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Low sclareol by-product of clary sage concrete: chemical analysis of a waste product of the perfume industry

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ABSTRACT: In line with the sustainable development objectives of the Claryssime® research programme, a by-product of *Salvia sclarea* concrete (low sclareol by-product) obtained from industrial scalreol purification, was subjected to full chemical fractionation, characterization and assessment of its economic potential as a by-product synergy candidate. Fractionation and intensive semi-preparative high-performance liquid chromatography isolation led to the characterization of several compounds that belong to different metabolite families, including flavonoids [salvigenin (3), 1.8%], diterpenoids [sclareol (6), 4.6%] and triterpenoids [oleanolic acid (10), 6.2%], and triacylglycerols (60.8%). The triacylglycerol fraction was further studied: its total fatty acid content was analysed by gas chromatography–mass spectrometry and all the triacylglycerols were described by ultra-performance liquid chromatography–quadrupole time of flight mass spectrometry (UPLC-QToF) analysis. The reproducibility of the low sclareol by-product chemical composition was assessed and validated by quantitative high-performance liquid chromatography–evaporative light scattering detector analysis and its evolution during the process was investigated via the analysis of a rich sclareol by-product. Such an analytical approach, from the improved fractionation to the triacylglycerol analysis via UPLC-QToF, can be applied to many industrial concretes or other by-products from natural ingredient extraction. Copyright © 2012 John Wiley & Sons, Ltd.

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Keywords: by-product valorization; Salvia sclarea; concrete; TAG; UPLC-QToF

Introduction

The principle of by-product synergy consists in the use of an industry's waste stream as another's primary resource.[1,2] Such a principle is a cornerstone for industrial companies driven by a policy of sustainable development.[3] By-product synergy is really important for the natural product industry. Such waste still contains numerous substances which can fulfill some market needs of the fragrance, cosmetic, nutraceutical and pharmaceutical industries.[4] The development of an analytical methodology applicable to the characterization of such by-products is essential for many societies. Our interest was turned to the fragrance industry and, more specifically, to the process of sclareol extraction from clary sage (*Salvia sclarea*). Nowadays, clary sage is mainly cultivated for the production of sclareol, which is used as a key starting material for the industrial synthesis of Ambrox®, a fundamental ingredient of most modern amber-based fragrances.[5,6] The high demand for sclareol makes its extraction from clary sage a crucial issue for the flavour and fragrance industry. Therefore, clary sage is widely extracted on an industrial scale with apolar solvents to generate extracts called concretes (Figure 1). Production of the concrete starts with a random mixture of fresh, wilt-dried and/or distilled clary sage. Different types of physical purification processes can be applied to these overall concretes to yield purified sclareol. The remaining low sclareol by-product (LSB) constitutes an industrial waste that has not previously been analysed. An attempt to valorize a *S. sclarea* by-product (extracted straw, not LSB) as an antioxidant ingredient was reported by Mela and Gaydou.[7] Recent studies have revealed that several raw extracts and pure compounds from *Salvia* species are active against a wide range of biological targets. Such activities could be expected from LSB.[8–12] The amount of LSB produced annually is sizable given the sclareol content of clary sage concretes (45–65% w/w). According to Bontoux SA, one of the biggest French producers of sclareol, the amount of LSB can reach several tens of tons a year in France.

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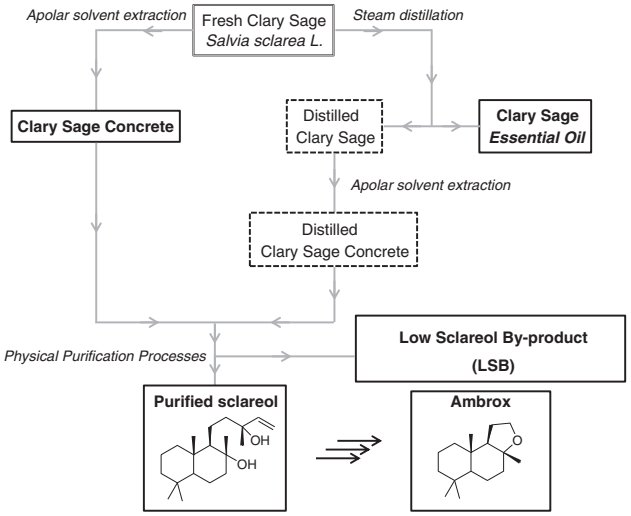


Figure 1. Sclareol extraction process from clary sage

Driven by the goals of sustainable development and the strengthening of the French sclareol competitiveness described in the Claryssime® research programme (http://www.claryssime.fr), an analysis of the constituents of LSB was undertaken to evaluate its market potential. This paper reports the first qualitative and quantitative analyses of LSB and a rich sclareol by-product (RSB; an earlier by-product in the sclareol purification process analysed here to evaluate the chemical changes of the concrete during the process). Analytical and semi-preparative high-performance liquid chromatography with a diode array evaporative light scattering detector (HPLC-DAD-ELSD), gas chromatography–mass spectrometry with a flame ionization detector (GC-MS-FID), nuclear magnetic resonance (NMR), and ultra-performance liquid chromatography–quadrupole time of flight mass spectrometry (UPLC-QToF) were used to analyse all the molecules. This methodology can be used as a template for the analysis of all concretes and concrete extraction by-products of the fragrance industry.

Methods

Chemicals

Acetonitrile (ACN, Chromasolv® for HPLC, > 99.9%), boron trifluoride diethyl etherate (BF_3–Et_2O, purified and redistilled), hydrochloric acid (HCl, 37% vol.) dichloromethane (DCM, Chromasolv® for HPLC, > 99.8%), diethyl ether (Et_2O, Puriss, purity > 99.5%), formic acid (puriss, > 98%), isopropanol (iPrOH, Chromasolv® for HPLC, > 99.9%), light petroleum (LP, 40–60 °C Puriss), methanol (MeOH, Chromasolv® for HPLC, > 99.9%), tetrahydrofuran (THF, Chromasolv® for HPLC, > 99.9%), standard fatty acid methyl esters (F.A.M. E. Mix C14–C22), and water (H_2O, Chromasolv® plus) were supplied by Sigma–Aldrich (XXXXX, XXXXX).

Ethanol (EtOH, 96% vol.) and magnesium sulfate ($MgSO_4$, technica) were supplied by VWR (XXXXX, XXXXX). Potassium hydroxide (KOH, 85%) was supplied by Panreac Quimica Sau (XXXXX, XXXXX). Leucine enkephalin (3 ± 0.15 mg) and SPE cartridges Oasis® were supplied by Waters (XXXXX, XXXXX).

All solvents and buffers for UPLC-MS analyses, acetic acid (Optima), ammonia (NH_3, 21% vol. optima), acetonitrile (ACN, Optima for HPLC-MS), methanol (MeOH, Optima for HPLC-MS), water (H_2O, Optima for HPLC-MS), and isopropanol (iPrOH, Optima for HPLC-MS) were supplied by Fischer Scientific (XXXXX, XXXXX). High-purity sclareol (99.7%) was supplied by Bontoux SA.

Materials

Several clary sage concretes were produced by Bontoux SA during the campaign of summer 2011. Different qualities of raw concretes were provided according to their cropping qualities (fresh or wilt-dried) and distillation process. The industrial purification processes were carried out by Bontoux SA, following exclusively solventless physical processes on the previous concretes, to remove most of the sclareol. Ten different batches of LSB from 10 different concretes were studied. RSB is an intermediate product from an earlier step of the industrial process provided by Bontoux SA to study the chemical composition changes during the sclareol extraction process.

High-performance Liquid Chromatography Analyses

Samples were first weighed into a 10 ml vial, solubilized in THF (four times the final concentration), 250 µl of this solution was added in a new 2 ml vial, completed to 1 ml with isopropanol and filtered to generate samples at the working concentrations. All HPLC analyses were performed on an Agilent 1200 series HPLC (XXXXX, XXXXX) equipped with a ELSD and DAD detectors using a Phenomenex Luna C18 column (4.6 × 150 mm, 5 µm). The ELSD was used under the following conditions: temperature: 40 °C; nebulizing gas pressure: 3.6 bar; gain: 3; sampling time: 100–10 Hz; filter: 3 s. The DAD provided three characteristic UV wavelengths: 210, 254 and 280 nm. A standard HPLC method was assessed and corresponds to a solvent system gradient H_2O/ACN/iPrOH from 50:50:0 (v/v/v) to 0:20:80 (v/v/v) in 50 min (standard HPLC method). The compound isolations were carried out by semi-preparative HPLC on the same HPLC Agilent 1200 series with a Phenomenex Luna C18 column (10 × 250 mm, 5 µm).

Fractionation of the Low Sclareol By-product and Isolation of its Compounds

LSB (17 g) was solubilized in THF (100 ml), silica gel (20 g) was added to the mixture, and the solvent was evaporated. The dry load obtained was poured on the top of a 200 g silica gel column. Fractions were eluted according to a solvent gradient of increasing polarity (LP, Et_2O, MeOH; 1 litre of each solvent system). Each fraction was analysed on HPLC-DAD-ELSD via the standard HPLC method. Moreover, the ^{1}H NMR spectra of each fraction provided information on its overall composition.

The fractions from LP to LP/Et_2O 6:4 (v/v) were combined to obtain a lipidic fraction (9.4 g, majority of triglyceride assessed by ^{1}H NMR) and the other fractions were further studied to isolate the major polar compounds of LSB and to elucidate their structures.

Pure oleanolic acid (**8**, 22 mg) was isolated from the fraction LP/Et_2O 5:5 (v/v) by semi-preparative HPLC according to a solvent system gradient ACN/iPrOH from 100:0 to 40:60 in 20 min.

Pure salvigenin (**3**, 14.5 mg) was isolated from the fraction Et_2O/MeOH 8:2 (v/v) eluted in semi-preparative HPLC by a solvent system gradient H_2O/ACN from 30:70 (v/v) to 0:100 (v/v) in 20 min.

The sclareol (**6**) was identified in the fraction Et_2O 100% by comparison of its retention time in HPLC-DAD-ELSD with the recrystallized compound supplied by Bontoux SA.

These studies also led to the isolation of minor compounds. The fraction LP/Et_2O 6:4 (v/v) provided several pure compounds: the 3-oxo-oleanolic acid (**10**, 7 mg) by semi-preparative HPLC according to a solvent system gradient ACN/iPrOH from 90:10 (v/v) to 40:60 (v/v) in 30 min and the 13-episclareol (**7**, 5 mg), 3-hydroxysclareol, 3-oxosclareol, and the 2α,3α-dihydroxy-24-nor-4(23),12-oleanadien-28-oic acid (**4**, 1.3 mg) were isolated from the fraction Et_2O/MeOH 8:2 by reversed-phase flash chromatography (gradient from 100% H_2O to 100% MeOH) followed by a semi-preparative HPLC eluted with a solvent system gradient H_2O/MeOH from 50:50 (v/v) to 0:100 (v/v) in 30 min. The taraxasterol acetate (**11**, 5 mg) and α-amyrin acetate (**12**, 4.3 mg) were isolated from the fraction LP/Et_2O 7:3 (v/v) by semi-preparative HPLC according to the solvent gradient system ACN/iPrOH from 80:20 (v/v) to 40:60 (v/v) in 30 min.

Fractionation of the Rich Sclareol By-product and Isolation of its Compounds

RSB (1 g) was solubilized in THF (10 ml), silica gel (1 g) was added to the mixture, and the solvent was evaporated. The dry load obtained was poured on the top of a 75 g silica gel column. Fractions were eluted according to a solvent gradient of increasing polarity (LP, Et_2O, MeOH; 100 ml of each solvent system). After analysing each fraction according to the standard HPLC method, the LP/Et_2O 4:6 (v/v) (191 mg) was subjected to a semi-preparative HPLC according to the solvent gradient system H_2O/ACN from 50:50 (v/v) to 0:100 (v/v) in 20 min to afford 7,4′-dimethoxyapigenin (**5**, 2 mg), and the Et_2O fraction was further fractionated by SPE (RP-C18, 2 g) according to a decreasing polarity step gradient from MeOH/H_2O, 0:1 (v/v) to 1:0 (v/v), to DCM/MeOH, 0:1 (v/v) to 1:0 (v/v). The ursolic acid (**9**, 1 mg) was isolated from the MeOH/H_2O 4:1 (v/v) fraction by semi-preparative HPLC according to the solvent gradient system ACN/iPrOH from 100:0 (v/v) to 90:10 (v/v) in 30 min.

Structural Elucidation

Structural elucidations were carried out by comparison of the literature spectral data listed in Table 1 with the ^1H and ^{13}C NMR spectra of the compounds recorded with a 200 MHz or 500 MHz Bruker Avance NMR spectrometer and their masses obtained on a Bruker Esquire 3000 plus on electrospray ionisation mode (Bruker, XXXXX, XXXXX). Volatile compounds, sclareol (**6**) and 13-episclareol (**7**), were assigned by GC-MS analysis by comparison of their retention index and mass spectra. The ^1H and ^{13}C NMR spectra of each isolated compound are available as supporting information.

Improved Fractionation of the Low Sclareol By-product

The LSB fractionation was optimized to afford the TAGs in a single fraction named the TAG fraction. Different proportions of LP and Et_2O were assessed and all the fractions were analysed by HPLC-DAD-ELSD according to the same standard HPLC method. The optimization was relevant by adding 3 g of silica gel to 1 g of LSB to afford the dry load, 50 g of stationary phase was used, and each fraction was eluted with 250 ml of different solvent systems. Figure 2 shows the HPLC chromatograms of each fraction and the corresponding conditions of elution. This optimization afforded three distinct fractions which contained, respectively, the TAG fraction, oleanolic acid (**8**), and sclareol (**6**) along with salvigenin (**3**). This fractionation was useful to enhance the isolation yield of each major compound in order to use them as standard for their quantification.

Quantification of the Major Polar Compounds

The quantification of salvigenin (**3**), sclareol (**6**), and oleanolic acid (**8**) was carried out in HPLC-ESLD using the standard HPLC method. The quantification was obtained using calibration curves with a range of four concentrations from 0.25 mg/ml to 2 mg/ml for oleanolic acid (**8**) and sclareol (**6**), and from 0.125 to 1 mg/ml for salvigenin (**3**). The previously isolated compounds were used as standards and each sample was triplicated. Table 2 shows the calibration curve equations presenting the logarithm of the concentration of each compound in LSB samples as a function of the logarithm of ELSD peak areas corresponding to a 30 µl injection. The concentration of each compound in the sample and their proportion in LSB can be calculated via the equations:

Table 1. Composition of the low sclareol by-product

N°	Retention time, t_R (min)	Compound name	Literature reference for identification	Presence in *S. sclarea*	Presence in *Salvia* species	Literature reference
1	2.9	3-Hydroxy-sclareol	13	—	—	—
2	4.1	3-Oxo-sclareol	13	—	—	—
3	10.5	Salvigenin	14	✓	—	15, 16
4	11.1	2α,3α-Dihydroxy-24-nor-4(23),12-oleanadien-28-oic acid	17	—	*S. carduacea*	17
5	12.8	7,4′-Dimethoxyapigenin	18	✓	—	15, 16
6	15.5	Sclareol	GC-MS[a]	✓	—	—
7	16.2	13-Episclareol	GC-MS[a]	✓	—	—
8	20.3	Oleanolic acid	19, 20[b]	✓	—	21
9	21.2	Ursolic acid	19, 20	✓	—	21
10	28.7	3-Oxo-oleanolic acid	22	✓	—	23
11	30.2	Taraxasterol acetate	24	—	—	—
12	31.1	α-Amyrin acetate	25	—	*S. cyanyscens*	26

[a]Identification confirmed by GC-MS analysis and retention index comparison.
[b]Oleanolic acid NMR were recorded in DMSO-d_6 and compared with the data recorded for a commercial standard.

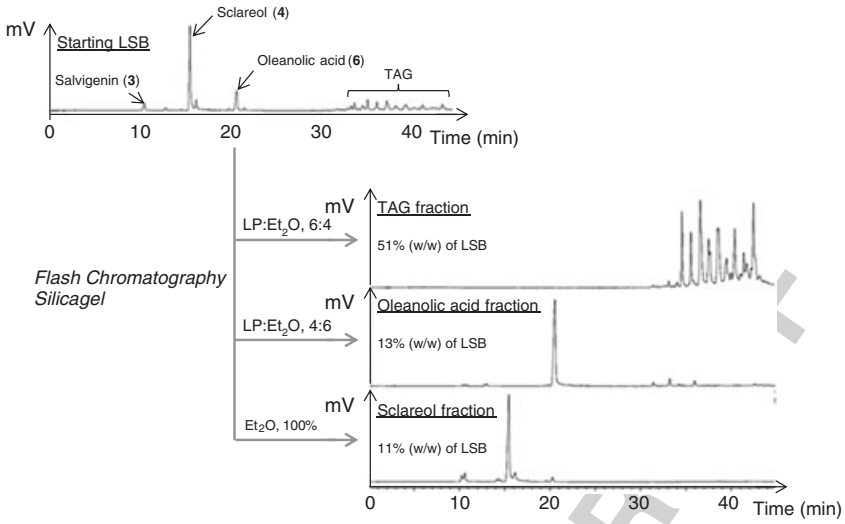


Figure 2. High-performance liquid chromatography with evaporative light scattering detection (HPLC-ELSD) chromatograms of the optimized fractions of the low sclareol by-product (LSB)

Table 2. Calibration curve equations

Low sclareol by-product compound	Slope of the curve, α	Intercept of the curve, β	R^2
Salvigenin (**3**)	0.596 ± 0.08	-1.98 ± 0.18	0.9992
Sclareol (**6**)	0.716 ± 0.04	-2.44 ± 0.10	0.9993
Oleanolic acid (**8**)	0.657 ± 0.04	-1.91 ± 0.12	0.9995
Triacylglycerol fraction	0.639 ± 0.06	-1.48 ± 0.16	0.9980

Results are given as the mean ± standard deviation.
The standard deviation is based on the injection of three different aliquots for each concentration.

$$\log C_i = \alpha \log A_i + \beta$$

and

$$\text{LSB}\ (\%) = \frac{C_i}{C_{LSB}}$$

where C_i is the concentration of compound i in the sample, C_{LSB} is the concentration in LSB of the analysed sample, A_i is the area of the ELSD signal of compound i, α is the slope of the calibration curve and β is its *y*-intercept. The R^2 coefficients were all acceptable above 0.999. The limit of detection and limit of quantification were not of interest because of the good availability of LSB and the high sensitivity of the ELSD.

Quantification of the Triacylglycerol Fraction

With the aim of estimating the quantity of lipids in LSB, the TAG fraction obtained after optimized fractionation was used to build a calibration curve showing the logarithm of the TAG fraction concentrations (mg/ml) in the samples as a function of the logarithm of the ELSD TAG domain integration area from 31 to 47.5 min. Therefore, a concentration range from 2.5 to 20 mg/ml was injected (30 µl) three times following the previous conditions. The lipid concentration in the sample against the TAG fraction and its proportion in LSB were calculated with the previous formula.

As previously mentioned, the R^2 coefficients were all acceptable above 0.998 and the limit of detection and limit of quantification were not of interest because of the good availability of LSB and the high sensitivity of the ELSD.

Synthesis of Fatty Acid Methyl Esters

The TAG fraction (100 mg) was solubilized in THF (15 ml) and treated with an excess of KOH in EtOH (2 M, 5 ml). The mixture was stirred at room temperature for 4 h. The reaction mixture was evaporated to neat and solubilized in DCM (20 ml). The organic layer was washed twice with 15 ml of HCl (1 N). The organic layer was dried under $MgSO_4$, filtered, and evaporated to yield 130 mg of a crude material. This material was solubilized in a MeOH:DCM 1:3 v/v mixture (20 ml) before the addition of BF_3–Et_2O (200 µl). The reaction mixture was stirred overnight at room temperature. After evaporation, the crude material was solubilized in DCM and washed with H_2O. The organic layer was dried under $MgSO_4$, filtered and evaporated to yield 80 mg of fatty acid methyl esters.

Analysis of the Fatty Acid Methyl Esters

A 30 mg/ml solution of fatty acid methyl esters in DCM was prepared for GC-MS and GC-FID analyses. GC-MS analyses were carried out using an Agilent 6890 N/5973 N system equipped with a DB-WAX column (25 m × 0.18 mm; film thickness, 0.3 µm) and operated using the following conditions: carrier gas: helium; constant flow: 1 ml/min; injector temperature: 250 °C; injected volume: 1 µl and split ratio: 1:100. GC oven temperature was set to 150 °C and increased to 220 °C with a rate of 5 °C/min and remained at 220 °C for 20 min. Transfer line temperature: 270 °C. Electron impact mass spectra were recorded at 70 eV with a mass range from *m/z* 30 to 350 amu. Their proportions were assessed by GC-FID using an Agilent 6890 N system equipped with a DB-WAX column (25 m 0.18 mm; film thickness, 0.3 µm) and operated using the following conditions: carrier gas: hydrogen; constant flow: 1 ml/min; injector temperatures: 250 °C; injected volume: 1 µl and split ratio: 1:100. GC oven temperature was set to 150 °C and increased to 220 °C with a rate of 5 °C/min and remained at 220 °C for 20 min. The conditions for FID were: detector temperature: 300 °C; hydrogen flow:

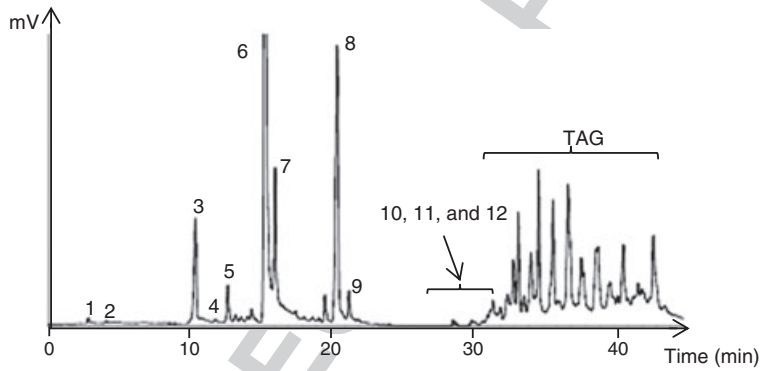
40 ml/min; air flow: 450 ml/min and make-up flow N_2 45 ml/min. The characterization of each fatty acid methyl ester was performed by comparison with a mixture of standards, F.A.M.E. Mix C14–C22. Their relative proportions were obtained using FID signal integrations according to the equation:

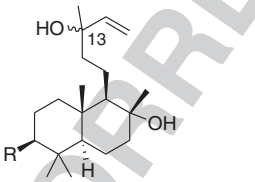
$$FA_i\ (\%) = \frac{A_i}{\sum_j A_j} \times 100$$

where FA is fatty acid, A_i is the FID area of the fatty acid methyl ester i, and A_j is XXXXX.

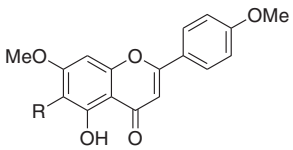
UPLC-QToF-MS^E and MS-MS Analyses of the Triacylglycerols

The analysis of each TAG was performed by UPLC–high-resolution electrospray ionization MS (HRESIMS) analysis on an ACQUITY UPLC®/Xevo™ G2 QToF (Waters) equipped with an ACQUITY UPLC® BEH C_{18} column (2.5 × 100 mm, 1.7 µm) according to an isocratic solvent system solvent A:solvent B 70:30, where solvent A was ACN/MeOH/H_2O 19:19:2 (v/v/v) + 0.1% acetic acid + 0.022% NH_3, and solvent B was iPrOH + 0.1% acetic acid + 0.022% NH_3 at a flow rate of 0.3 ml/min. The TAG fraction was analysed by injecting 1 µl of a solution in iPrOH of the TAG fraction at a concentration of 100 µg/ml. Mass spectrometry was performed on a G2-QToF (Waters, UK) operated in the positive electrospray ionization mode (ESI+). The capillary voltage was set at 3 kV and the cone voltage at 40 V. Source and desolvation temperatures were, respectively, 120 °C and 300 °C. The nebulization and the cone gas were adjusted to 500 l/h and 10 l/h. Samples were analysed by MS^E (alternating between low and high energy to capture both molecular ion and fragment exact masses in a single run) and MS-MS modes; the collision energy was kept constant at 30 V and argon was used as collision gas. Data were collected in centroid mode, using the $[M+H]^+$ ions of leucine-enkephalin (10 µl/min of a 400 ng/µl solution) at *m/z* 556.2771 and 120.0813 Da as the

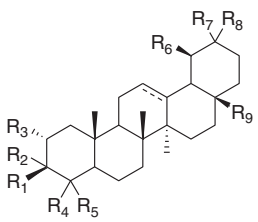




Name	R
3-hydroxysclareol (**1**)	OH (13-R isomer)
3-oxosclareol (**2**)	=O (13-R isomer)
sclareol (**6**)	H (13-R isomer)
13-episclareol (**7**)	H (13-S isomer)



Name	R
salvigenin (**3**)	OMe
7,4-dimethoxyapigenin (**5**)	H



Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	Insat.
2α,3α-dihydro-24-nor-4(23),12-oleanadien-28-oic acid (**4**)	H	OH	OH	$=CH_2$	-	H	CH_3	CH_3	COOH	Δ^{12}
oleanolic acid (**8**)	OH	H	H	CH_3	CH_3	H	CH_3	CH_3	COOH	Δ^{12}
ursolic acid (**9**)	OH	H	H	CH_3	CH_3	CH_3	CH_3	H	COOH	Δ^{12}
3-oxo-oleanolic acid (**10**)	=O	-	H	CH_3	CH_3	H	CH_3	CH_3	COOH	Δ^{12}
taraxasterol acetate (**11**)	AcO	H	H	CH_3	CH_3	CH_3	$=CH_2$	-	CH_3	Δ^{30}
α-amyrin acetate (**12**)	AcO	H	H	CH_3	CH_3	CH_3	CH_3	H	CH_3	Δ^{12}

Figure 3. High-performance liquid chromatography with evaporative light scattering detection (HPLC-ELSD) chromatogram of the low sclareol by-product (LSB) and compounds identified in the LSB

lockmasses to ensure the accuracy and reproducibility of mass measurement. The raw data were processed by MassLynx Applications Manager 4.1 (Waters, UK). HRMS deviations (Δ in ppm) of each TAG fragment ([DAG-OH] and [MAG-OH]) and ^{1}H and ^{13}C NMR spectra of each isolated compound are listed in the supporting material.

Results and Discussion

Chemical Composition of the Low Sclareol By-product

LSB is mainly composed of a flavonoid, salvigenin (**3**), a triterpenoid, oleanolic acid (**8**), a diterpenoid, sclareol (**6**), and triacylglycerols (TAGs) as presented in Figure 3 and Table 1. Salvigenin (**2**) and oleanolic acid (**8**) were already described in *S. sclarea*. Further LSB studies led to the identification of several minor triterpenic compounds listed in Figure 3 and Table 1, such as 3-oxo-oleanolic acid (**10**), 2α,3α-dihydroxy-24-nor-4(23),12-oleanadien-28-oic acid (**4**), taraxasterol acetate (**11**), and α-amyrin acetate (**12**). The 3-oxo-oleanolic acid (**10**) was already described from *S. sclarea*. Compounds **4** and **12** were only described in *Salvia carduacea* and *Salvia cyanyscens*, respectively.[17,26] Taraxasterol acetate (**11**) was never isolated from a *Salvia* species. The occurrence of compounds **1** and **2**, which are oxidized derivatives of sclareol, was surprising. Such compounds, hydroxylated and carboxylated at the C-3 position, were only known as fungal and bacterial biotransformation products of sclareol.[13,27] The biosynthetic origin of 3-hydroxysclareol (**1**) and 3-oxosclareol (**2**) was investigated by analysing different industrially made concretes and freshly harvested *S. sclarea* concretes. The freshly harvested *S. sclarea* extracts did not contain any detectable amount of compounds **1** and **2**, even after fractionation to concentrate minor constituents. Their presence in the industrial concretes and LSB samples previously analysed suggests that compounds **1** and **2** may have been generated through the microbial-based biotransformation of sclareol during the storing of the harvested plant material.

Chemical composition of the Rich Sclareol By-product: from concrete to Low Sclareol By-product

Salvigenin (**3**) is usually described in *S. sclarea* along with apigenin, luteolin, and their methoxylated derivatives.[15,16] Likewise, oleanolic acid (**8**) is often described with its isomer ursolic acid (**9**).[28,29] The analysis of a RSB, which is an earlier intermediate by-product in the sclareol extraction process, revealed the presence of a flavonoid, 7,4′-dimethoxyapigenin (**5**), and triterpenoid, ursolic acid (**9**). The comparison of the LSB and RSB HPLC chromatograms confirmed the presence of traces of 7,4′-dimethoxyapigenin (**5**) and ursolic acid (**9**) in LSB. Their relative proportions (ratio between their ELSD signal integration) were investigated at different steps of the process: in the starting concrete, in RSB and in LSB. The ratio of the content of 7,4′-dimethoxyapigenin (**5**) over salvigenin (**3**) remained at 60% in RSB, as described in the literature, and was 80% in LSB.[15] The changes in the relative proportions of oleanolic (**8**) and ursolic acid (**9**) were more significant since they were only 30% in the concrete against 95% in LSB. Ursolic acid (**9**) was reported to be more predominant than oleanolic acid (**8**) in the study of *Salvia sclarea* and seems to be extracted or transformed during the sclareol extraction process.[28]

Quantification of Major Polar Compounds and of the Triacylglycerol Fraction

The quantification of the major compounds provided the results given in Table 3. The analysis of 10 different LSB samples revealed

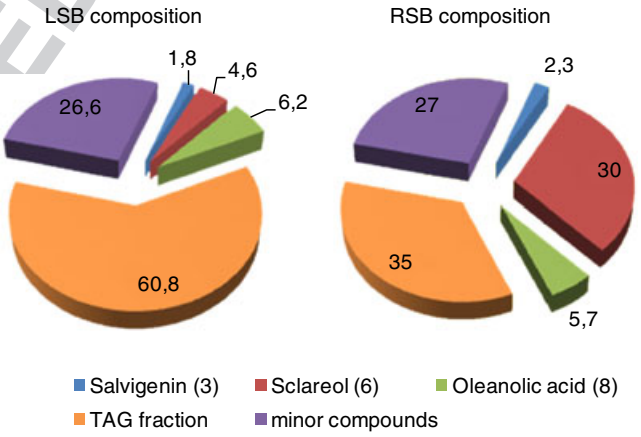


Figure 4. Composition of the the low sclareol by-product (LSB) and the rich sclareol by-product (RSB)

Table 3. Proportion of salvigenin, sclareol, oleanolic acid and triacylglycerol fraction in the low sclareol by-product (LSB)

Sample of LSB	% Salvigenin (**3**)[a]	% Sclareol (**6**)[a]	% Oleanolic acid (**8**)[a]	% TGA[a]
1	2.7	5.8	7.9	59.1
2	2.3	4.6	7.9	52.9
3	1.7	4.5	6.1	56.6
4	1.7	6.4	6.9	58.8
5	1.6	5.7	8.0	64.9
6	1.8	6.0	7.9	67.6
7	1.7	1.0	6.0	76.4
8	1.6	6.0	6.0	67.4
9	1.2	1.6	2.8	51.3
10	1.4	4.5	2.7	53.2
Mean ± 95% CI[b]	1.8 ± 0.3 (17%)	4.6 ± 1.2 (26%)	6.2 ± 1.2 (19%)	60.8 ± 5 (8%)

[a]Quantification method standard deviation based on the injection of three different aliquots of each sample: salvigenin, 11%; sclareol, 8%; oleanolic acid, 11%; triacylglycerol fraction 10%.
CI, confidence interval; TGA, triacylglycerol.

that salvigenin (**3**), sclareol (**6**), oleanolic acid (**8**), and the TAG fraction represented 1.8 ± 0.3, 4.6 ± 1.2, 6.2 ± 1.2, and 60.8 ± 5% of LSB, respectively. The process standard deviation on LSB composition was determined at 17% for salvigenin (**3**), 26% for sclareol (**6**), 19% for oleanolic acid (**8**) and 8% for TAG fractions, which is acceptable for such an industrial process using natural resources from different origins. As presented in Figure 4, 26.6% of LSB was composed of minor compounds already described as 7,4′-dimethoxyapigenin (**5**), 13-episclareol (**7**), ursolic acid (**9**), 3-oxo-oleanolic acid (**10**), 2α,3α-dihydroxy-24-nor-4(23),12-oleanadien-28-oic acid (**4**), taraxasterol acetate (**11**) and α-amyrin acetate (**12**). Major compounds of RSB were also quantified with the aim of evaluating potential changes in the chemical composition during the industrial sclareol extraction process. As presented in Figure 4, the relative proportions of salvigenin (**3**) and oleanolic acid (**8**) remained the same during this process and the proportion of TAGs increased for 25.8% while sclareol decreased from 30% to 4.6%.

Table 4. Total fatty acid content of the triacylglycerol fraction[a]

Fatty acid		*m/z*	R_t (min)	Content (%)[b]	95% CI
Saturated fatty acids					
16:0	Palmitic	270	7.7	15.4	±1.2 (8%)
18:0	Stearic	298	11.4	4.9	±0.2 (4%)
20:0	Arachidic	326	14.7	6.4	±1.9 (30%)
Total saturated fatty acids content		—	—	26.7	—
Unsaturated fatty acids					
18:1, ω9	Oleic	296	11.7	20.3	±1.1 (5%)
18:2, ω6	Linoleic	294	12.5	18.1	±2.7 (15%)
18:3, ω3	Linolenic	292	13.6	34.9	±5.9 (17%)
Total unsaturated fatty acids content		—	—	73.3	—

[a]The relative quantification method standard deviations were below 3% for each FAME.
[b]Relative content.
Values for *m/z* and R_t are given for the relevant methyl ester.

Table 5. Triacylglycerol (TAG) composition of the low sclareol by-product

Total chain length	Total degree of unsaturation	*m/z* ($[M+NH_4]^+$)	Δ (ppm)	TAG molecular species identified[a]	s_T (min)	n.[b]
54	9	890.72	0.4	18:3–18:3–18:3	4.25	1
	8	892.74	0.1	18:3–18:3–18:2	5.03	2
	7	894.76	−0.7	18:3–18:2–18:2	6.02	3
			0.3	18:3–18:3–18:1	6.21	4
	6	896.77	0.1	18:2–18:2–18:2	7.23	6
			0.8	18:3–18:2–18:1	7.5	7
	5	898.78	−0.6	18:2–18:2–18:1	9.14	9
			−0.2	18:3–18:1–18:1	9.49	11
			0	18:3–18:2–18:0	9.95	13
	4	900.8	−1.2	18:2–18:1–18:1	11.63	15
			0.8	18:2–18:2–18:0	12.22	17
			−0.3	18:3–18:1–18:0	12.65	19
	3	902.82	−1.6	18:1–18:1–18:1	14.5	20
			−1.9	18:2–18:1–18:0	15.05	22
	2	904.83	−2.2	18:1–18:1–18:0	17.76	24
52	6	868.74	−0.6	18:3–18:3–16:0	6.35	5
	5	870.75	0.1	18:3–18:2–16:0	7.68	8
	4	872.77	−1.6	18:2–18:2–16:0	9.37	10
			−1.7	18:3–18:1–16:0	9.74	12
	3	874.78	−2.5	18:2–18:1–16:0	11.95	16
	2	876.8	−1.1	18:1–18:1–16:0	14.8	21
50	3	846.75	0.4	18:3–16:0–16:0	9.98	14
	2	848.77	−0.8	18:2–16:0–16:0	12.27	18
	1	850.78	−4.0	18:1–16:0–16:0	15.15	23

[a]TAG fatty acid composition was determined by MS-MS analysis, as detailed in the supporting information.
[b]Numbering is according to the retention time.

Lipid Constituents of the Low Sclareol By-product

Total fatty acid composition of triacylglycerol fraction

The TAGs were mainly composed of the saturated fatty acids (FAs) palmitic acid (16%), stearic acid (4.9%) and arachidic acid (6.1%), along with the high market value unsaturated FAs oleic acid (20.2%), linoleic acid (18%) and linolenic acid (34.8%) (Table 4). The FA composition of RSB was found to be the same. These results are in agreement with data reported in the literature.[30] The FA composition standard deviation fluctuated between 4% and 30% which represented a good reproducibility of the LSB production. A total of 52.8% of the total FA content is considered essential for human health (ω3 and ω6). Moreover, the ω6/ω3 ratio is lower than 4 which reinforces the potential of LSB as dietary supplements or active ingredients for the pharmaceutical and cosmetic industries.

Triacylglycerol composition of the triacylglycerol fraction

With the aim of fully characterizing LSB, TAG composition analyses were undertaken with UPLC-HRESIMS analyses. The analysis of the TAG fraction by UPLC-QToF in MS^E mode provided the molecular ion of each TAG (summarized in Table 5). The TAGs were assigned by ion peak extraction of the TAG mass previously calculated according to the total fatty acid content. Each extracted ion was further checked for not being an isotopic peak of a TAG isomer. As described by Ikeda *et al.*, the molecular ion corresponded to the NH_4^+ adduct which can lose one or two fatty acid moieties to afford the fragment $[DAG\text{-}OH]^+$ and $[MAG\text{-}OH]^+$ after collision-induced fragmentation directly in the source or in the collision cell (Figure 5).[31] The comparison of these molecular formulae directly led us to the constitution of the individual fatty acid chains and allowed us to separately describe each TAG isomer. In total, 24 TAGs were analysed with four pairs of FA position isomers (TAGs n. 3–4, 6–7, 10–12 and 20–22) and two triplets of FA position isomers (TAGs n. 9–11–13 and 15–17–19). This TAG composition was consistent with the FA total content analysis and confirmed the large predominance of linolenic acid (34.9%) followed by oleic (20.3%), linoleic (18.1%) and palmitic (15.4%) acids. Stearic and arachidic acids were not detected here and are probably present in minor TAGs as confirmed by their low content of 4.9% and 6.4%, respectively, in the TAG fraction.

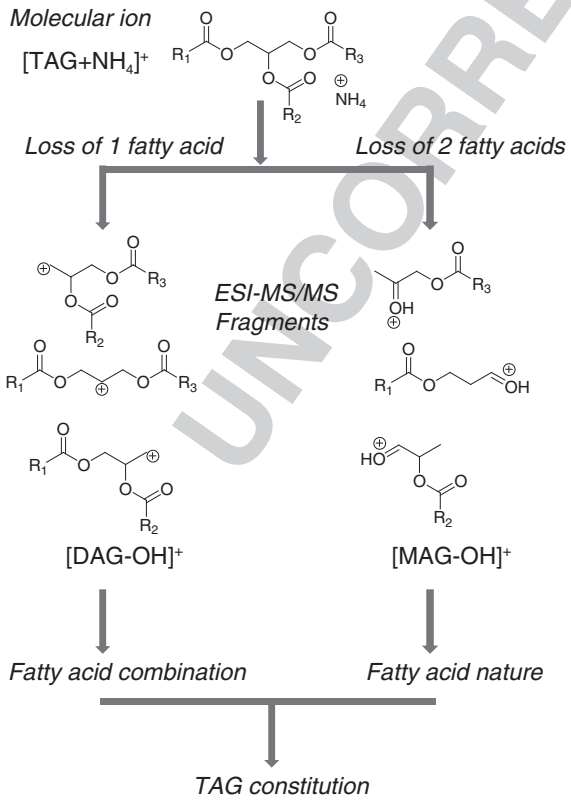


Figure 5. Triacylglycerols (TAGs) fragmentation used for their structural elucidations

The Involvement of the Low Sclareol By-product in By-product Synergy

As a source of active compounds

Flavonoids are well known for providing biological activities such as antioxidant, anti-inflammatory and antimicrobial activities and contribute to the prevention of cardiovascular diseases and cancer.[32] The use of hydroxylated polymethoxyflavones as dietary supplements, food additives, pharmaceutical components, nutraceutical components and cosmetic components has been patented.[33] Bibliographical data on salvigenin (**3**) and 7,4′-dimethoxyapigenin (**5**) do not report such biological activities.[34,35] Indeed, even if a structure–activity relationship cannot be guessed, some studies on polymethoxyflavone asserted that flavonoid activities depend on the presence of hydroxyl and methoxyl moieties which do not fit with our compounds.[36] The triterpenic compounds isolated from LSB were already reported to possess interesting therapeutic properties, such as anti-inflammatory, hepatoprotective, gastroprotective, cardiovascular, anti-tumour, anti-HIV, analgesic and anti-hepatitis among many.[37–43] Nowadays, triterpenoids do not provide enough 'druggability' as medicinal chemistry leads. However, their activities could be a value-added to natural raw material used in cosmetic and dermo-pharmaceutical preparations such as a skincare product. LSB fractions were tested for skin treatment. Some activities were revealed for the oleanolic acid (**8**) fraction against acne with an IC_{50} (human HSD11B1) of 8 μg/ml. Oleanolic acid (**8**), purified or in titrated olive tree leaf extract, has already been described as an active constituent of cosmetic and dermo-pharmaceutical compositions for skin prone to acne.[44]

As lipidic raw material

Polyunsaturated FA (PUFA) based raw materials are widely used as dietary supplements or as active ingredient in pharmaceutical and cosmetic compositions and in painting and lubricant products.[45,46] Besides fish oil, linseed oil is well known as plant sources of ω3 PUFA.[45,46] Some early studies on *Salvia hispanica* seeds recently complemented on *Salvia sclarea* seeds disclosed their potential as linseed oil substitute.[48] Indeed, the oil content of *S. sclarea* seed is 25–30% including 50% of ω3 linolenic acid (giving an overall content of 12–15%). According to our study, LSB contains 60% of lipids including 35% of ω3 linolenic acid which affords an overall content of 20%. As part of the by-product synergy policy, LSB represents a good candidate as a source of ω3.

Conclusion

With the aim of satisfying an ever-increasing demand for natural ingredients by industries and, more precisely, in the flavour and fragrance industry, the whole chemical analysis of a concrete by-product was undertaken. Its potential as by-product synergy

candidates was also assessed. Even if its valorization as a source of active compound seems to be economically unviable, LSB is still under investigation for further valorization as a cosmetic raw material or as an analytical chemistry standard. This by-product is described here for the first time and such an analytical approach could be used for the analysis of other industrial by-products generated by perfume, or more generally, natural ingredient industries.

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Supporting Information

Supporting information may be found in the online version of this article.

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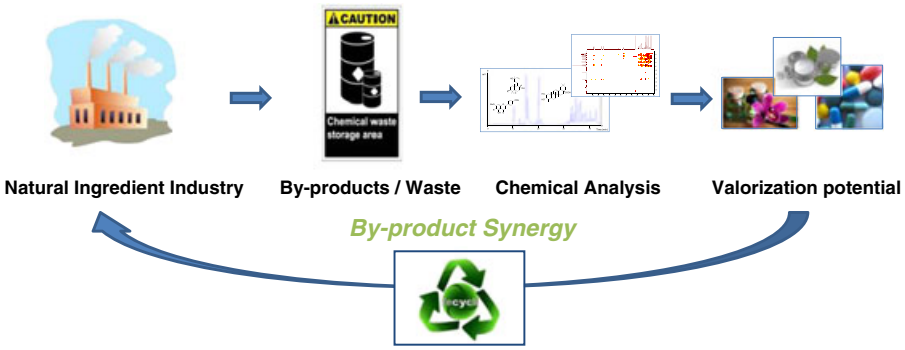
Research Article

Low sclareol by-product of clary sage concrete: chemical analysis of a waste product of the perfume industry

Rémi Laville, Cécilia Castel, Karine Fattarsi, Celine Roy, Laurent Legendre, Claire Delbecque, Pierre-Philippe Garry, Arthur Audran and Xavier Fernandez



In line with the objectives of sustainable development, a by-product of *Salvia sclarea* concrete, obtained from industrial scalreol purification, was subjected to full chemical fractionation, characterization and assessment of its economic potential as a by-product synergy candidate. Several compounds belonging to different metabolite families, including flavonoids, diterpenoids and triterpenoids, and triacylglycerols, were found. The analytical approach can be applied to many industrial concretes or other by-products from natural ingredient extraction.

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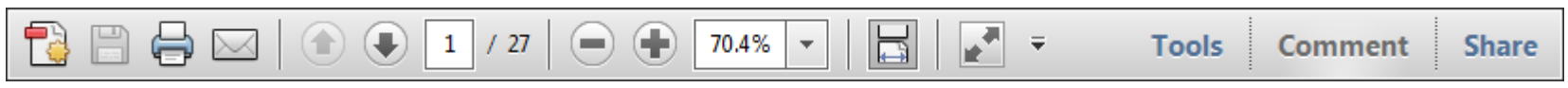
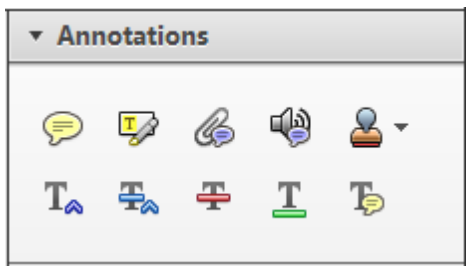
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

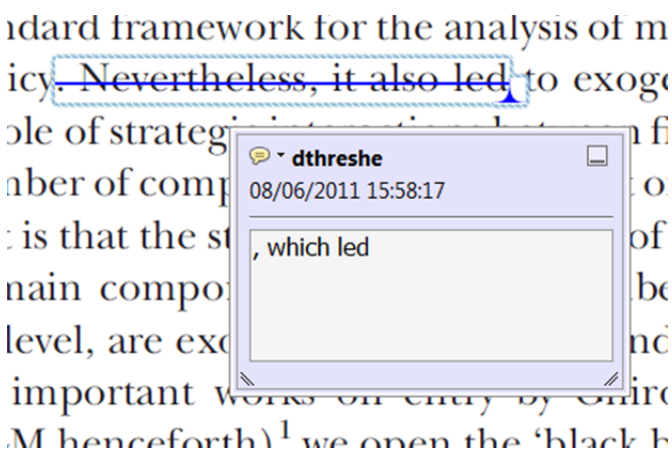


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

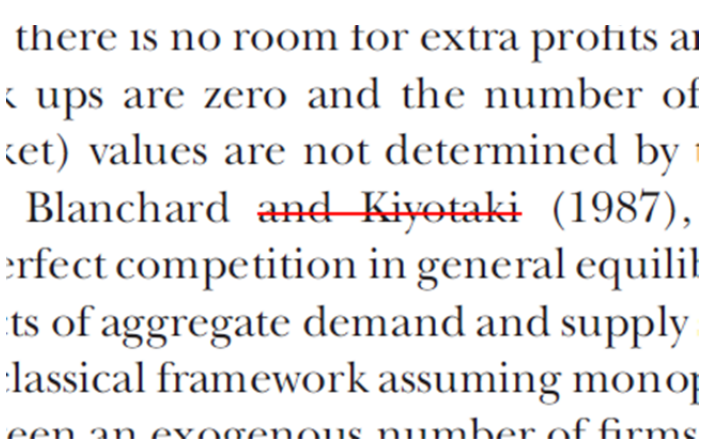


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

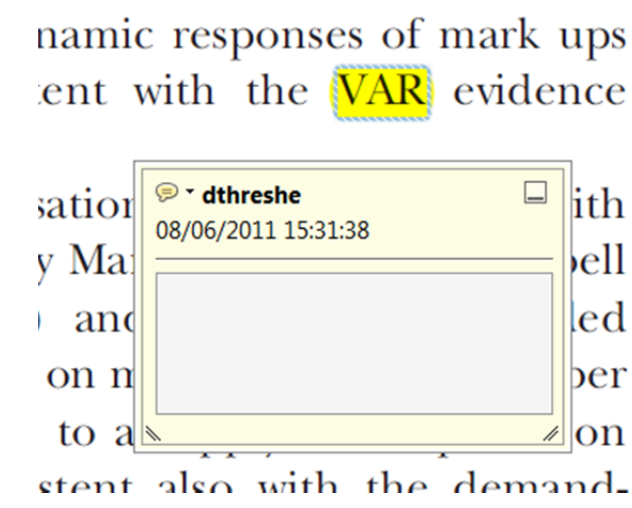


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

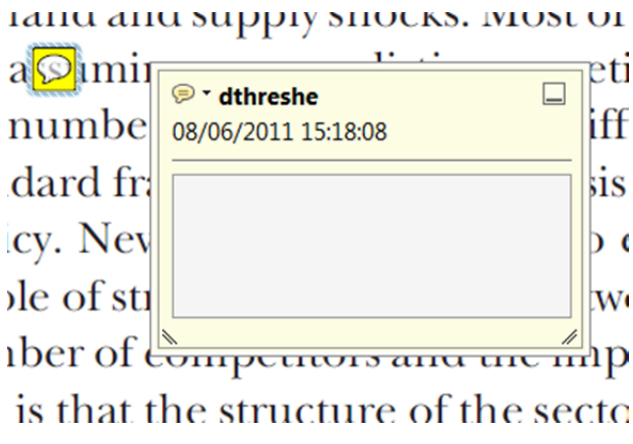


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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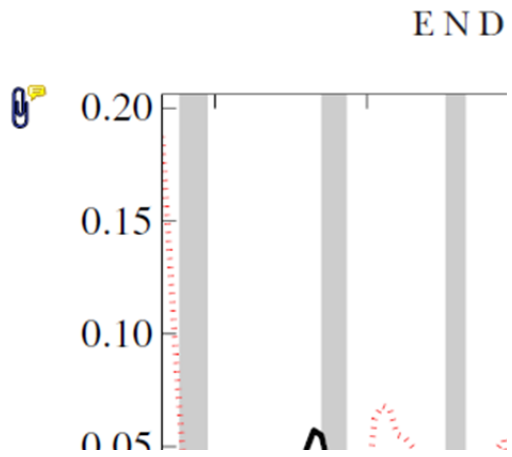
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

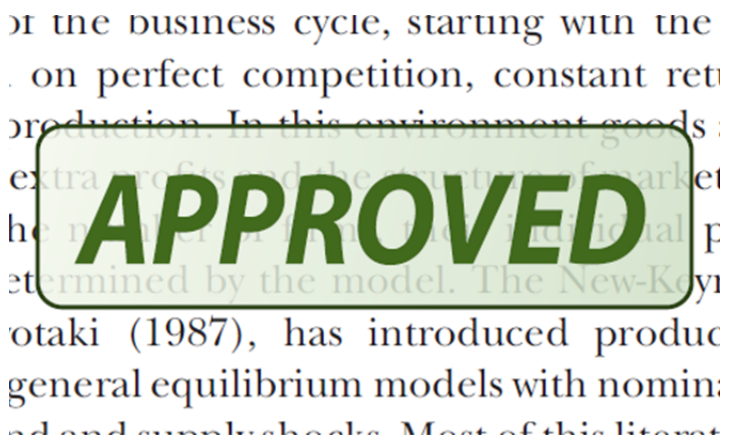


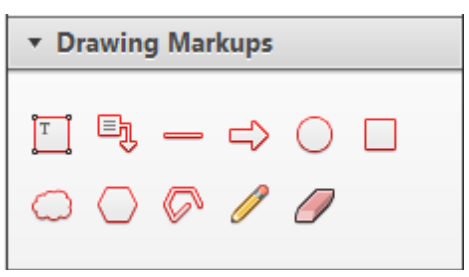
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



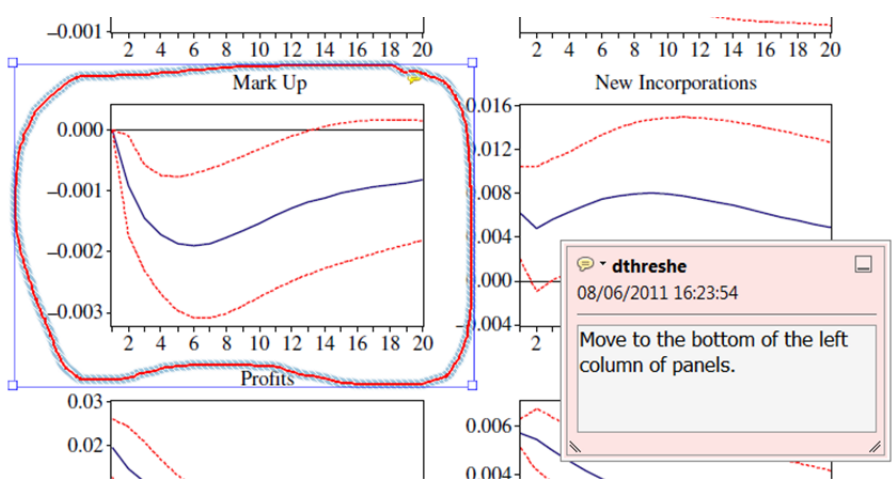


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: