Hyphenation of ultra-high performance supercritical fluid chromatography with atmospheric pressure chemical ionisation high resolution mass spectrometry: Part 2. Study of chromatographic and mass spectrometry parameters for the analysis of natural non-polar compounds

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Hyphenation of ultra-high performance supercritical fluid chromatography with atmospheric pressure chemical ionisation high resolution mass spectrometry: Part 2. Study of chromatographic and mass spectrometry parameters for the analysis of natural non-polar compounds.

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Abstract

An analytical method based on Ultra-High-Performance Sub/Supercritical Fluid Chromatography (UHPSFC) coupled with High-Resolution Mass Spectrometry (HR-MS) equipped with an Atmospheric Pressure Chemical Ionisation source (APCI-Q-TOF-HRMS) was developed for the screening of various compounds in oily samples. The hyphenation was achieved using a hybrid UHPSFC system for the vegetable oil analysis, mainly composed of fatty acids, diacylglycerols, triacylglycerols but containing also some minor bioactive compounds. No split was used with this ionisation source in this optimized configuration, allowing the introduction of all compounds in the mass spectrometer, ensuring a better sensitivity. This configuration is preserving peak shapes and efficiency because the dead volume of this interface is optimized by the supplier for the hyphenation with open cell detectors such as Evaporative Light Scattering Detector (ELSD) or MS detectors. The influence of analytical conditions (UHPSFC and APCI parameters) on MS response was studied to understand the behaviour of analytes in UHPSFC-APCI-MS. The tested parameters were: the corona discharge value, the nebulising gas pressure, the drying gas flow rate, MS source temperature and the mobile phase flow rate (at constant modifier percentage). A factorial experimental design was carried out for this study, which displayed the major and negative role of increased nebulising gas pressure. The factorial design was renewed with the four remaining parameters, allowing to enlighten and explain their different effects on MS responses. Finally, some vegetable oils were analysed by UHPSFC-APCI-HRMS with these optimal conditions to determine the chemical structures of unknown compounds from oily samples.

Key words: Detector response, High Resolution, Hyphenation, Mass spectrometry, Supercritical Fluid Chromatography, Vegetable oil.

Highlights:

- UHPSFC-APCI-HRMS used for the complete study of vegetable oils
- No split between UHPSFC and APCI-HRMS for a better sensitivity
- Understanding of MS parameters on MS responses of lipid compounds
- Application to Sunflower and *Calophyllum inophyllum* (Tamanu) oils

1. Introduction

For several years, Supercritical Fluid Chromatography (SFC) has rapidly grown in analytical chemistry with the introduction of new and more robust SFC (and HPLC/SFC hybrid) systems on the analytical market. Amongst application areas, the lipidomic field draws attention [1–3]. In lipidomics, analytical strategies generally are supported by Gas Chromatography (GC), Normal phase liquid chromatography (NPLC), Non-Aqueous Reversed-Phase Liquid Chromatography (NARP-LC) or Thin-Layer Chromatography (TLC). However, these analytical techniques have encountered several difficulties such as experimental, economic or environmental problems. For instance, the disadvantages of GC are the use of high temperatures for elution, which could favour degradation of thermo-sensitive analytes. Also, in GC, derivatization procedures are time-consuming steps which require methodological developments. TLC (or High Performance TLC) is accurate, fast and versatile but it also requires derivatization agents for lipid detection [2]. Besides, the hyphenation of TLC with MS is not so obvious. Indeed, for this kind of coupling, it is necessary to desorb compounds from the stationary phase. Consequently, several bleeding phenomena may occur that could impact the MS sensitivity. In NARP-LC, only organic and (often) toxic solvents (Acetonitrile (ACN), Methanol (MeOH), Isopropyl alcohol (iPrOH), methylene chloride, or hexane) are used to elute lipids from non-polar stationary phases [4].

In SFC, the mobile phase is mainly composed of CO_2 in super/subcritical conditions (depending on the pressure and temperature values, and it has a lower viscosity and a higher diffusivity than liquid mobile phases used in Reversed-Phase-HPLC (RP-LC). Consequently, it is possible to use high flow rates (at least 3 mL/min), while maintaining a high chromatographic efficiency. This green and powerful high-throughput technique has demonstrated a great interest for the screening of complex oily mixtures [1,5,6]. Packed

column supercritical fluid chromatography (pcSFC) often improves separations relative to HPLC. It has been successfully applied for the analysis of triacylglycerols (TAG) [7], tocopherols / tocotrienols [8], vitamin D [9], phospholipids, sterols, fatty acids or prenols [5,10] and appears as a good alternative for lipidomics.

In SFC, numerous detectors can be used. Closed-cell detectors such as UV detectors can be used but they need to have a pressure-resistant cell. Indeed, the Back-Pressure Regulator (BPR) module that maintains the supercritical state of the mobile phase in the chromatographic system is located after these detectors and this one is set at higher values than the critical value (typically above 12 MPa). Open-cell detectors such as MS, ELSD or Corona Aerosol Detector (CAD) can be directly connected after the BPR without additional interface (when working with short tube length and with modifier mixed or added to CO_2). Indeed, the fluid depressurization provides a natural spray at the detector entrance, which is favourable to these detectors [11].

Nowadays, in SFC-MS, the most prevalent MS sources are Atmospheric Pressure Ionisation (API) sources with Electrospray (ESI) and Atmospheric Pressure Chemical Ionization (APCI). During the SFC-MS hyphenation, some difficulties might occur: analyte precipitation in the capillary connector when using low modifier content, carbon ice formation (due to the CO_2 decompression cooling – endothermic reaction- after the BPR), or the lack of adequate solvent to assist the analyte ionization (CO_2 would be inert in ion formation process). However, this technique is gradually introduced in laboratories and is now most commonly used [12]. A make-up solvent is often added (after or before the BPR) to solve some of these problems. This make-up solvent ensures the good solubilisation of compounds after the BPR (for a proper transfer from SFC to MS) and maintains (in most cases) the chromatographic efficiency. Also, it helps to the ion formation (*i.e.* pseudomolecular ion). Moreover, some SFC systems also have a heated BPR. This particularity is very useful (for a direct connection with SFC and MS) to avoid the ice formation or to prevent the compound precipitation as was well explained elsewhere [13,14].

In the literature, ESI is the most popular ionization source for SFC-MS due to numerous LC-ESI-MS pharmaceutical applications [14]. The use of a splitter between the column and the ESI source was extensively discussed, especially regarding the interface configuration and the modelling of the splitter behaviour when used with compressible fluids [14]. Today, this trend still continues in the lipidomic field, for polar lipids (phospholipids, glycosylated lipids) [15–19] and pesticides [20]. However, for nonpolar lipids the ESI source may not always be the best choice. In LC, for lipidomic studies, APCI or Atmospheric

Pressure Photo-Ionisation (APPI) are complementary to ESI by their specific ionisation mechanisms [8-9]. Some papers related to SFC-APCI-MS [8-9,19] have been published but few of them have demonstrated the influence of SFC and MS parameters on MS responses. Thus, an important gap of fundamental knowledge is highlighted for lipid analysis in SFC-APCI-MS.

In the first part of this series of papers [21], we described the effect of the addition of a make-up solvent in terms of location (in relation to the BPR), flow-rate and chemical nature, by using direct Ultra-High-Performance SFC (UHPSFC) coupled to MS with no-split. The direct coupling is expected to favour the MS response with APCI source [22]. Whatever the type of coupling interface, the make-up solvent allowed the introduction of a protic solvent supposedly favouring the ionisation process. However, as was shown, with the use of a mobile phase of CO_2/MeOH (90/10, v/v), we obtained satisfactory responses for lipids from plant seeds without the need for a make-up solvent in a UHPSFC-APCI-MS configuration.

The aim of this second part is to have a better understanding of the influence of UHPSFC and MS parameters on MS signals in directly hyphenated UHPSFC-APCI-MS when no additional make-up solvent is used. An oily matrix, extracted by Supercritical Fluid Extraction (SFE) from *Kniphofia uvaria* seeds, which includes fatty acids (FA), diacylglycerols (DAG), hydroxylated TAG (TAG+H_2O), TAG and bioactive (and very minor) anthraquinone (AQ) compounds, was used to study the influence of these parameters. The selected parameters were then applied for a deeper study of sunflower and Calophyllum oils.

2. Materials and methods

2.1 Chemicals

Methanol (MeOH) was purchased from Sigma (St. Quentin Fallavier, France) and it was a reagent grade for LC-MS. For extraction, CO_2 was supplied by Linde (Munich, Germany) with a purity of 99.95%. For UHPSFC-MS analysis, CO_2 was purchased from Messer (Bad Soden, Germany) with a purity of 99.995 %.

2.2 Sample preparation of the oily extract by Supercritical Fluid Extraction (SFE)

Kniphofia uvaria and Sunflower seeds were collected and prepared at LVMH-Recherche. Seeds of *Kniphofia uvaria* (300 g) were grinded with a Braun blender of 1 L. They were extracted by Supercritical Fluid Extraction (SFE) with a Separex SFE-500

apparatus with pure CO_2 for *Kniphofia uvaria* and CO_2/EtOH 97.5/2.5 (v/v) for Sunflower, both at 29 MPa and 60°C. The extraction time depended on the mass of CO_2 eluted through the extraction cell. When the CO_2 mass reached 3 kg, the extraction was stopped. The oil was dried in vacuum with some milliliters of ethanol in order to facilitate the drying. The oil extract was stored at 4°C and under nitrogen to prevent oxidation of compounds. For analysis, the extract was weighed and diluted in MeOH / methylene chloride (1/1, v/v) at 0.1 mg/mL for UHPSFC-HRMS analysis.

The *Calophyllum inophyllum* (Tamanu) oil was provided by the French Polynesian University. This oil was stored and prepared for analysis with the same specific protocol as described above.

2.3 UHPSFC-HRMS system and analytical conditions

Analytical chromatographic separations were carried out using an equipment purchased from Jasco (Tokyo, Japan) and Thermo-Dionex (Villebon-sur-Yvette, France). The UHPSFC-HRMS system was composed of a CO_2-2080 pump and a back-pressure regulator (BPR) BP-2080 from Jasco and an ultimate 3000 RSLC system (binary pump, autosampler and thermostated column compartment) from Thermo-Dionex [21]. To configure this system, the autosampler controlled the start to run analysis and it was connected by a contact closure to the CO_2 pump. The BPR and CO_2 pump were set to comply with the analysis time with a time program.

While previous developments had been conducted on a 75 cm-column (five 15 cm-columns connected in series) to achieve very high efficiency, the present analysis was performed with only one Kinetex C18 column (150 x 4.6 mm, core shell particle 2.6 µm) (Phenomenex, Le Pecq, France) in order to shorten the analysis time (<5 min). The mobile phase was composed of CO_2:MeOH (90/10; v/v) with isocratic elution mode. The column was placed in the LC thermostated column compartment at 9°C [21]. The outlet pressure was equal to 10 MPa and the BPR was thermostated at 70°C. The volume of the BPR was 10 µL. The BPR outlet was directly connected to the MS detector by a simple peek capillary (32 cm x 125 µm). The transfer volume from BPR to MS was 4 µL. MS detection was carried out using a maXis UHR-Q-TOF mass spectrometer (Bruker, Bremen, Germany) in the negative and positive chemical ionisation modes with an APCI source from Bruker. Mass spectra were recorded for *m/z* ranging from 50 to 1400 and the acquisition frequency was 1 Hz in Full Scan mode.

At the end of the analysis, the CO_2 pump was turned off and the system depressurized to reach atmospheric pressure. The binary pump continued to deliver 100% MeOH to ensure system equilibration. It was confirmed that no carry-over occurred.

2.4 Evaluation and optimisation of the SFC and MS conditions by an experimental design

The aim of this experimental design was to enhance our knowledge of the SFC-APCI-MS hyphenation. This study identified the most influential parameters on the MS responses for FA, DAG, TAG and two main AQ (rhein and aloe-emodin) included in the oily extract of *Knifophia Uvaria* and selected the best parameter values (from the tested ones) for a lipid screening. As explained in the introduction and in this publication [21], no make-up solvent was necessary to obtain good results. The five variables were: the corona discharge current, the ionisation source temperature, the nebulising gas pressure, the drying gas and the SFC flow rate. Following the recommendations in the user's guide of maXis UHR-Q-TOF mass spectrometer, the following parameters were used: corona discharge (+/- 2µA and +/- 6µA), nebulising gas pressure (N_2) (0.04 and 0.2 MPa), drying gas flow (N_2) (3 and 8 L/min) and drying gas temperature (APCI temperature) included (250 and 450°C). The UHPSFC flow rate was adjusted at 1 and 3 mL/min.

Table 1 illustrates the values and their coding for each variable of this experimental design. This factorial design was defined by 35 experiments (Table 2)(2^k with k = 5; 32 experimental points and a central point (n°33 with source temperature at 350°C, drying gas flow rate at 5.5 L/min, nebulising gas pressure at 0.12 MPa, SFC flow rate at 2 ml/min and Corona discharge at 4 µA) (done in triplicate) only used for studying the relative standard deviations for peak area (RSD). These RSD were respectively from 3.4 to 7.8% (measured for lipids) and from 3.5 to 4.8% (measured for AQ). These low SD values show the relevance of the experimental design.

The responses measured were peak areas of: TAG (LLL and OLL), DAG (LL and OL), FA (linolenic acid) and AQ (anthraquinones with rhein and aloe emodin). The statistical analysis was carried out with Open Office. The values reported in table 3,4 and 5 for the five studied factors are the average values from the 32 experiments, each of the 32 values being calculated as the product of the encoding value -1 or +1 (from table 2) and the experimental peak area.

This calculation allows to get negative or positive average values, explaining the respective effect of the lower (negative) or higher (positive) experimental parameters.

2.5 Study of vegetable oil

The analyses of sunflower and Tamanu oil were carried out with optimized SFC-MS conditions by using a total column length of 75 cm to improve the separation quality [7].

3. Results and discussions

3.1 Understanding the effects of the SFC and MS parameters on MS responses

The negative and positive APCI ionisation modes were used. The effects of different parameters were individually studied for TAG, DAG, FA and AQ families under SFC conditions (CO_2/MeOH, 90/10, v/v). However, in the negative mode, only the AQ family was studied. The carboxylic acid group (for rhein) or alcohol groups (for aloe-emodin) induce a high ionic current by the proton abstraction. Consequently, we decided to use the APCI (+) for an exhaustive analysis and APCI (-) to reveal minor compounds (AQ) not well detected in APCI (+).

3.1.1 Study in SFC-APCI-MS: APCI (+)

3.1.1.1 First attempt to streamline the SFC-APCI-MS hyphenation

In Fig.1, the peak areas of LLL, OLL, LL, OL (TAG and DAG), linolenic acid and LLL+H_2O (hydroxylated TAG) are plotted for each experiment from the experimental design. Identification of peaks from mass spectra was described in previous papers [21, 23]. Rhein and aloe emodin are not presented in Fig.1 because their peak areas were much lower than other compounds and their MS signals were unobservable with this graphic scale.

On Fig. 1, a half of the experiments had no MS responses (or very low MS responses). Thus, one of the experimental parameters has a binary effect on the MS response of lipids, which acts as a switching signal. The variation in the MS response due to the change in the studied parameters was calculated to identify this “binary parameter” (Table 3).

As observed in Table 3, the nebulising gas is the key descriptor. This parameter had the highest values (negative) for most of the studied compounds. Thus, the nebulising gas rules this binary trend.

For a value fixed at 0.2 MPa (high level), the MS signals dramatically dropped whereas at 0.04 MPa (low level), the MS signals significantly increased. This strong variation in MS responses evidences that the depressurised CO_2 acts as a nebulising gas and is sufficient to ensure the effluent transport by the gaseous phase in the restrictor from the column to the ionization chamber of the mass spectrometer. This effect had already been described in the literature [24]. The high volatility of CO_2 may have assisted the nebulization process for SFC-APCI. Here, the addition of gaseous CO_2 and nebulizing gas (N_2) probably results in too high a rate of compounds in the high wattage chamber at the exit of the spraying needle to allow for good ionization. This high rate of the spray could reduce the compound ionisation by the corona discharge needle in the spray chamber, due to a lower ionisation of the solvent vapor (by removal of an electron). On the other hand, it is more likely that a low transfer of the heaviest compounds into the mass analyser is the reason of this MS response decrease. Indeed, a spray shield (-3.6 kV) is applied for the ion extraction and it could be optimized in order to increase the number of compounds introduced into the MS analyser. Moreover, with this APCI source, the inlet capillary is directed towards the spray needle with an angle of around 120 degrees. This angle obviously favours the ion extraction from the non-ionised compounds but could limit the transfer for heavy compounds (lipids) when a high total gas flow rate (depressurised CO_2 + nebulising gas) is applied. Thus, for lipid analysis, and with the mass spectrometer configuration used in this study, it is necessary to set the nebulising gas pressure at a very low value to improve the MS signals (whatever the SFC flow rate from 1 to 3 mL/min).

Consequently, in order to improve understanding of the effect of the four remaining parameters, the nebulising gas pressure was set at the lowest value (0.04 MPa) to favour the mass responses. Then, the 16 remaining experiment data were used to perform a new statistical analysis from a reduced set of data (experiments n°1, 2, 3, 4, 9, 10, 11, 12, 17, 18, 19, 20, 25, 26, 27 and 28 in Table 2).

3.1.1.2 Statistical study relevance for the interpretation

Fig. 2 shows the new variations of MS responses related to the four selected factors (ionisation source temperature; drying gas flow rate; SFC flow rate and corona discharge) at a constant low value of the nebulising gas pressure (0.04 MPa). From the reduced data set (16)

of the experimental design, a new calculation of the mass response variation was achieved to determine the influence of parameters.

Table 4 shows the effect of the four individual factors on the response of selected compounds. However, because there was no central point for this new matrix of data, the validity of the statistical approach is evaluated with a normal probability plot (Henry's graph) (Fig. 3). Henry's line is a tool to check a theoretical distribution. This graph is obtained by plotting the experimental values (product of peak area and encoding value) *vs*. the centred normalised values (see in legend of figure 3). If the values are perfectly aligned, as is the case here (Fig. 3), the distribution follows a normal distribution. Consequently, this linear distribution allows validating this factorial plan, making it possible to interpret the results with confidence.

Table 4 shows that factor 4 (the SFC flow rate) significantly acts on the MS responses for any compound. According to these values, this flow rate is now the first key parameter to optimize for lipids (FA, DAG, TAG). Indeed, it greatly modifies the MS peak area just after the nebulising gas pressure (factor 3 in Table 4). For AQ, this parameter also seems important to optimize but it may be ranked at the third place. The negative values obtained for this parameter indicate that the lowest SFC flow rate value (1 mL/min) will enhance the MS responses. Similar results have already been reported by Hsieh and co-workers [19] for analysis of pharmaceuticals in SFC-APCI-MS by using a total introduction of the mobile phase in MS. They reported that the increase of SFC flow rate from 3 to 6 mL/min had a negative effect on MS responses, which could be due to a less effective heat transfer at higher flow rates.

Because ELSD is also an open cell detector, several results obtained in SFC-ELSD could be compared to the SFC-APCI-MS coupling. The first step (the nebulisation process), consisting in mixing the chromatographic eluent with nitrogen (or another gas), is similar in ELSD and APCI-MS detectors. Then, the evaporation of solvent droplets is performed in the drift tube (ELSD) or in the chamber (APCI) at a controlled temperature. One study [25] dealing with the parameter effect on the chromatographic responses in SFC-ELSD also showed that the increase of the SFC flow rate reduced the peak area of caffeine.

The ionisation source temperature is described by factor 1 in Table 4. These values are negative for TAG and DAG, but positive for FA and AQ. Thus, for the TAG and DAG analysis in SFC-APCI-MS, it is recommended to use a low APCI temperature (250 °C). This result could be surprising. Indeed, the TAG/DAG boiling points are high so the vaporization process should require a higher temperature. However, TAG and DAG might be thermally

fragile analytes and so they could be degraded by high temperature. Other authors have already studied the thermolability properties of glycerides [26,27].

Moreover, a TAG analysis by SFC-APCI-MS demonstrated that the APCI temperature was an essential parameter which affected the TAG fragmentation [28]. Consequently, 250°C as APCI temperature seems beneficial to the detection of pseudo-molecular ions. For AQ and FA, the temperature must be set at 450 °C. Hsieh *et al.* [19] published similar results with an APCI source : the rise in temperature had a positive effect on the MS signals. It may be due to a better desolvation of molecules and/or a greater thermal agitation. This last effect would lead to a closer contact between analytes and solvent. Compared to TAG and DAG, AQ and FA possess numerous acessible hydroxyl or acid groups, which can be involved in interactions with the polar MeOH of the mobile phase, resulting in a higher solvation sphere. This hypothesis could explain the behavior of these compounds with relation to the APCI temperature. As reported from these results, the source temperature is also a key parameter (the 3rd parameter to optimize for lipids and 2nd parameter for AQ), which must be optimized depending on the compounds studied.

The effect of the APCI corona discharge value is described by factor 5 (Table 4). The corona discharge is an electric discharge (electron emission), which ionizes molecules in the APCI source. These positive values highlight that the use of the highest value for the corona discharge (+6 µA) favour the mass response. Consequently, the increase in discharge current enhances the proportion of ionized molecules in the plasma, enhancing the MS sensitivity.

In Table 4, the drying gas flow rate is described by factor 2. These values are negative for all compounds, TAG, DAG and AQ. Thus, the MS optimum signals are achieved for 3 L/min. Because the drying gas is opposed to the chromatographic effluent in the MS inlet, the rise in the drying gas flow rate may reduce the proportion of molecules reaching the MS detector. This behaviour could be related to the effect of the nebulising gas previously discussed.

However, opposite effects of the drying gas flow rate, obtained with pharmaceutical compounds, were reported in SFC-ESI-MS by using a split and a make-up fluid before the BPR [14,29]. However, according to other previous studies [30], the use of an auxiliary fluid nebulized by an auxiliary gas (N_2) in an interface used with an APCI source and a split configuration enhances the mass response in SFC-MS.

Besides, in LC-MS, the drying gas is supposed to favour the reduction of droplets in order to improve the ionisation recovery. However, in UHPSFC-MS, due to the low liquid content in the mobile phase (10% of methanol in this work), the increase in the drying gas

flow rate could have an opposite behaviour. This effect may depend on the modifier percentage in the UHPSFC mobile phase, which most often varies from 2 to 50%.

In order to better understand how the effects of the analytical parameters are related to the structure of compounds, we can have à look on Ion Mobility Spectrometry (IMS) [31]. In IMS, the separation of analytes that takes place in a gaseous state is ruled by size, shape, and charge. This separation is provided in part by the drift gas (opposite to the MS inlet), and the interaction between ions and the neutral gas is calculated by the Collision Cross-Section (CCS). This CCS depends on structures and three-dimensional conformations. Consequently, molecules with large size (e.g. TAG and DAG) or with planar/rigid 3D conformation (e.g. AQ) may be more affected by the drying gas flow rate, as possibly occurs in the present study after the fluid depressurization.

These different conclusions show the effects of mass parameters on the sensitivity, which is strongly related to the conditions, especially the type of ionisation, the use of a split, the addition of a make-up or the total amount of liquid solvent used for the analysis (modifier + make-up solvent), with regards to the size and chemical nature of the studied compounds, and also probably to the ionization source geometry.

3.1.1.3 Summary in APCI (+) for the UHPSFC-MS hyphenation

According to this statistical study and to streamline the hyphenation, the first parameter to optimize is the nebulising gas pressure (whatever the analytical conditions). This parameter seems to depend on the type of configuration, the modifier percentage in CO_2 and the amount of make-up solvent delivered. In the present case, there was only 10 % MeOH (isocratic conditions) with direct BPR-MS introduction and no make-up fluid. Consequently, for a lipid analysis (AQ, FA, DAG, TAG) and the use of a small amount of modifier (from 0.1 to 0.3 mL/min of MeOH), a low value of nebulising gas pressure is recommended in UHPSFC-APCI-MS (+).

The UHPSFC flow rate should be studied. For this statistical study, the use of the lowest flow rate (1 mL/min) yielded the optimal MS responses. Thus, for a quantitative lipid analysis, we recommend using a low flow rate to reach the optimal sensitivity or to screen unknown samples. However, a flow rate of 3 mL/min UHPSFC is also possible for the screening of known samples in a high-throughput context. In this case, it could be applied for Quality-Control laboratories (QC lab).

For a UHPSFC-APCI-MS (+) method development in lipidomics, the APCI temperature will be the next challenge. This parameter should be optimized in function of the heat-resistance and chemical structure of the targeted compounds. This lipid analysis of TAG and DAG highlighted that the lowest temperature values (250°C) should be favoured, whereas for AQ and FA, 450°C was the best APCI temperature. Consequently, for a first lipid screening (with a low MeOH amount from 0.1 to 0.3 mL per min) and to obtain an exhaustive response, we recommend using a low APCI temperature. Indeed, several molecular families have structural similarities with DAG and TAG as for instance phospholipids, glycolipids etc… and as shown in [21], this strategy would permit to ensure a fast and reliable response.

Finally, based on this lipid strategy, the analytical conditions selected in the following sections 3.2 and 3.3 are: source temperature 450°C (to detect bioactive and minor polar compounds), nebulising gas 0.04 MPa, and UHPSFC flow rate 1 mL/min, with 10% MeOH. Thus, satisfactory results are obtained with both of the other two parameters: the corona values (2 and 6 µA) and the drying gas flow rate (3 and 8 L/min). This means that these two parameters are not as significant as the three previous ones. We thus selected a high corona value (6 µA) and a low drying gas flow rate (3 L/min) to favour the mass responses for minor and polar AQ compounds.

Figure 4 shows the area differences measured for LLL and OLL at 250°C and 0.04 MPa (optimal MS responses of TAG). Whatever the drying gas flow rate (3 or 8 L/min) and the corona value (2 and 6 µA), the response differences are reduced at high mobile phase flow rate (3 mL/min) whereas they are greater at low mobile phase flow rate (1 mL/min). It clearly shows that the ionisation recovery, which is related to the compound structure (here these two TAGs differ by one double bond) is dramatically different with regards to the mobile phase flow rate.

3.1.2 Study in UHPSFC-APCI-MS: APCI (-)

The negative mode in APCI was applied to study the AQ family because a higher MS response was obtained in APCI (-) for these hydroxylated or acidic compounds.

Table 5 describes the effect of the five individual factors on the MS response (peak area) for rhein and aloe emodin. According to this table, the drying gas flow rate (factor 2) is

the first parameter to optimize in a method development. Thus, 3 L/min as drying gas flow rate should be selected (negative values).

It is difficult to rank the other parameters. However, these data may be interpreted. The APCI temperature is illustrated by factor 1. It is necessary to apply a high APCI temperature (450°C) to improve ionisation efficiency. Corona current (factor 5) and nebulising gas pressure (factor 3) parameters should be respectively fixed at 6 µA and 0.04 MPa. These results are consistent with the above study in APCI (+), despite a different ionisation mechanism. However, the UHPSFC flow rate (factor 4) has a different impact for the AQ detection. Therefore a UHPSFC flow rate of 3 mL/min should be set up for aloe emodin and 1 mL/min for rhein. These opposite effects of the UHPSFC flow rate for rhein and aloe emodin in APCI (-) could be related to the different chemical structure between aloe emodin (hydroxyl group) and rhein (carboxylic group), which react differently. Differential behaviours in the effect of the UHPSFC flow rate on the MS response in UHPSFC-APCI-MS were also reported in the analysis of cytarabine and clofazimine in mouse plasma [32].

3.2 Application to sunflower oil in UHPSFC-APCI-MS (+)

Sunflower oil is often used in cosmetics as a component of the formulations or as a green extraction solvent to extract various active compounds from plants. Thus, to evaluate the extraction recovery, the study of the vegetable oil composition should be achieved.

Figure 5 shows the chromatograms for a sunflower oil, obtained by SFE or by cold-pressed extraction. Between 40 and 80 minutes, the TAG composition shows some composition differences between the two samples. This SFE extraction seems to have favoured the recovery of the most unsaturated TAG, *i.e.* LLL, PLL, OLL, or OOL, whereas cold-pressed extraction favours the recovery of the most saturated species, POO, OOO and SOO. This result is not unexpected due to the use of 2.5% ethanol in CO_2 for the SFE extract, which induces a higher solubility of TAG having double bonds in the alkyl chains.

The most surprising is the presence of several peaks between 30 and 40 minutes for the SFE extract. The MS identification for one of the major peaks was achieved by the study of the mass spectrum (Fig. 6). The peak at *m/z* 896.73984 is due to $[LLL(OH)+H]^+$ whereas the peak at *m/z* 877.72926 corresponds to the loss of a water molecule from the previous one. These peaks show that the SFE extraction favoured the recovery of hydroxylated triglycerides with regards to cold-pressed extraction.

3.3 Application to *Calophyllum inophyllum* oil (Tamanu oil) in UHPSFC-APCI-MS (+)

The UHPSFC-HRMS-APCI (+) coupling was also applied to the *Calophyllum inophyllum* oil, which is used in topical application by Polynesians, or as a traditional medicine ingredient or as a well-being ingredient [33, 34]. This analysis was achieved at 450°C (for the mass source) to improve the response of the first eluted peaks, but this was detrimental to the peak area of TAG due to a probable thermo-sensitivity of the tri-esters (Fig. 7).

Figure 7 shows three peak areas. Between 40 and 80 minutes, the TAG found in this oil were in accordance with previous reports [35,36]. DAG eluted around 20 minutes whereas several compounds eluted around 10 minutes, in which the following were identified: Tamanolide ($C_{24}H_{28}O_5$), Calophyllolide ($C_{25}H_{22}O_5$), and Inophyllum (E or C) ($C_{25}H_{20}O_5$)). These three compounds were previously reported in Tamanu oil ethanolic extracts [34], *i.e.* an extract free of the main lipids. The analysis of these ethanolic extracts was achieved with normal phase liquid chromatography, with a mobile phase composed of cyclohexane-ethylacetate and a silica gel stationary phase [34]. The UHPSFC approach allows a most complete oil analysis, including both lipid composition and the study of the main bioactive compounds, without fractionation of the ethanol-soluble compounds. However, the separation of some isomers (Inophyllum C and E) was not reached with these C18 stationary phases in UHPSFC, and an improvement of this separation should be performed in the future.

4. Conclusions

The direct coupling of UHPSFC to the APCI-MS interface has showed a great potential for lipid analyses of various matrices containing bioactive compounds (ex: AQ, coumarins) with a mixture of methanol and CO_2 in isocratic conditions. The positive and negative ionisation mode were studied. The main advantages of this kind of direct hyphenation (UHPSFC to MS with no split) is to have a better MS sensitivity without losing the chromatographic resolution of the MS peak (on condition of working with an optimised BPR design).

As described in section 3.1.2, for UHPSFC method development in APCI (+) focused on lipids, the nebulising gas pressure had a dramatic effect on the MS responses. Due to this direct interface by the introduction of the total flow in the APCI source, this pressure should be set at the lowest value. In addition, the mobile phase and the drying gas flow rates should both be reduced to improve the sensitivity. Besides, the ionisation source temperature also had

an impact, but this parameter must be optimized for each targeted compound. In our case, this parameter was related to a probable thermo-sensitivity of the lipid esters. As usually observed, the increase in the corona discharge value enhanced peak area for all compounds.

The negative APCI mode favoured the MS response for AQ with regards to the positive mode, but the lipid part of the oil could not be observed. Consequently, this ionisation mode will permit to have a better sensitivity and selectivity for some compounds and it will be used in a second approach.

With the UHPSFC-APCI-HRMS, we could elucidate chemical structures ($\Delta < 2$ ppm) and screen several raw materials for the cosmetic field to detect minor bioactive compounds. We showed the presence of hydroxylated TAG in sunflower oil and this approach could be useful for a better total characterisation of the *Calophyllum inophyllum* oil.

Acknowledgments.

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Figure captions

Figure 1: Descriptive analysis of 33 experiments from the experimental design in function of FA, DAG, TAG molecular mass from a *Kniphofia uvaria* oily extract (0.5 mg/mL) – Analytical conditions: 1 Kinetex C18 column (150 x 4.6 mm, 2.7 µm), 9°C, 10 MPa, V_{inj} = 5 µL.

Figure 2: Descriptive analysis of 16 experiments from the experimental design in function of TAG, DAG, FA molecular mass from a *Kniphofia uvaria* oily extract (0.5 mg/mL) – see Fig. 1 for analytical conditions.

Figure 3: Evaluation of the distribution of experimental results (from 16 experiments) performed by the Henry graph for each compound. x axis: product of the experimental peak area and the endcoding value (-1 or +1) ; y axis: normalized data ($y = (x-x_{average})/\sigma$)

Figure 4: The MS response peak area differences between LLL and OLL in UHPSFC-HRMS-APCI (+) according to varied analytical conditions (drying gas flow rate, mobile phase flow rate, corona discharge) applied in APCI-MS with a source temperature equal to 250°C.

Figure 5: Chromatographic profile of sunflower oil analysed by UHPSFC-HRMS-APCI (+). For analytical conditions see in Text. Green chromatogram: oil obtained from mechanical cold pressure; Orange chromatogram: oil obtained from SFE extraction (see conditions in the text).

Figure 6: Mass spectra of the main additional peak obtained by SFE for the sunflower oil (see pink arrow in Fig. 5). Columns: 4 Kinetex C18 + 1 Accucore C18 (each with dimensions of 150 mm x 4.6 mm; particle size respectively 2.7 and 2.6 µm); Mobile phase: CO_2/MeOH (90/10; v/v); T°C = 9°C; P_{out} = 10 MPa; F = 1 ml/min. HRMS-APCI+: T° = 450°C; corona discharge value = +6 µA; 0.04 MPa (N_2); Drying gas flow rate = 3 L/min; V_{inj} = 5 µL, oil concentration = 0.1 mg/ml dissolved in MeOH/CH_2Cl_2 (50/50, v/v).

Figure 7: Chromatogram of *Calophyllum inophyllum* oil by UHPSFC-HRMS-APCI+.
Columns: 4 Kinetex C18 + 1 Accucore C18 (each with dimensions of 150 mm x 4.6 mm; particle size respectively 2.7 and 2.6 µm); Mobile phase: CO_2/MeOH (90/10; v/v); T°C =

9°C; P_{out} = 10 MPa; F = 1 ml/min. HRMS-APCI+: T° = 450°C; corona discharge value = +6 µA; 0.04 MPa (N_2); Drying gas flow rate = 3 L/min; V_{inj} = 5 µL, oil concentration = 0.1 mg/ml dissolved in MeOH/CH_2Cl_2 (50/50, v/v).

Tables

Table 1: Experimental design

Table 2: Variable encoding for the experimental design (see significance of -1 and +1 values in table 1). For experiment 33, the 0 encoding values correspond to 350° for source temperature; 5.5 L/min for drying gas; 0.12 MPa for Nebulising gas; 2 ml/min for SFC flow rate, and 4 µA for corona discharge).

Table 3: Average values obtained from the products of the peak area and the encoding value (-1; +1) of the parameter for the anthraquinones (Rhein, Aloe emodin) and the lipidic compounds (Linolenic acid, LL, OL, LLL+ H_2O, LLL,OLL) for the 32 experiments. Analytical conditions: 1 Kinetex C18 column (150 x 4.6 mm, 2.7 µm), 9°C, 10 MPa, 5 µL, APCI +.

Table 4: Average values from the products of the peak area and the encoding value of the parameter for the anthraquinones (Rhein, Aloe emodin) and the lipidic compounds (Linolenic acid, LL, OL, LLL+ H_2O, LLL,OLL) for 16 experiments with a nebulizing gas equal to 0.04 MPa Analytical conditions identical to table 3.

Table 5: Average values obtained from the products of the peak area and the encoding value (-1; +1) of the parameter for the anthraquinones (Rhein, Aloe emodin) for the 32 experiments. Analytical conditions: 1 Kinetex C18 column (150 x 4.6 mm, 2.7 µm), 9°C, 10 MPa, 5 µL; APCI -.

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Factor	Definition	Low value (-1)	High value (+1)
1	Ionisation source temperature (°C)	250	450
2	Drying gas (L/min)	3	8
3	Nebulising gas (MPa)	0.04	0.2
4	SFC flow (mL/min)	3	1
5	Corona (mA)	2	6

Table 1

Experiment	Ionisation source temperature (APCI) (°C)	Drying gas (L/min)	Nebulising gas (MPa)	SFC flow (mL/min)	Corona (μA)
1	-1	-1	-1	-1	-1
2	-1	-1	-1	-1	+1
3	-1	-1	-1	+1	-1
4	-1	-1	-1	+1	+1
5	-1	-1	1	-1	-1
6	-1	-1	1	-1	+1
7	-1	-1	1	+1	-1
8	-1	-1	1	+1	+1
9	-1	+1	-1	-1	-1
10	-1	+1	-1	-1	+1
11	-1	+1	-1	+1	-1
12	-1	+1	-1	+1	+1
13	-1	+1	1	-1	-1
14	-1	+1	1	-1	+1
15	-1	+1	1	+1	-1
16	-1	+1	1	+1	+1
17	+1	-1	-1	-1	-1
18	+1	-1	-1	-1	+1
19	+1	-1	-1	+1	-1
20	+1	-1	-1	+1	+1
21	+1	-1	1	-1	-1
22	+1	-1	1	-1	+1
23	+1	-1	1	+1	-1
24	+1	-1	1	+1	+1
25	+1	+1	-1	-1	-1
26	+1	+1	-1	-1	+1
27	+1	+1	-1	+1	-1
28	+1	+1	-1	+1	+1
29	+1	+1	1	-1	-1
30	+1	+1	1	-1	+1
31	+1	+1	1	+1	-1
32	+1	+1	1	+1	+1
33	0	0	0	0	0

Table 2

	Rhein	Aloe emodin	Linolenic acid	LL	OL	LLL+H_2O	LLL	OLL
Source temperature	6 916	15 795	36 205	-1 118 818	-987 628	39 846	-800 203	-1 032 659
Drying gas	-3514	-2175	13113	-144 150	-102 884	-489 059	-544 015	-539 312
Nebulising gas	-5 761	-12 152	-81 306	-3 002 108	-266 0372	-3 251 657	-4 812 258	-3 311 593
SFC flow rate	-4 560	-11 196	-39 464	-1 212 755	-352 486	-802 373	-1 863 651	-1 343 699
Corona discharge	1 777	4 196	11 692	413 659	347 223	175 412	802 329	699 808

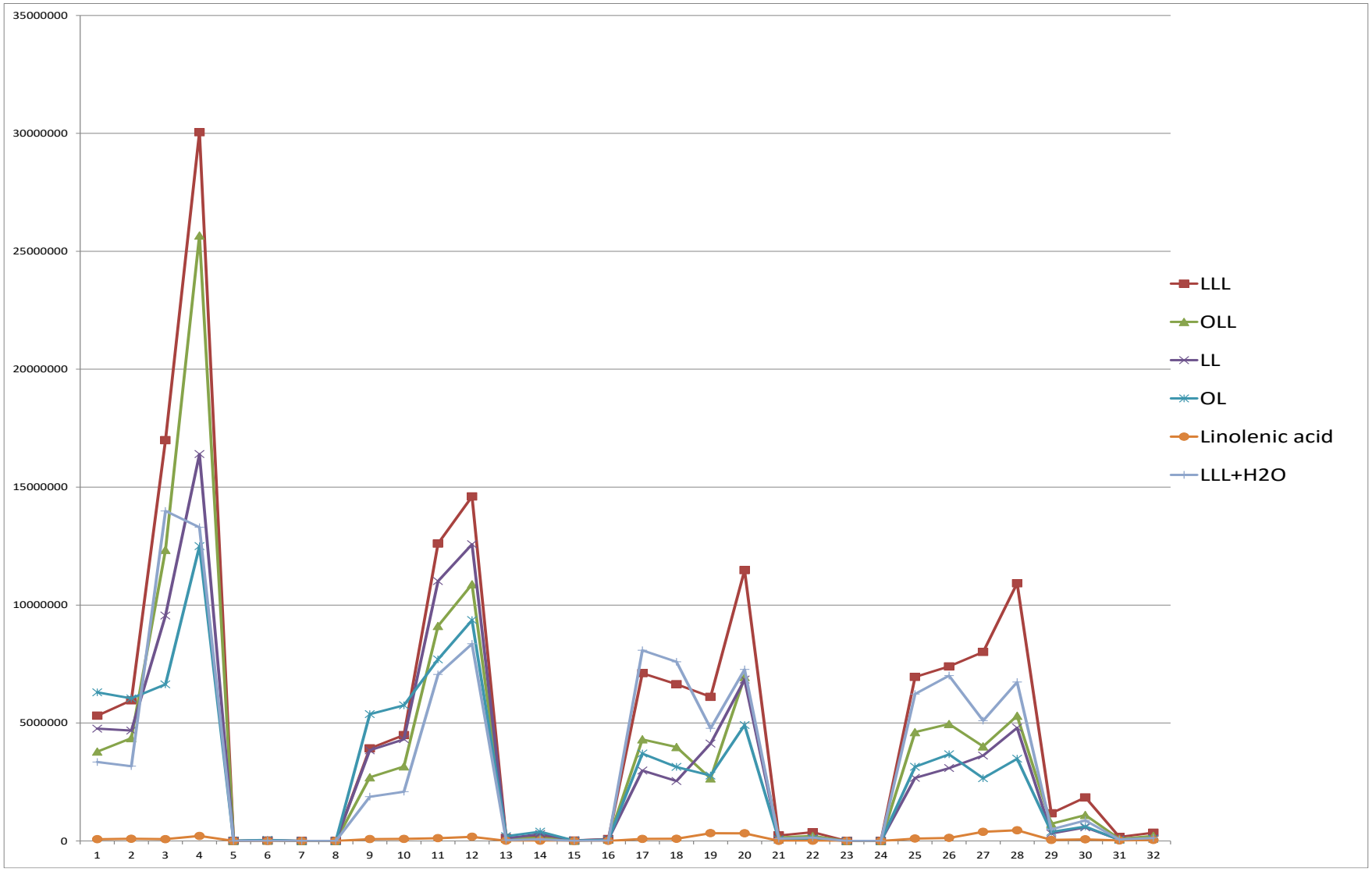
Table 3

	Rhein	Aloe emodin	Linolenic acid	LL	OL	LLL+H_2O	LLL	OLL
Source temperature	12 676	27 947	61 842	-2 279 823	-2 011 281	-24 124	-1 831 241	-2 201 975
Drying gas	-8 133	-7 994	14 564	-372 909	-303 673	-1 067 344	-1 296 931	-1 206 690
SFC flow rate	-7 866	-18 802	-83 744	-2 502 880	-806 069	-1 699 192	-3936921	-2 816 809
Corona discharge	2 399	4 749	19 841	790 520	659 953	316 666	1 533 559	1 359 095

Table 4

	Rhein	Aloe emodin
Source temperature	138 214	159 212
Drying gas	-207 151	-2 177 873
Nebulising gas	-78 034	-533 165
SFC flow rate	-82 257	606 331
Corona discharge	18 547	201 154

Table 5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LLL
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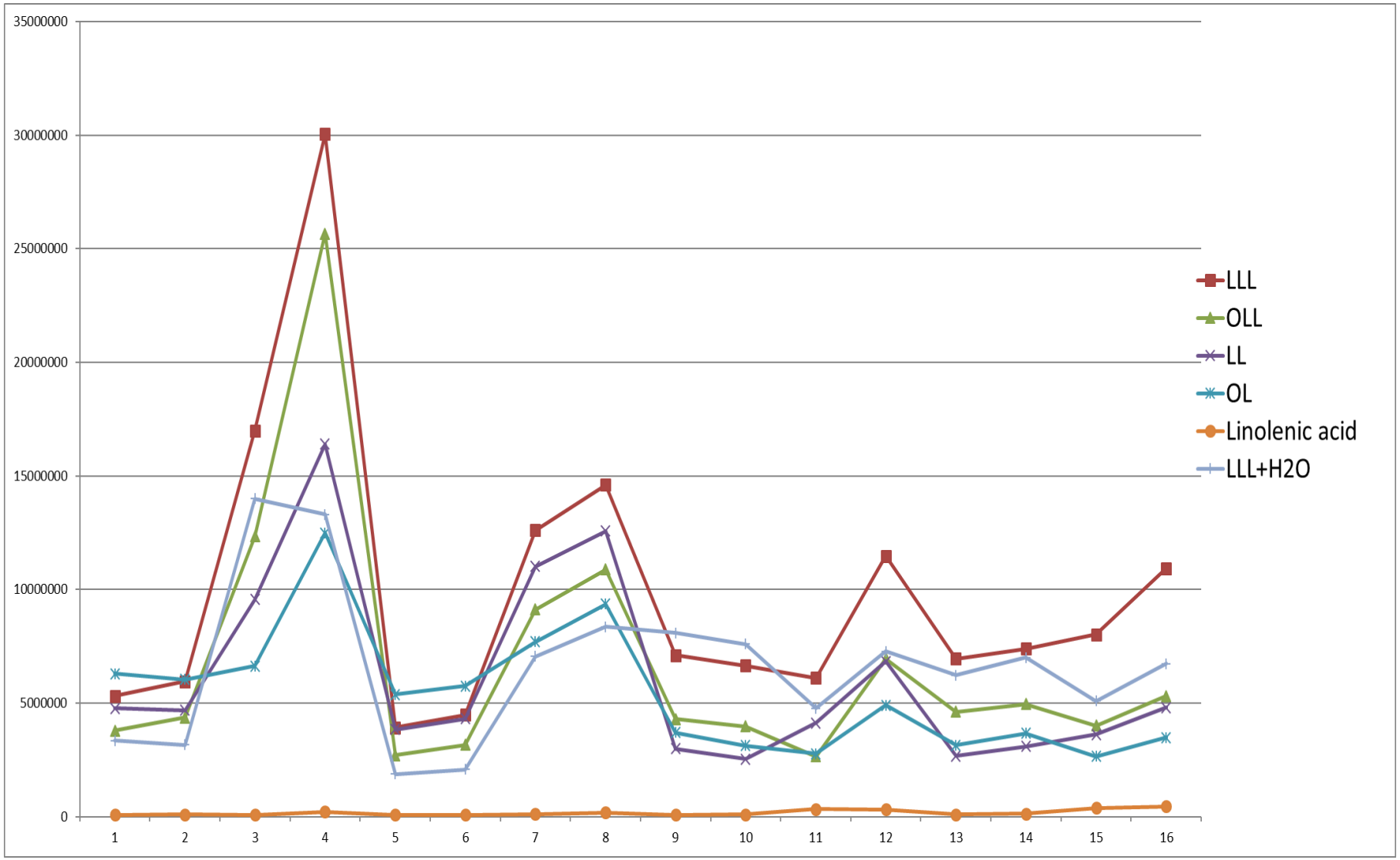
LL
OL
Linolenic acid
LLL+H2O

Figure 1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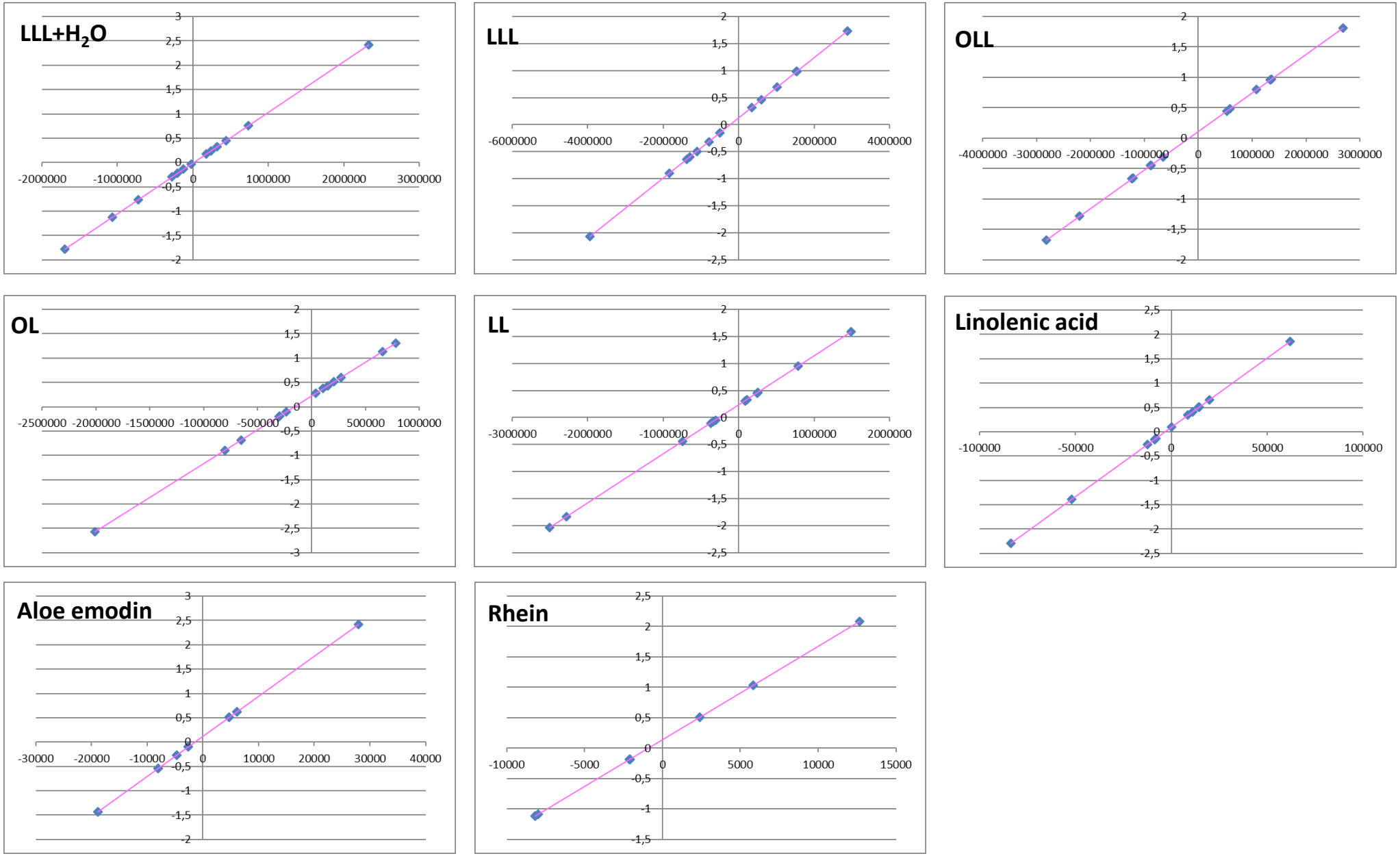
Linolenic acid
LLL+H2O

Figure 2



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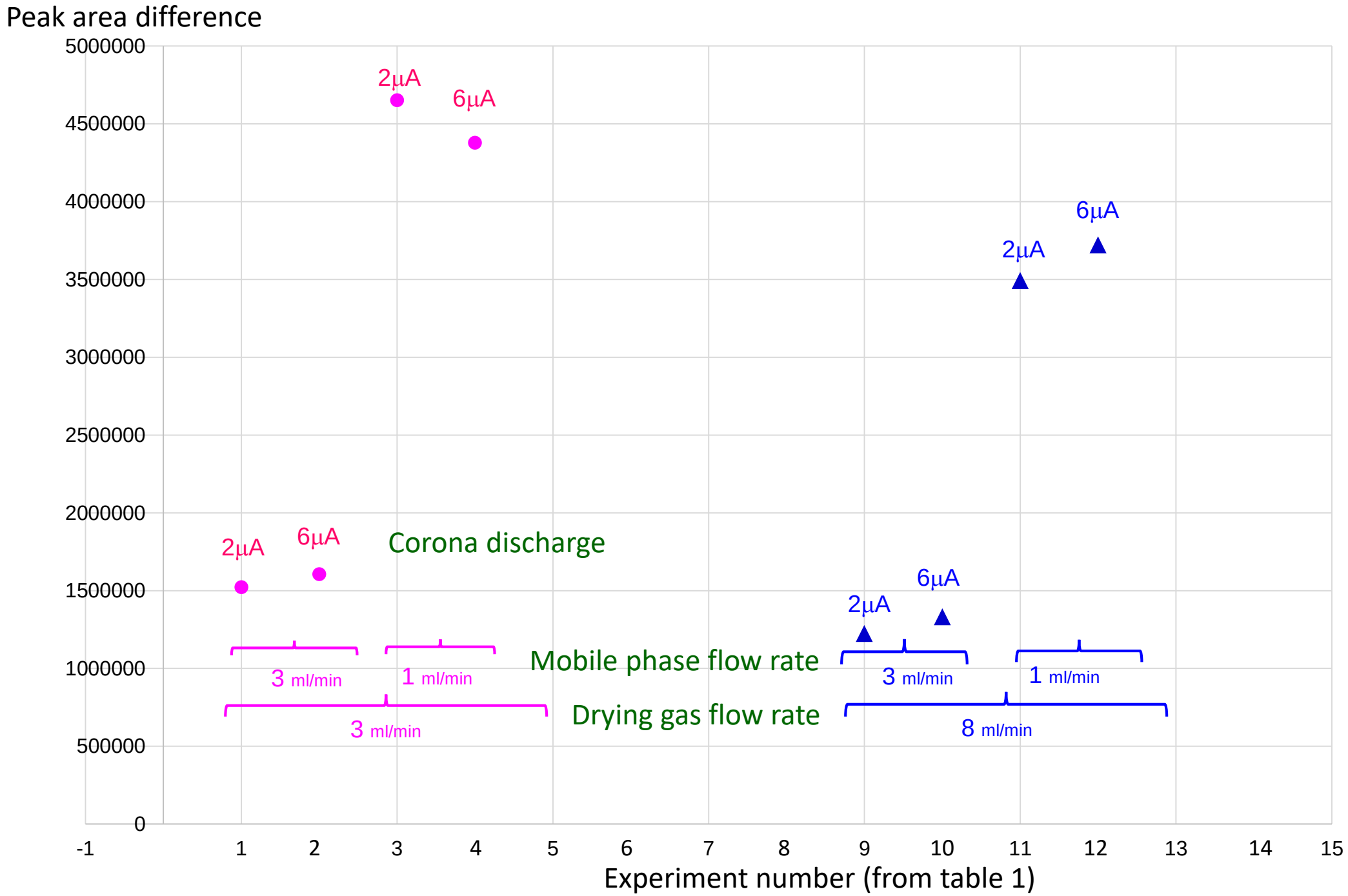
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Experiment number (from table 1)
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6µA
2µA
6µA
Corona discharge
2µA
6µA
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Drying gas flow rate
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Figure 4

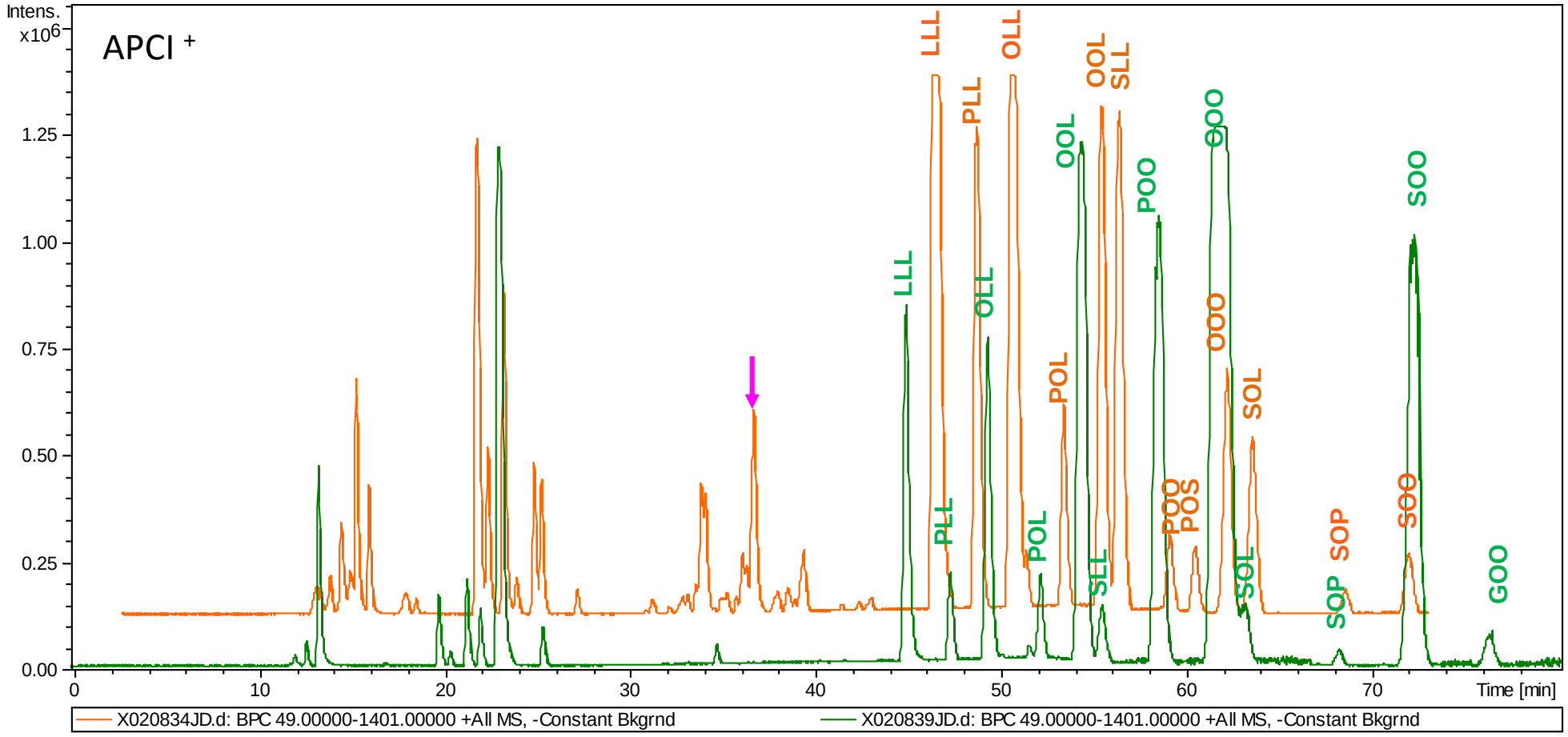
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Figure 5

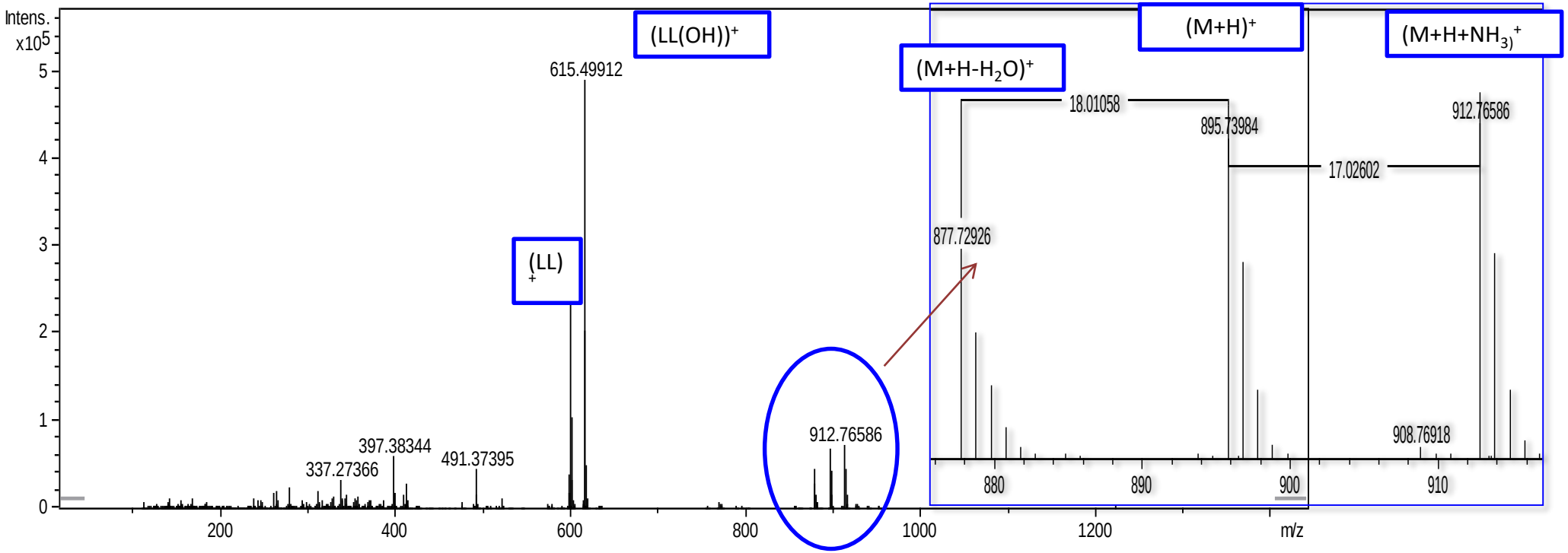
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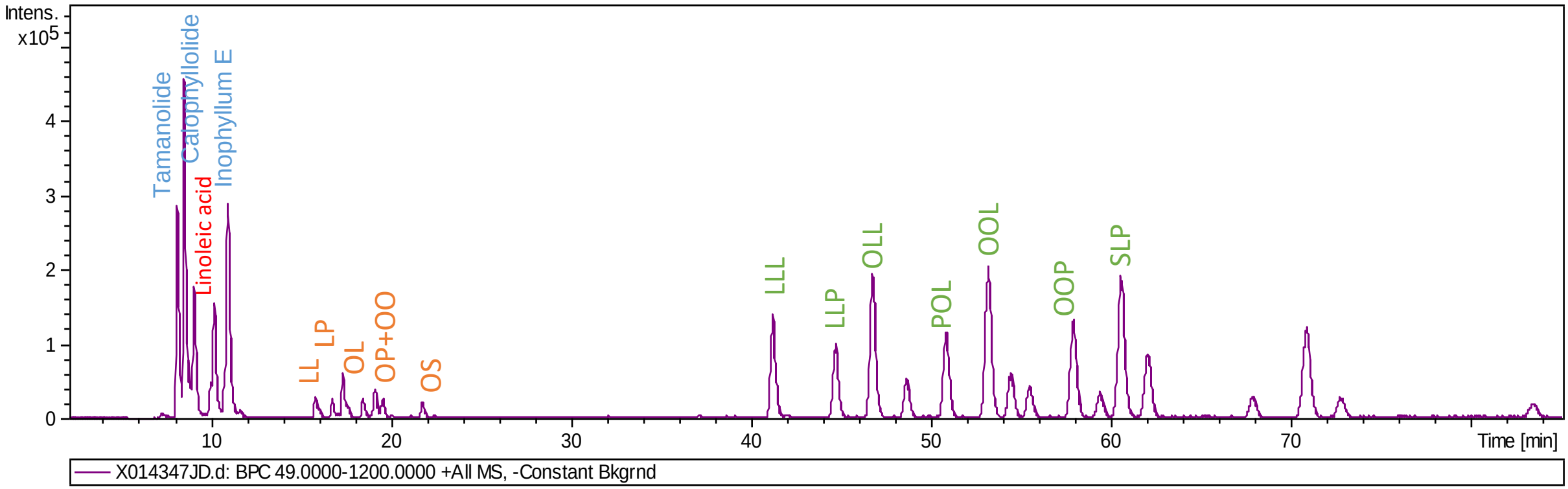
Figure 6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Time [min]
X014347JD.d: BPC 49.0000-1200.0000 +All MS, -Constant Bkgrnd

Figure 7