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

Rémi Laville, a,*, Cécilia Castel, a Karine Fattarsi, b Celine Roy, c Laurent Legendre, d Claire Delbecque, e Pierre-Philippe Garry, e Arthur Audran e and Xavier Fernandez a,*

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 sclareol 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.

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

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

Introduction

The principle of by-product synergy consists in the use of industry's waste stream as another's primary resource. 5,6 Such a principle is a cornerstone for industrial companies driven by a policy of sustainable development. 5,6 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. 5,6 The development of an analytical methodology applicable to the characterization of such by-products is essential for many societies. 5,6 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. 7,8 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. 9-13 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.

* Correspondence to: Rémi Laville and Xavier Fernandez, Institut de Chimie de Nice, UMR 7727, Université de Nice-Sophia Antipolis, CNRS, Parc Valrose, 06108 Nice Cedex 2, France. E-mails: remilaville@unice.fr and xavier.fernandez@unice.fr

9 Institut de Chimie de Nice, UMR 7727, Université de Nice-Sophia Antipolis, CNRS, Parc Valrose, 06108 Nice Cedex 2, France

10 Laboratoire de Biotechnologies Végétales appliquées aux Plantes Aromatiques et Médicinales, Faculté de Sciences et Techniques, 23 rue Dr Paul Michelon, 42023 Saint-Etienne Cedex 2, France

11 European Research Institute on Natural Ingredients, Espace Jacques-Louis Lions, 4 Traverse Dupont, 06130 Grasse, France

12 Ecologie Microbienne, CNRS, UMR 5557, Université de Lyon 1, Villeurbanne, F-69622, Lyon, France

13 Bontoux S.A., Quartier Aguzon, Lieu-dit Le Clos, 26 170 Saint Aubans-sur-l'Ouveze, France

Flavour Fragr. J. 2012 Copyright © 2012 John Wiley & Sons, Ltd.
Driven by the goals of sustainable development and the strengthening of the French sclareol competitiveness described in the Claryssime\textsuperscript{TM} 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) 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\textsuperscript{TM} for HPLC, > 99.9%), boron trifluoride diethyl etherate (BF\textsubscript{3}-Et\textsubscript{2}O, purified and redistilled), hydrochloric acid (HCl, 37% vol.) dichloromethane (DCM, Chromasolv\textsuperscript{TM} for HPLC, > 99.8%), diethyl ether (Et\textsubscript{2}O, Puriss, purity > 99.5%), formic acid (puriss, > 98%), isopropyl alcohol (iPrOH, Chromasolv\textsuperscript{TM} for HPLC, > 99.9%), light petroleum (LP, 40–60 °C Puriss), methanol (MeOH, Chromasolv\textsuperscript{TM} for HPLC, > 99.9%), standard fatty acid methyl esters (F.A.M. E. Mix C14–C22), and water (H\textsubscript{2}O, Chromasolv\textsuperscript{TM} plus) were supplied by Sigma–Aldrich (XXXXX, XXXXX). Ethanol (EtOH, 96% vol.) and magnesium sulfate (MgSO\textsubscript{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\textsuperscript{TM} were supplied by Waters (XXXXX, XXXXX). All solvents and buffers for UPLC-MS analyses, acetic acid (Optima), ammonia (NH\textsubscript{3}, 21% vol. optima), acetonitrile (ACN, Optima for HPLC-MS), methanol (MeOH, Optima for HPLC-MS), water (H\textsubscript{2}O, 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 an 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\textsubscript{2}O/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\textsubscript{2}O, MeOH; 1 litre of each solvent system). Each fraction was analysed on HPLC-DAD-ELSD via the standard HPLC method. Moreover, the \textsuperscript{1}H NMR spectra of each fraction provided information on its overall composition. The fractions from LP to LP/Et\textsubscript{2}O 6:4 (v/v) were combined to obtain a lipidic fraction (9.4 g, majority of triglyceride assessed by \textsuperscript{1}H NMR) and the other fractions were further studied to isolate the major polar compounds of LSB and to elucidate their structures.

Pure oleic acid (8, 22 mg) was isolated from the fraction LP/Et\textsubscript{2}O 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\textsubscript{2}O/MeOH 8:2 (v/v) eluted in semi-preparative HPLC by a solvent system gradient H\textsubscript{2}O/ACN from 30:70 (v/v) to 0:100 (v/v) in 20 min. The sclareol (6) was identified in the fraction Et\textsubscript{2}O 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/Et₂O 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-episcclareol (7, 5 mg), 3-hydroxysclareol, 3-oxosclareol, and the 2x,3z-dihydroxy-24-nor-4(23),12-oleanadien-28-oic acid (4, 1.3 mg) were isolated from the fraction Et₂O/MeOH 8.2 by reversed-phase flash chromatography (gradient from 100% H₂O to 100% MeOH) followed by a semi-preparative HPLC eluted with a solvent system gradient H₂O/MeOH from 50:50 (v/v) to 0:100 (v/v) in 30 min. The taraxasterol acetate (11, 5 mg) and a-amyрин acetate (12, 4.3 mg) were isolated from the fraction LP/Et₂O 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, Et₂O, MeOH; 100 ml of each solvent system). After analysing each fraction according to the standard HPLC method, the LP/Et₂O 4.6 (v/v) (191 mg) was subjected to a semi-preparative HPLC according to the solvent gradient system H₂O/ACN from 50:50 (v/v) to 0:100 (v/v) in 20 min to afford 7A,4'-dimethoxyapigenin (5, 2 mg), and the Et₂O fraction was further fractionated by SPE (RP-C18, 2 g) according to a decreasing polarity step gradient from MeOH/H₂O, 0:1 (v/v) to 1:0 (v/w), to DCM/MeOH, 0:1 (v/v) to 1:0 (v/v): The ursolic acid (9, 1 mg) was isolated from the MeOH/H₂O 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 13C 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, XXXX, XXXXX). Volatile compounds, sclareol (6) and 13-episcclareol (7), were assigned by GC-MS analysis by comparison of their retention index and mass spectra. The 1H and 13C 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 Et₂O 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

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time, t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>Compound name</th>
<th>Literature reference for identification</th>
<th>Presence in S. sclarea</th>
<th>Presence in Salvia species</th>
<th>Literature reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.9</td>
<td>3-Hydroxy-sclareol</td>
<td>13</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>4.1</td>
<td>3-Oxo-sclareol</td>
<td>13</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>10.5</td>
<td>Salvigenin</td>
<td>14</td>
<td>✓</td>
<td>—</td>
<td>15, 16</td>
</tr>
<tr>
<td>4</td>
<td>11.1</td>
<td>2x,3z-Dihydroxy-24-nor-4(23),12-oleanadien-28-oic acid</td>
<td>17</td>
<td>—</td>
<td>S. carduacea</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>12.8</td>
<td>7A,4’-Dimethoxyapigenin</td>
<td>18</td>
<td>✓</td>
<td>—</td>
<td>15, 16</td>
</tr>
<tr>
<td>6</td>
<td>15.5</td>
<td>Sclareol</td>
<td>GC-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>✓</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>16.2</td>
<td>13-Episcclareol</td>
<td>GC-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>✓</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>20.3</td>
<td>Oleanolic acid</td>
<td>19, 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>✓</td>
<td>—</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>21.2</td>
<td>Ursolic acid</td>
<td>19, 20</td>
<td>✓</td>
<td>—</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>28.7</td>
<td>3-Oxo-oleanolic acid</td>
<td>22</td>
<td>✓</td>
<td>—</td>
<td>23</td>
</tr>
<tr>
<td>11</td>
<td>30.2</td>
<td>Taraxasterol acetate</td>
<td>24</td>
<td>✓</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>31.1</td>
<td>α-Amyrin acetate</td>
<td>25</td>
<td>✓</td>
<td>S. cyanescens</td>
<td>26</td>
</tr>
</tbody>
</table>

<sup>a</sup>Identification confirmed by GC-MS analysis and retention index comparison.

<sup>b</sup>Oleanolic acid NMR were recorded in DMSO-d₆ and compared with the data recorded for a commercial standard.
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 (7.5 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 (1N). The organic layer was dried under MgSO4, 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 BF3-Et2O (200 μl). The reaction mixture was stirred overnight at room temperature. After evaporation, the crude material was solubilized in DCM and washed with HzO. The organic layer was dried under MgSO4, 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: hydrogen; 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: 20 ml/min; detector temperature: 300 °C; hydrogen flow: 20 ml/min.

### 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 was calculated with the previous formula.

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

and

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

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, $\alpha$ is the slope of the calibration curve and $\beta$ 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.

### Table 2. Calibration curve equations

<table>
<thead>
<tr>
<th>Low sclareol by-product compound</th>
<th>Slope of the curve, $\alpha$</th>
<th>Intercept of the curve, $\beta$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salvigenin (3)</td>
<td>0.596 ± 0.08</td>
<td>-1.98 ± 0.18</td>
<td>0.9992</td>
</tr>
<tr>
<td>Sclareol (6)</td>
<td>0.716 ± 0.04</td>
<td>-2.44 ± 0.10</td>
<td>0.9993</td>
</tr>
<tr>
<td>Oleanolic acid (8)</td>
<td>0.657 ± 0.04</td>
<td>-1.91 ± 0.12</td>
<td>0.9995</td>
</tr>
<tr>
<td>Triacylglycerol fraction</td>
<td>0.639 ± 0.06</td>
<td>-1.48 ± 0.16</td>
<td>0.9980</td>
</tr>
</tbody>
</table>

Results are given as the mean ± standard deviation. The standard deviation is based on the injection of three different aliquots for each concentration.
A chemical analysis method for perfume industry wastes

40 ml/min; air flow: 450 ml/min and make-up flow N\textsubscript{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\textsuperscript{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\textsuperscript{E}/Xevo \textsuperscript{TM} 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\textsuperscript{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 ng/min of a 400 ng/µl solution) at m/z 556.2771 and 120.0813 Da as the BEH C\textsubscript{18} column (2.5 × 100 mm, 1.7 µm) according to an isocratic solvent system solvent Asolvent B 70:30, where solvent A was ACN/MeOH/H\textsubscript{2}O 19:19:2 (v/v/v) + 0.1% acetic acid + 0.022% NH\textsubscript{3} and solvent B was iPrOH + 0.1% acetic acid + 0.022% NH\textsubscript{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\textsuperscript{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 ng/min of a 400 ng/µl solution) at m/z 556.2771 and 120.0813 Da as the

**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 ¹H and ¹³C 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), 2,3,12-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 cyanescens, 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 triterpenoids, 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) and salvigenin (3) remained at 60% in RSB, as described in the literature, and was 80% in LSB.[25] 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

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

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

¹Quantification method standard deviation based on the injection of three different aliquots of each sample: salvigenin, 11%; sclareol, 8%; oleanolic acid, 11%; triacylglycerol fraction 10%.

²CI, confidence interval; TGA, triacylglycerol.
that salvigenin (3), sclareol (6), oleanic 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 oleanic 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), 23,32-dihydroxy-24-nor-4(23),12-oleanadien-28-oic acid (4), taraxasterol acetate (11) and 2-amyris 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 oleanic 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

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>m/z</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>Content (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0 Palmitic</td>
<td>270</td>
<td>7.7</td>
<td>15.4</td>
<td>±1.2 (8%)</td>
</tr>
<tr>
<td>18:0 Stearic</td>
<td>298</td>
<td>11.4</td>
<td>4.9</td>
<td>±0.2 (4%)</td>
</tr>
<tr>
<td>20:0 Arachidic</td>
<td>326</td>
<td>14.7</td>
<td>6.4</td>
<td>±1.9 (30%)</td>
</tr>
<tr>
<td>Total saturated fatty acids content</td>
<td>—</td>
<td>—</td>
<td>26.7</td>
<td>—</td>
</tr>
<tr>
<td><strong>Unsaturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1, o9 Oleic</td>
<td>296</td>
<td>11.7</td>
<td>20.3</td>
<td>±1.1 (5%)</td>
</tr>
<tr>
<td>18:2, o6 Linoleic</td>
<td>294</td>
<td>12.5</td>
<td>18.1</td>
<td>±2.7 (15%)</td>
</tr>
<tr>
<td>18:3, o3 Linolenic</td>
<td>292</td>
<td>13.6</td>
<td>34.9</td>
<td>±5.9 (17%)</td>
</tr>
<tr>
<td>Total unsaturated fatty acids content</td>
<td>—</td>
<td>—</td>
<td>73.3</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup>The relative quantification method standard deviations were below 3% for each FAME.

<sup>b</sup>Relative content.

Values for m/z and R<sub>t</sub> are given for the relevant methyl ester.

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

<table>
<thead>
<tr>
<th>Total chain length</th>
<th>Total degree of unsaturation</th>
<th>m/z ([M + NH₄]&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>Δ (ppm)</th>
<th>TAG molecular species identified&lt;sup&gt;a&lt;/sup&gt;</th>
<th>s&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>n&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>9</td>
<td>890.72</td>
<td>0.4</td>
<td>18:3–18:3–18:3</td>
<td>4.25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>892.74</td>
<td>0.1</td>
<td>18:3–18:3–18:2</td>
<td>5.03</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>894.76</td>
<td>−0.7</td>
<td>18:3–18:2–18:2</td>
<td>6.02</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>896.77</td>
<td>0.1</td>
<td>18:2–18:2–18:2</td>
<td>7.23</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>898.78</td>
<td>−0.6</td>
<td>18:2–18:2–18:1</td>
<td>9.14</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>900.8</td>
<td>−1.2</td>
<td>18:2–18:1–18:1</td>
<td>11.63</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>902.82</td>
<td>−1.6</td>
<td>18:1–18:1–18:1</td>
<td>14.5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>904.83</td>
<td>−1.9</td>
<td>18:2–18:1–18:0</td>
<td>15.05</td>
<td>22</td>
</tr>
<tr>
<td>52</td>
<td>6</td>
<td>868.74</td>
<td>−0.6</td>
<td>18:3–18:3–16:0</td>
<td>6.35</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>870.75</td>
<td>0.1</td>
<td>18:3–18:2–16:0</td>
<td>7.68</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>872.77</td>
<td>−1.6</td>
<td>18:2–18:2–16:0</td>
<td>9.37</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>874.78</td>
<td>−1.7</td>
<td>18:2–18:1–16:0</td>
<td>9.74</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>876.8</td>
<td>−2.5</td>
<td>18:1–18:1–16:0</td>
<td>11.95</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>850.78</td>
<td>−4.0</td>
<td>18:1–16:0–16:0</td>
<td>15.15</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup>TAG fatty acid composition was determined by MS-MS analysis, as detailed in the supporting information.

<sup>b</sup>Numbering is according to the retention time.
Lipid Constituents of the Low Sclareol By-product

Total fatty acid composition of triacylglyceral 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 LSB was found to be the same. These results are in agreement with data reported in the literature.\(^{33}\)

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 (\(\omega 3\) and \(\omega 6\)). Moreover, the \(\omega 6/\omega 3\) 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\(^5\) 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-OH]\(^+\) and [MAG-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.

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.\(^{32,33}\)

The use of hydroxylated polymethoxyflavones as dietary supplements, food additives, pharmaceutical components, nutraceutical components and cosmetic components has been patented.\(^{34}\)

Bibliographical data on salvigenin (3) and 7,4’-dimethoxyapigenin (5) do not report such biological activities.\(^{35,36}\) 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.\(^{37}\)

The triterpene 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 antioxidant activities and constitute an active constituent of cosmetic and dermo-pharmaceutical compositions for skin prone to acne.\(^{43}\)

As lipidic raw material

Polysaturated 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 \(\omega 3\) PUFA.\(^{47,48}\) Some early studies on Salvia hispanica seeds recently complemented on Salvia sclarea seeds disclosed their potential as linseed oil substitute.\(^{49}\)

Indeed, the oil content of S. sclarea seed is 25–30% including 50% of \(\omega 3\) linolenic acid (giving an overall content of 12–15%). According to our study, LSB contains 60% of lipids including 35% of \(\omega 3\) 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 \(\omega 3\).

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.

Acknowledgements
This work was funded by the FUI (Fond Unique Interministériel). It is part of the Claryssime® programme (http://www.claryssime.fr) registered by the French ‘Pôle de compétitivité Parfums, Arômes, Senteurs, Saveurs’. We are grateful to Galderma for the dermo-pharmaceutical bioassay.

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

References
45. J. L. Piersinsard, Y. Millou, K. Fontes, C. Tourel, XXXXXXXX2008, XX, XXX.
In line with the objectives of sustainable development, a by-product of *Salvia sclarea* concrete, obtained from industrial scalpereol 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.
Dear Author,

During the copyediting of your paper, the following queries arose. Please respond to these by annotating your proofs with the necessary changes/additions.

- If you intend to annotate your proof electronically, please refer to the E-annotation guidelines.
- If you intend to annotate your proof by means of hard-copy mark-up, please refer to the proof mark-up symbols guidelines.

If manually writing corrections on your proof and returning it by fax, do not write too close to the edge of the paper. Please remember that illegible mark-ups may delay publication.

Whether you opt for hard-copy or electronic annotation of your proofs, we recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

<table>
<thead>
<tr>
<th>Query No.</th>
<th>Query</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>AUTHOR: Please check that I have correctly assigned all the affiliations.</td>
<td></td>
</tr>
<tr>
<td>Q2</td>
<td>AUTHOR: Please give the location (town/city and state/country) for each of Sigma, VWR, Panreac, Waters and Fischer.</td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>AUTHOR: Please give the location (town/city and state/country) of Agilent.</td>
<td></td>
</tr>
<tr>
<td>Q4</td>
<td>AUTHOR: Please give the location (town/city and state/country) of Bruker.</td>
<td></td>
</tr>
<tr>
<td>Q5</td>
<td>AUTHOR: Please define A_j.</td>
<td></td>
</tr>
<tr>
<td>Q6</td>
<td>AUTHOR: Reference “14” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q7</td>
<td>AUTHOR: Reference “18” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q8</td>
<td>AUTHOR: Reference “19” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q9</td>
<td>AUTHOR: Reference “20” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q10</td>
<td>AUTHOR: Reference “21” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q11</td>
<td>AUTHOR: Reference “22” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q12</td>
<td>AUTHOR: Reference “23” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Query No.</td>
<td>Query</td>
<td>Remark</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Q13</td>
<td>AUTHOR: Reference “24” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q14</td>
<td>AUTHOR: Reference “25” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q15</td>
<td>AUTHOR: There seems to be a letter missing in this name. Please check.</td>
<td></td>
</tr>
<tr>
<td>Q16</td>
<td>AUTHOR: Please give the title of the journal, the volume number, and the page span.</td>
<td></td>
</tr>
<tr>
<td>Q17</td>
<td>AUTHOR: Reference “47” has not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.</td>
<td></td>
</tr>
<tr>
<td>Q18</td>
<td>AUTHOR: I cannot locate this reference in the paper. Please insert it at the appropriate point(s).</td>
<td></td>
</tr>
</tbody>
</table>
Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)

The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

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.
   - Strikethrough (Del) Tool – for deleting text.
   - Add note to text Tool – for highlighting a section to be changed to bold or italic.
   - Add sticky note Tool – for making notes at specific points in the text.

   **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.
   - Click on the Strikethrough (Del) 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.

   **How to use it**
   - Highlight a word or sentence.
   - Click on the Strikethrough (Del) icon in the Annotations section.
   - Type instruction on what should be changed regarding the text into the yellow box that appears.
   - 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.
5. **Attach File Tool** – for inserting large amounts of text or replacement figures.

   **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.

   **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.

   **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: