1998). Enzymology of UDP-glucose:sinapic acid glucosyltransferase from *Brassica napus*. *Phytochemistry, 49*, 307–318.

Weselake, R. J., Taylor, D. C., Rahman, M. H., Shah, S., Laroche, A., McVetty, P. B. E., et al. (2009). Increasing the flow of carbon into seed oil. *Biotechnology Advances, 27*, 866–878.

Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M., Knox, C., Liu, Y., et al. (2013). HMDB 3.0 – the human metabolome database in 2013. *Nucleic Acids Research, 41*, D801–D807.