Method development for a quantitative analysis performed without any standard using an evaporative light-scattering detector

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Abstract

A new method for quantitative analyses using an evaporative light-scattering detector (ELSD) is proposed. It is based on the preliminary determination of the calibration curve of an ELSD which correlates coefficient b and log a, the two coefficients from the equation: \( A = a m^b \), that characterize the law of the quantitative response for an ELSD. Dilutions of the mixture to be analyzed allow the determination of coefficient \( b \) for each component of the mixture. The knowledge of the \( b \) value and the experimental relationship correlating \( b \) with log \( a \) allows to determine the log \( a \) value and consequently to quantify each compound of the mixture. This method is an alternative to the quantitative method which uses an internal normalization without any response coefficient. This internal normalization method used with an ELSD provides inaccurate results and this inaccuracy increases when the analytes are in very different proportions. The relevance of the new method proposed in this work lies in the quantification of all the components present in a complex mixture when some of them are not available as standards.

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1. Introduction

The evaporative light-scattering detector (ELSD) is usually used in liquid chromatography [1–3]. Its ability to detect almost all eluted compounds in isocratic mode as well as gradient elution allows a large range of applications. The process is based on three successive steps: nebulization, evaporation of the eluent and measurement of the scattered light created by the particles. This complex mechanism leads to a non-linear empirical quantitative law described by the relation:

\[ A = a m^b \]  

where \( A \) is the area of the chromatographic peak, \( m \) the mass of the compound, \( a \) the response factor and \( b \) is the response index measured from the slope of the curve \( \log A = f(\log m) \) [i.e. \( \log A = b \log m + \log a \)]. The coefficients \( a \) and \( b \) depend on many parameters, such as the average size, the shape and the distribution of the particles, the nature, the volatility and the concentration of the analyte, the nature of the mobile phase and nebulizing gas, the liquid and gas flowrates, etc. The dependence of \( b \) and \( a \) values with these numerous parameters involves the need for a calibration curve which is specific for each compound and each condition of nebulization. This involves first the impossibility of obtaining precise quantitative results when the analyte is not available as a standard, and second it explains the recent studies related to the literature which propose quantitative methods using an ELSD to get round this problem [4–8].

For analytes with similar or different structures, we showed in a recent article that even if the parameters \( a \) and \( b \) change with the experimental conditions, there is a linear relationship between \( b \) and log \( a \) for a given ELSD, whichever analyses are performed using an aqueous or a non-aqueous eluent [9].

In this article, it is shown that establishing the correlation between \( b \) and log \( a \) is the preliminary condition for a quantitative analysis. It allows a quantitative analysis of a complex mixture, without having the corresponding standards.
2. Experimental

2.1. Chemicals

Triacylglycerols (see Table 1) were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France) or Nu Chek Prep (Cluzeau, Sainte-Foy-la-Grande, France).

Acetonitrile (Acros, Noisy le Grand, France) and methylene chloride (Carlo Erba, Milan, Italy) were of HPLC grade.

The seeds of Citrullus lanatus oil were bought from a market of Brazzaville, Congo. After crushing the seeds, the lipids were extracted with hexane (Carlo Erba) using a Soxhlet extraction system.

2.2. Equipment

The chromatographic system consisted of a Model LC-6A pump (Shimadzu, Kyoto, Japan), a Model 7125 injection valve with a 10 µL loop (Rheodyne, Rohnert Park, CA, USA) and a Model Sedex 75 light-scattering detector (Sedere, Alfortville, France). The nebulizing gas was air set at 3 bar, the evaporation temperature was set at 40 °C and the gain (PM) at 11. The column temperature was controlled using an Igloo-cil column oven (Cluzeau, Sainte-Foy-la-Grande, France) and set at 30 °C. A Kromasil C18 (5 µm), 250 mm × 4.6 mm column was used (Touzart et Matignon, Les Ulis, France). Chromatograms were recorded using an Azur (v 3.0) data acquisition software (Datalys, Saint-Martin d’Hères, France).

The mobile phase gradient was MeCN/CH2Cl2 (70/30 to 40/60 in 35 min) and the flowrate was 1 mL/min.

2.3. Methods

The calibration curves were established using five concentration levels of triacylglycerols (from 5 to 50 ppm). Value of each area was the average of three reproducible injections. For each compound, the concentration range was measured at least in duplicate.

These curves allow the determination of the parameters a and b for each triacylglycerol, to plot b versus log a and to obtain the experimental calibration curve of the detector under these conditions:

\[ b = a \log a + \beta \]  \hspace{1cm} (2)

2.3.1. Solutions for validation and oil from C. lanatus

Two types of solutions for validation were prepared. The unsaturated triacylglycerol mixture consisted of the following: LnLnLn, LLL, OOO, PLL and OPO at concentrations of 30, 30, 35 and 43 ppm for the highest level, respectively. The saturated triacylglycerol mixture consisted of: CyCyCy, CCC, LaLaLa, MMM, PPP and SSS each at 30 ppm for the highest level.

Two dilutions were carried out. For each compound, the coefficient b was obtained from the following plot: log(A) versus log(x), with x being the dilution factor.

For each compound, when b is determined, parameter a can be calculated from Eq. (2) using the plot: b versus log a.

When coefficients b and a are known, the mass (m) can be consequently determined using Eq. (1). Therefore, the mass percentage (mass% calculated) of each analyte can be obtained.

In the case of the solution for validation, a relative error was calculated:

\[ \text{Error} (\%) = 100 \times \frac{\text{mass% calculated} - \text{mass% real}}{\text{mass% real}} \]  \hspace{1cm} (3)

2.3.2. Comparison of the results obtained by the method of internal normalization and the method developed in this work

The mass percentage of the components in a complex mixture is equal to the percentage of the areas of the chromatographic peaks only if simultaneously the coefficient a in Eqs. (1) and (4), is the same for all the analytes and if the coefficient b is equal to 1 for each analyte, which is very rare for mixtures of natural compounds.

\[ \text{area} (\%) = A (\%) = \frac{A_i}{\sum A_i} = \frac{a_im_i}{\sum a_im_i} \]  \hspace{1cm} (4)

In order to evaluate the error when the area percentage is considered to give the real mass percentage, we therefore calculated the percentage difference between the results obtained by the internal normalization without coefficient of response (area%) and the method developed in this work (mass% calculated) for the

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**Table 1**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Formula</th>
<th>Molecular mass (g/mol)</th>
<th>Purity</th>
<th>Provenance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyCyCy</td>
<td>Glyceryl trioctanoate</td>
<td>C27H50O6</td>
<td>470.70</td>
<td>&gt;99%</td>
<td>Nu Chek Prep</td>
</tr>
<tr>
<td>CCC</td>
<td>Glyceryl tridecanoate</td>
<td>C31H52O6</td>
<td>554.86</td>
<td>&gt;99%</td>
<td>Sigma Prep</td>
</tr>
<tr>
<td>LaLaLa</td>
<td>Glyceryl tridecanoate</td>
<td>C35H54O6</td>
<td>639.02</td>
<td>≥99% (GC)</td>
<td>Sigma</td>
</tr>
<tr>
<td>MMM</td>
<td>Glyceryl trimyristate</td>
<td>C41H66O6</td>
<td>723.18</td>
<td>≥99% (GC)</td>
<td>Sigma</td>
</tr>
<tr>
<td>PPP</td>
<td>Glyceryl tripalmitate</td>
<td>C41H66O6</td>
<td>807.32</td>
<td>≥99% (GC)</td>
<td>Sigma</td>
</tr>
<tr>
<td>SSS</td>
<td>Glyceryl tristearate</td>
<td>C45H70O6</td>
<td>891.51</td>
<td>≥99% (GC)</td>
<td>Sigma</td>
</tr>
<tr>
<td>OOO</td>
<td>Glyceryl triooleate</td>
<td>C43H68O6</td>
<td>885.46</td>
<td>&gt;99%</td>
<td>Nu Chek Prep</td>
</tr>
<tr>
<td>LLL</td>
<td>Glyceryl trilinoleate</td>
<td>C45H70O6</td>
<td>873.34</td>
<td>&gt;99%</td>
<td>Nu Chek Prep</td>
</tr>
<tr>
<td>LnLnLn</td>
<td>Glyceryl trilinolenate</td>
<td>C47H72O6</td>
<td>854.7</td>
<td>&gt;99%</td>
<td>Nu Chek Prep</td>
</tr>
<tr>
<td>PLL</td>
<td>1,3 Dioleoyl-2,3 linoleyl-rac glycerol</td>
<td>C49H80O6</td>
<td>854.7</td>
<td>≥99% (GC)</td>
<td>Sigma</td>
</tr>
<tr>
<td>OPO</td>
<td>1,3 Dioleoyl-2,3 palmitoyl-rac glycerol</td>
<td>C51H80O6</td>
<td>859.4</td>
<td>≥99% (GC)</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
solutions of validation and for the analyzed vegetable oil. A significant difference (Eq. (5)) will justify the use of the proposed method for quantitative analysis, even if the mass percentage calculated by our method is slightly different from the real mass percentage, which will be confirmed by the results obtained from the solutions of validation.

\[
\text{Variation (\%)} = 100 \left( \frac{\text{mass\% calculated} - \text{area\%}}{\text{mass\% calculated}} \right)
\] (5)

3. Results and discussion

Triacylglycerols (TAG) are the principal components of vegetable oils. In order to emphasize their nutritional interest, it is important to be able to identify and directly measure each TAG from a mixture. Practically no standard is available for these heterogeneous TAG. In addition, ELSD suits the detection of these compounds which have a very low volatility, particularly well. This is why these analytes have been used in this work.

As mentioned in the introduction, a linear relationship was found between \( b \) and \( \log a \) for several compounds belonging to different chemical classes (TAG, ethoxylated alcohols, wax monoesters) [9].

In the particular case of TAG, the plot: \( b = f(\log a) \) is given in Fig. 1.

Thus, if the original Eq. (1) is combined with Eq. (2), which is deduced from the plot given in Fig. 1, and corresponds to the calibration curve of the ELSD, the new Eq. (6) becomes a function of only one unknown factor \( b \):

\[
A = 10^{(b-\beta)/a} m^b.
\] (6)

Determining \( b \) for each compound of the mixture allows therefore the quantitative analysis.

Eq. (1) can be transformed into logarithmic coordinate curves to obtain a linear relationship. By definition, the slope of the curve \( \log A = \log a + b \log m \) is given by the following relation:

\[
b = \frac{\log A_1 - \log A_2}{\log m_1 - \log m_2}
\] (7)

If \( m_2 \) corresponds to a dilution of \( m_1 \): \( m_2 = m_1/x \), where \( x \) is the dilution coefficient.

Thus, the slope is calculated as follows:

\[
b = \frac{\log A_1 - \log A_2}{\log m_1 - \log(m_1/x)} = \frac{\log A_1 - \log A_2}{\log x}
\] (8)

Therefore, \( b \) is easily obtained experimentally from at least three levels of concentration and without knowing the initial value \( m_1 \).

Once the value of \( b \) is obtained, the value of \( \log a \) can be determined using Eq. (2) and then the value of \( m \) using Eq. (1).

By this way, there is no approximation of coefficient \( b \) since it is systematically given for all the compounds of the mixture and whatever is their initial concentration. Even if the compounds of the mixture have very different \( b \) values, these are obtained with precision. The error which can be made comes from the dispersion of the experimental points giving the plot: \( b = f(\log a) \) obtained for the ELSD. \( \alpha \) and \( \beta \) values from Eq. (2) will vary slightly according to the number of the analytes tested to establish the response curve of the detector, as well as the chemical nature of the compounds. Consequently, the precision with which the mass of the analyte will be obtained will depend on the precision of the calculated value of \( \log a \) deduced from the \( b \) value determined by dilutions.

3.1. Validation of the method

Thus, it is necessary to check that the calibration curve used to determine \( \log a \) provides calculated mass percentages equal or close to the real mass percentages of a mixture of a known composition.

In order to validate this step, we used TAG mixtures of known composition and analyzed them just like mixtures of unknown composition. The mixtures were composed of the following TAG: LnLnLn, LLL, OOO, PLL and OPO for unsaturated TAG and CyCyCy, CCC, LaLaLa, MMM, PPP and SSS for saturated TAG.

Using the plot: \( b = f(\log a) \) from Fig. 1, three possibilities appear:

- to use the equation by taking all the saturated and unsaturated TAG into account \( b = -0.37 \log a + 1.85 \);
- to use the equation established with only unsaturated TAG \( b = -0.37 \log a + 1.81 \);
- to use the equation established with only saturated TAG \( b = -0.63 \log a + 2.3 \).

Each one of these three equations was tested to see whether only one suits to determine the real mass percentages of the TAG in the solutions of validation, or if all these equations are suitable. Thus, the step described above was used to determine the mass percentages of the TAG from two dilutions of the solutions of validation (three levels of concentration). This mass percentage obtained from the experiment (mass\% calculated) was compared with the real mass percentage (mass\% real), and a relative error was calculated in each case. The results are given in Table 2.

In the last row of Table 2 the error made by using the internal normalization without any response coefficient is listed for
Table 2
Relative errors (%) between the mass percentages obtained from the three equations in the new method (mass% calculated) or the area percentages (area%) obtained with the internal normalization and the real known mass percentage of the analytes in the solution of validation (mass% real)

<table>
<thead>
<tr>
<th>Unsaturated TAG</th>
<th>Saturated TAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LnLnLn</td>
<td>LLL</td>
</tr>
<tr>
<td>From equation with all TAG</td>
<td>+1.4</td>
</tr>
<tr>
<td>From equation with unsaturated TAG</td>
<td>+0.7</td>
</tr>
<tr>
<td>From equation with saturated TAG</td>
<td>−10.6</td>
</tr>
<tr>
<td>Obtained from area%</td>
<td>−28</td>
</tr>
</tbody>
</table>

Table 3
Deviation (%) between the area percentages obtained with the internal normalization (area%) and the mass percentages obtained with the new method (mass% calculated)

<table>
<thead>
<tr>
<th>Unsaturated TAG</th>
<th>Saturated TAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LnLnLn</td>
<td>LLL</td>
</tr>
<tr>
<td>From equation with all TAG</td>
<td>+29</td>
</tr>
<tr>
<td>From equation with unsaturated TAG</td>
<td>+29</td>
</tr>
<tr>
<td>From equation with saturated TAG</td>
<td>+19</td>
</tr>
</tbody>
</table>

each TAG, i.e. using only the area percentage of each chromatographic peak (Eq. (4)). Assuming that these area percentages are equal to the mass percentage of each TAG, their respective relative deviation was calculated and compared to the real known mass percentage.

As expected with ELSD, the errors obtained with the method based on the measurement of the areas can be relatively high because all the compounds do not answer in the same way and there is no direct proportionality between the area and the corresponding mass. However, during the analysis of complex mixtures, when the standards are not available, it represents a means of getting an estimation of the mixture composition.

With regard to the results obtained with the method developed here, the errors are definitely smaller but, as expected, depend on the equation: \( b = f(\log a) \). Indeed, for the saturated TAG, the error is relatively small when it was calculated using the plot obtained from the saturated TAG (from 0.6 to 9.6%). On the other hand, for the unsaturated TAG, homogeneous or heterogeneous, the smallest errors are obtained using the equation taking all the saturated and unsaturated TAG into account (from 1.4 to 9.7%) or only the unsaturated TAG (from 0.7 to 10.4%).

Of course, the errors obtained even in the most favorable case may seem rather high to provide a quantitative result. However, it should be stressed that these analyses of mixtures are performed without any available standard. In this case, the only way of getting a quantitative result so far was to use the area percentages. The deviations (expressed as percentages) between the area percentages obtained by the internal normalization (area%) and the mass percentages obtained by the method developed here (mass% calculated) are reported in Table 3. For the unsaturated TAG, when the equation taking all the TAG or only the unsaturated TAG into account, i.e. when the relative error was the smallest, the calculated deviation is relatively high, which shows that it is better to give the results obtained from the proposed method and not from the area percentages. In the case of the saturated TAG, the variations obtained when using the equation established from the saturated TAG are definitely smaller, which means that giving a result using the area percentages could lead to acceptable results for the analytes that have a very low volatility.

3.2. Application of the validated method to real samples

The abovementioned procedure was used for the quantitative analysis of oil seeds from cucurbitaceous plants grown in sub-Saharan Africa, in order to study the nutritional interest of their constitutive lipids.

![Fig. 2. Chromatogram of triacylglycerols in Citrullus lanatus oil (see Section 2 for the chromatographic conditions).]
The oil analyzed in this work is from the variety *C. lanatus*. The LC-ELSD chromatogram is shown in Fig. 2 and the result of the quantitative analysis using the method described above is given in Table 4. This oil is mainly composed of unsaturated heterogeneous TAG which are not available as standards, and shows the applicability of the method developed here. This quantitative method can be applied for all vegetable oils, using non-aqueous reversed-phase chromatography, because they hardly contain any saturated TAG. Using area percentages can produce large deviations in the principal TAG concentrations (2.8–28.2%), and can become very large at trace level (up to 300%) which is pleading for the new method described here.

The method developed in this work provides a more precise knowledge of the respective quantities of each compound. In addition, ELSD is known for its lack of reproducibility from one day to another, which results in a variation of the parameters $a$ and $b$ with the experimental conditions, such as the composition of the mobile phase, the temperature of the evaporation tube or with a partially clogged nebulizer [9]. The method developed here allows to outrule the effect of these phenomena on the quantitative results, since for each value of $b$, even fluctuating from one analysis to another for any reason mentioned above, a value of log $a$ is obtained from the calibration curve of the detector. It allows in every case to determine the mass percentages without having to create new calibration curves. The method that was proposed here leads to the development of a robust method for the quantitative analysis using ELSD.

### 4. Conclusion

A method for the quantitative analysis using an ELSD is proposed, which allows the determination of compounds in a mixture without the corresponding standards. This method requires a calibration of the ELSD, i.e. the establishment of the plot: $b = f(\text{log } a)$. This calibration should be done with compounds having structures as close as possible to those of the compounds to be analyzed. The method was validated with calibrated solutions of TAG standards and led to the quantitative analysis of oil seeds from cucurbitaceous plants grown in sub-Saharan Africa, in order to study the nutritional interest of their constitutive lipids.

### References