Nano- and rapid resolution liquid chromatography-electrospray ionization-time of flight mass spectrometry to identify and quantify phenolic compounds in olive oil

Rocío García-Villalba[1], Alegría Carrasco-Pancorbo[1*], Gabriela Zurek[2], Marina Behrens[2], Carsten Bäβmann[2], Antonio Segura-Carretero[1], Alberto Fernández-Gutiérrez[1*]

[1] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/ Fuentenueva s/n, E-18071 Granada, Spain

[2] Bruker Daltonik GmbH, Bremen, Germany

[*]Author to whom correspondence should be addressed:

Dr. Alegría Carrasco-Pancorbo or Alberto Fernández Gutiérrez, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain.

E-mail: alegriac@ugr.es or albertof@ugr.es

Fax: +34 958 249510

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Abstract

The applicability of nano-liquid chromatography coupled to electrospray ionization-time of flight-mass spectrometry (nanoLC-ESI-TOF MS) for the analysis of phenolic compounds in olive oil was studied and compared with a HPLC method. After the injection, the compounds were focused on a short capillary trapping column (100 μm i.d., effective length 20 mm, 5 μm particle size) and then nanoLC analysis was carried out in a fused silica capillary column (75 μm i.d., effective length 10 cm, 3 μm particle size) packed with C18 stationary phase. The mobile phase was a mixture of water + 0.5% acetic acid and acetonitrile eluting at 300 nL/min in a gradient mode. Phenolic compounds from different families were identified and quantified. The quality parameters of the nanoLC method (linearity, limits of detection and quantification, repeatability) were evaluated and compare to those obtained with HPLC. The new methodology presents better sensitivity (reaching LOD values below 1 ppb) with less consumption of mobile phases, but worse repeatability, especially inter-day repeatability, doing more difficult to get highly accurate quantification. The results described in this paper open up the application fields of this technique to cover a larger variety of compounds and its advantages will make it especially useful for the analysis of samples containing low concentration of phenolic compounds, as for instance, in biological samples.

1 Introduction

Miniaturization of analytical techniques has recently become one of the most important areas of research and several groups have paid great attention to the study and development of new miniaturized separation methods. Among these, nano-liquid chromatography (nanoLC), firstly introduced by Karlsson and Novotny in 1988 [1], has emerged as a new powerful analytical tool, complementary and/or competitive to conventional HPLC, providing a wide number of important applications, especially in proteomics and related fields [2-5], mainly due to the very low sample requirements. Other applications of nanoLC can be also found in fields such as pharmaceutical [6], environmental [7,8], and enantiomeric analysis [9,10]. The use of nanoLC for food analysis has not been so widely extended so far, although in the last years some interesting works have demonstrated its potential in this field [11-15]. The analyses are carried out in capillaries of small internal diameter (10-100 μm), in most of the cases either of fused silica or peek material and containing selected stationary phases usually used in HPLC with particle sizes of 3-5 μm. In theory, by reducing the internal diameter of the capillary column, better sensitivity can be achieved because the lower flow rate causes a reduction of the chromatographic dilution. However, due to the low injected sample volumes required in column with smaller diameter, the sensitivity will not be high. The loss of sensitivity can be avoided using large volume injections with specific techniques that prevent column overloading: on-column and extra column focusing techniques. In the first one, solutes are dissolved in a solvent of lower eluting power compared to the mobile phase [16] and the second solution consists of using a pre-column combining with a switching system [11]. With both techniques large injection volumes could be used, increasing the sensitivity.

As far as detection system is concerned, UV and, in some cases, fluorescence detection are the most commonly used, although when sensitivity is of paramount importance, MS detection is gaining more interest due to its easy coupling to nanoLC instrumentation. For coupling nanoLC to mass spectrometry, several nanospray interfaces have been tested (sheatliquid and sheatless), some of them commercially available and, in many occasions, homemade [6,17].

Extra virgin olive oil (EVOO) is a valuable component of the traditional Mediterranean diet, unique among other vegetable oils because of its fatty acid composition (characterized by a high monounsaturated-to-polyunsaturated fatty acid ratio) and its high concentration level of phenolic compounds. The phenolic fraction of EVOO consists of a heterogeneous and very complex mixture of compounds, mainly simple phenols, lignans, flavonoids and secoiridoids; every family of compounds varies in chemical properties and has a particular influence on the quality of EVOO [18,19]. There is evidence that phenolic compounds could play a major role in the healthy effects of EVOO, besides to be responsible of its antioxidant activity and organoleptic properties. Therefore, the determination of this family of compounds in olive oil is of special relevance. So far, different analytical methods (gas chromatography (GC) [20], HPLC [21,22], capillary electrophoresis (CE)) [23] coupled to different detectors (UV, fluorescence, mass spectrometry, etc) [24] have been developed to analyze olive oil phenols. However, although nanoLC have already been employed in food analysis, to the best of our knowledge it has not been applied to the analysis of polyphenols.

The aim of this work was to test and evaluate the potentiality of nanoLC coupled with mass spectrometry (ESI-TOF MS) for the analysis of phenolic compounds in olive oil. When an analytical technique is applied by first time to face a particular problem, it is quite interesting to compare its potential and performance with those of other techniques

more widely used. Therefore, a comparison between the performance of both nanoLC-ESI-TOF MS and HPLC-ESI-TOF MS methodologies for the separation and quantitation of this type of compounds was made.

2 Materials and methods

2.1 Chemicals and samples

Methanol and *n*-hexane of HPLC grade used during sample extraction and acetic acid were purchased from Panreac (Barcelona, Spain). Acetonitrile from Lab-Scan (Dublin, Ireland) was used in the mobile phase for the HPLC and nanoLC analysis. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA).

Standards of hydroxytyrosol, tyrosol, luteolin and apigenin were purchased by Sigma-Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Other phenolic compounds used as pure standard samples, elenolic acid and ligstroside aglycon, were isolated from EVOOs by semipreparative HPLC. Stock solutions at concentration of 500 mg/L for each polyphenol were prepared in MeOH and then serially diluted to working concentrations. For the nanoLC analysis the standards were dissolved in mobile phase (water + 0.5% acetic acid) with 10% MeOH. EVOO samples of three different olive fruit varieties so-called Picual, Hojiblanca and Arbequina used for the study were acquired from a supermarket (Granada, Spain).

2.2 Sample extraction

The extraction procedure was based on a specific solid phase extraction (SPE) method with Diol-cartridges which is used as routine extraction protocol in our research group [25]. Briefly, the extraction consisted of passing through a column, previously conditioned with 10 mL of methanol and 10 mL of hexane, 60 g of EVOO dissolved in

60 mL of hexane. After removing the non-polar fraction with 15 mL of hexane, the phenolic compounds were recovered with methanol (40 mL). The final volume was dried in a rotary evaporator under reduced pressure at 35°C and the residue was dissolved in 2 mL of methanol. After preparing the extracts, proper dilutions were made depending on the technique used in each case (nanoLC or HPLC). For the HPLC analysis a 1:10 dilution in MeOH was used and for the injection into the nanoLC, the sample was diluted 1:500 in mobile phase (water + 0.5% acetic acid) with 10% MeOH.

2.3 Nano- liquid chromatography analyses

Experiments were performed in a commercial available instrumentation EASY-nLC™ (Bruker Daltonik GmbH, Bremen, Germany), composed of one module and equipped with three pumps, three pressure sensors, four valves, two flowsensors, an autosampler and a touchscreen.

The chromatographic separation was performed in a capillary column BioSphere (75 μm i.d., packed length 10 cm and particle size 3 μm) packed with C18 particles. An on-line C18 trapping column (BioSphere (100 μm i.d., packed length 20 mm and particle size 5 μm)) was used before the nanoLC column in order to achieve both pre-concentration and clean up of samples.

Optima chromatographic conditions were achieved by using a mobile phase composed of water + 0.5% acetic acid (phase A) and acetonitrile (phase B) with the following gradient: 0 to 10 min, 20-33% B; 10 to 35 min, 33-40% B; 35 to 38 min, 40-95% B. Finally, the B content was decreased to the initial conditions (20%) within 2 min and the column rinsed with these conditions for 5 min. Before starting the following analysis the pre-column and column were re-equilibrated with phase A at 6 μL/min for 2 min and 0.6 μL/min for 8 min, respectively. A volume of 5 μL of the sample was injected into the

loop and later loaded onto the pre-column using the phase A (water + 0.5% acetic acid) at a flow rate of 6 μL/min during 1 min, to trap de compounds of interest and to clean the sample. Afterwards, the valve changed position and switched the pre-column in-line with the analytical column eluting the compounds of interest at a flow-rate of 300 nL/min and 25ºC (the column was at room temperature because of the lack of thermostatization system). Figure 1 shows a schematic figure of the nanoLC system, showing the moment when the sample is loading onto the pre-column using pump A.

All nanoLC parts were controlled by Hystar (version 3.1) software. The compounds separated were analyzed with a mass spectrometry detector.

2.4 High performance liquid chromatography analyses

An Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a UV-Vis detector was used for the chromatographic determination. Polyphenolic compounds were separated by using a Zorbax C18 analytical column (4.6 x 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing, operating at 30ºC and a flow rate of 1.5 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: 0 to 10 min, 5-30% B; 10 to 12 min, 30-33% B; 12 to 17 min, 33-38% B; 17 to 20 min, 38-50% B; 20 to 23 min, 50-95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 10 min. A volume of 10 μL of the 1:10 diluted methanolic extracts of olive oil was injected. The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass spectrometry detector.

2.5 Mass spectrometry

The nanoLC column and the RRLC system were coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using electrospray ionization (ESI).

In this study the nanoLC column was interfaced to the mass spectrometry using a commercial sheatless nano-spray interface with a tapered fused silica sprayer tip. The key parameters of the nano-ESI were adjusted for the flow rate used (300 nL/min) to achieve stable spray across the entire gradient range: pressure 0.4 bar, dry gas flow 4 L/min and dry gas temperature 150ºC.

The RRLC system was coupled to the mass spectrometer using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The flow rate used in the RRLC method 1.5 mL/min was too high for achieving an stable electrospray ionization (ESI) (maximum flow-rate is around 1 mL/min), therefore it was necessary to use a flow divisor 1:6, so the flow delivered into the mass spectrometer was reduced to 0.21 mL/min. According to this inflow the ESI parameters were chosen: nebulizer pressure was set at 2 bar, dry gas flow 9 L/min and dry gas temperature 190ºC.

The mass transfer parameters (radio frequencies and voltages in the different skimmers, hexapoles and lenses) were similar to those previously optimized in recent works where the same matrix (EVOO) was analyzed [26] acquiring spectra in the range of 50-800 m/z in the negative mode. So far, nanoflow ESI has become routine in the positive ion mode and just few applications have been developed in negative ion mode due to difficulties with spray instability. TOF analyzers provide greatly improved mass resolution (5,000–10,000 at 250 *m/z*) and significantly high sensitivity and accuracy when acquiring full-fragment spectra. In order to obtain high mass accuracy in TOF, mass calibration is

required. After a good instrument calibration, the accurate mass data of the molecular ions and the true isotopic pattern (TIP) can be processed by DataAnalysis 4.0 software (Bruker Daltonik GmbH) which provides information of elemental composition of compounds. The calibrant can either be measured within the sample itself (internal calibration) or, alternatively, can be introduced externally, for instance, with a pump at the beginning or at the end of the analysis (external calibration). In general, it is safest and more convenient to measure the calibrant externally to avoid signal suppression and contamination and to assure the calibrant signal is measured at an appropriate, controlled intensity level. However, the internal calibration provides better mass accuracy (less than 3 ppm error). With the instrumentation used in this work for the nanoLC analysis was not possible to do an external calibration because a system to introduce the calibrant at the beginning or the end of the chromatographic run has not been developed yet. Instead of this, an internal calibration was applied using a mixture of well-known phenols present in the olive oil extracts (Table 1), giving mass peaks throughout the desired range of 100-400 m/z. The seven phenolic compounds included in Table 1 (which were available as pure standards) were used to calibrate every analysis. First of all, we corroborated their presence in the analyzed samples (taking into account their retention time, MS spectra and also by analyzing spiked samples). After their unequivocal identification, we decided to use them to increase the accuracy of our results. So we made an average MS spectrum of the whole chromatogram and we re-calibrated every analysis by using the calibration list mentioned above. This procedure resulted in mass accuracies of less than 3 ppm.

2.6 Statistics

Results of phenolic compounds are the averages of at least three repetitions (n=3), unless otherwise stated. Tukey's honest significant difference multiple comparison (one-way ANOVA) and Pearson's linear correlations, both at $p < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK).

3 Results and discussion

3.1 NanoLC-ESI-TOF MS method

3.1.1. Development of the method

In order to develop the nanoLC method for the separation of the olive oil phenolic compounds, a capillary column BioSphere C18 (75 μm i.d., packed length 10 cm and particle size 3 μm) coupled to a C18 trapping column BioSphere (100 μm i.d., packed length 20 mm and particle size 5 μm) was used.

Capillary columns of 75 μm i.d. usually have an ideal injection volume of few nanoliters (20-60 nL) but the use of trapping columns before the analytical column, as mentioned in the introduction, allows injecting relatively high sample volumes, improving the sensitivity. In the case under study, where the sample amount was not limited, 5 μL of the sample was initially chosen for the injection.

Preliminary studies were done analyzing the best way to load the sample from the loop to the pre-column in order to trap the analytes. With the instrumentation used in this work the solvent used to load the samples is always phase A (in this case water + 0.5% acetic acid), and it is not possible to change it. Using this solvent, an appropriate loading time and speed were chosen. With a flow rate of 6 μL/min, a very low loading time led to some analytes, especially the most hydrophobic, not to reach the pre-column and to keep sorbed onto the tubing connecting the loop and the pre-column. On the contrary, if the time is too large, the analytes, principally the most hydrophilic was displaced from

the pre-column during the loading/washing phase of the analysis. Finally, the best loading conditions that allowed an optimum recovery of olive oil phenols into the separation columns were 6 µL/min for 1 min. This description results easier to understand observing Figure 1.

Once optimized the loading conditions, other experimental variables affecting nanoLC analysis were studied. Based on our previous studies with this type of compounds water + 0.5% acetic acid and acetonitrile were selected as mobile phases and different isocratic and gradient programs were tested. In general, because of the very different properties of the analyzed compounds, an isocratic elution at different percentages of organic solvents did not provide an appropriate separation of the selected compounds and thus, a gradient elution was required. Optimum separation was achieved by using the following gradient: 0 to 10 min, 20-33% B; 10 to 35 min, 33-40% B; 35 to 38 min, 40-95% B. Finally, the B content was decreased to the initial conditions (20%) within 2 min and the column rinsed with these conditions for 5 min. As it can be observed, the gradient is limited between 20 and 100% of organic solvent, as in most of the nanoLC-MS systems described in literature [14,27,28], in order to improve spray stability that can be an issue when predominantly aqueous solvents are used (due to the high surface tension of water).

Different flow rates were tested: 200, 300 and 400 nL/min (the maximum flow rate supported by the column is 600 nL/min). Worse efficiency and long retention times were obtained when lower flow rates were used, whilst for high flows we got shorter analysis time but a loss of sensitivity and resolution for some compounds. After the optimization, chromatographic separations were carried out at room temperature (25ºC) at a flow rate of 300 nL/min. Other lower injection volumes were also tested (500 nL, 1 µL, 2 µL) but the resolution did not improve and the sensitivity was lower.

As described in the experimental section the detection was carried out with mass spectrometry (TOF) using a sheathless nano-electrospray. MS was operated in the range between 50-800 m/z in negative polarity; however analyses of EVOO by nanoLC-ESI-TOF MS were performed in negative ion mode and in positive ion mode. In positive ion mode, in general, the profiles were worse and as far as the different families of phenolic compounds are concerned, flavonoids and lignans were ionized quite properly, whilst secoiridoids, simple phenols and phenolic acids (or very related compounds) were detected better in negative polarity. We decided to use negative polarity for the rest of the analyses. Figure 2 shows the chromatograms of the olive oil extract (Picual variety) obtained by using nanoLC-ESI-TOF MS and HPLC-ESI-TOF MS. As it can be observed, the nanoLC separation was successfully carried out in a relatively short time (less than 30 min), comparable to some results reported in literature for olive oil phenols. The resolution and efficiency for some compounds were a bit worse than in HPLC, particularly at the beginning of the chromatogram where predominantly aqueous portion is present.

3.1.2 Identification of the compounds under study

The optimized nanoLC-ESI-TOF MS method was applied for the identification and quantification of phenolic compounds in different olive oil samples.

The identification of the compounds was easily performed by comparing both migration time and MS spectral data obtained from olive oil samples and standards (commercial standards or isolated compounds by HPLC), and by using the information about the polarity of the compounds, the wide information previously reported in literature [26,29] and the information provided by the mass spectrometer with TOF analyzer. Table 2 summarizes the main phenolic compounds identified in Picual EVOO by nanoLC-ESI-

TOF MS including information about the retention time, product ions obtained spontaneously in the ionization source, m/z, molecular formula, error and sigma value. As shown in Table 2 calibration error for each mass was less than 3 ppm.

Figure 3 shows the base peak chromatogram (BPC) obtained by the developed nanoLC-ESI-TOF MS method operating at the optima conditions for the olive oil from Picual variety and the extracted ions chromatograms (EICs) of the main phenolic compounds identified.

A quite stable nanospray was obtained by using these optima conditions and, in general, we could observe minimal carryover in the samples. The most hydrophilic compounds (hydroxytyrosol and tyrosol) presented low efficiency and resolution, probably due to the higher aqueous content used at the beginning of the gradient. We tried to improve the first part of the chromatogram starting with higher organic solvent content (30 and 40% ACN), but with these conditions, the resolution in the rest of the chromatogram was quite worse. Compounds in the family of lignans (pinoresinol, acetoxipinoresinol and syringaresinol) and flavonoids (luteolin and apigenin) were detected with very high efficiency and good peak shape. Regarding secoiridoids, some of them (oleuropein aglycon, ligstroside aglycon, methyl oleuropein aglycon) showed several peaks corresponding to different isomeric forms some of them, previously described in literature [23,26]. The extra peaks that appear in the extracted ion chromatograms of tyrosol and elenolic acid correspond to the fragmentation of other compounds that elute later in the chromatogram.

Three different varieties of olive oil (Picual, Arbequina and Hojiblanca) were analyzed by using the optima conditions and the results are shown in Figure 4.

3.1.3 Analytical parameters and quantification of the compounds

The analytical method was then validated in terms of specificity, linearity and precision for the analysis of phenolic compounds in olive oil.

The specificity of the method was tested by screening analysis of blank (in terms of phenols) oil samples. There were no impurity peaks or contamination at the retention times corresponding to the analytes. In order to obtain the calibration curves, the analyte peak area was plotted versus the analyte concentration. Ten points of different concentrations level (n=10) were chosen for the different phenols standards and for each point the appropriate standard solution was injected three times. In general, wide linearity ranges were observed for each analyte with reasonable linearity and correlation coefficients (r^2) from 0.9670 to 0.9974. To test the sensitivity of the method, the mixture of the seven compounds were diluted several times and injected into the nanoLC system. The limits of detection (LODs) were determined as three times the signal to noise ratio (S/N) and were ranged between very low values: 0.7 and 0.9 ppb for the flavonoids, luteolin and apigenin respectively, and 30 ppb for Hydroxytyrosol. These LODs values are much lower than those described in literature for the same compounds by HPLC-MS. Regarding the repeatability, it was assayed out by three consecutive injections (n = 3) of the standard mixture of phenolic compounds in the same day (intra-day repeatability) and in four different days (inter-day repeatability) obtaining values of relative standard deviation (RSD%) on the peak area ratio above 4.3 and 15.6%, respectively. These results show fairly good intra-day repeatability but, as expected, worse inter-day repeatability that could be explained by the instability of the nanospray.

Table 3 shows the main quality parameters of the nanoLC-ESI-TOF MS method: linear range, calibration curve, correlation coefficients (r^2), limits of detection (LODs), limits of quantification (LOQs) and compares them with those obtained for HPLC-ESI-TOF MS. Both methods were compared taking into account the best chromatographic and

mass spectrometry conditions for each one. Better results were obtained using HPLC in terms of linearity and repeatability, especially inter-day repeatability. However, concerning the sensitivity, the nanoLC-ESI-TOF method provided a much higher S/N ratio for the compounds, and therefore, a better sensitivity.

The described method was applied to quantify the phenolic compounds under study in 3 different varieties of EVOO samples. The analyses were performed in triplicate and the concentration was determined using the area of each individual compound and making an interpolation in the corresponding calibration curve. Table 4 present the polyphenolic content found in commercial olive oils by nanoLC-ESI-TOF MS together with the results obtained with HPLC-ESI-TOF MS. The variability in the phenolic content among the studied varieties can be motivated by some environmental, genetic, geographical and agronomic factors; all those variables have been widely studied in literature.

Having a look at Table 4, we can say that, in general, the results obtained for the seven phenolic compounds quantified in terms of their standards in this study are in good agreement when we compare nanoLC-ESI-TOF MS and HPLC-ESI-TOF MS. Only for hydroxytyrosol and tyrosol - belonging to the family of simple phenols - the results achieved by both techniques were not statistically the same. This fact could be explained taking into account that simple phenols are the most hydrophilic compounds in the extracts from olive oil and they appear in the profile when aqueous proportion is more abundant in the mobile phase, resulting in poor spray stability. For the rest, nanoLC and HPLC quantitative results are statistically the same, except for the luteolin content in Arbequina olive oil.

From our point of view, it is pretty worth to highlight that as repeatability was higher in HPLC, the standard deviation of HPLC results was lower. Although nanoLC quantitative data were not excellent, we consider that it is very interesting the fact of evaluating that

technique and checking its performance and its capability to quantify accurately this kind of compounds. So far, in part due to the low reproducibility of the nanospray technology, nanoLC has been mainly used for qualitative analysis.

3.2 Comparison between NanoLC and HPLC results

With regards to the analytical parameters, nanoLC showed in general worse, although sufficient, resolution, efficiency and repeatability compared to HPLC. However, in terms of sensitivity, the LODs obtained with the nanoLC system used in this study are much lower that those reported previously by using HPLC methods. This high sensitivity could be explain because of the possibility to inject large volumes of samples using the on-line pre-column together with the reduction of the chromatographic dilution due to the use of small i.d. capillary columns [17].

The use of nanoLC can also offer other attractive advantages over classical HPLC. The use of small amounts of stationary phases made the columns cheaper (ten-folder lower) than a conventional C18 column and allows the use of expensive packing materials. In nanoLC, the use of pre-columns is highly recommended, since capillaries can be easily blocked at the inlet when real samples have to be analyzed. Besides, it allows samples to be both pre-concentrated and partially cleaned up. It should also be indicated, that after more than 500 injections of olive oil phenols, the column is still in perfect state for the analysis of this type of compounds.

Another important advantage of this miniaturized technique it is the use of relatively low flow rates (40-600 nL/min depending on the column). Mobile phases, especially acetonitrile, are quite expensive, and the small amount request for nanoLC makes this technique very attractive with lower cost and reduction of waste solvents. Similar results were obtained in both LC and nanoLC with 3000-fold reduction in reagent consumption.

The reduction of flow rate also allows a good coupling with MS transferring the entire effluent from the column into the MS instrument, whereas with HPLC we normally need a splitter. Both systems can be easily coupled to the mass spectrometer although the nanoLC coupling is most delicate and present more technical problems, requiring significant expertise, mainly because of the delicate plumbing and the use of fragile fused-silica ESI emitters. Besides, the mass spectrometer gets dirty earlier with nanoLC because the higher flows of solvents used in HPLC clean the surfaces of the equipment. Due to the low consumption of mobile and stationary phases, the nanoLC method seems to be cheaper than the traditional HPLC but, at the moment, because of the novelty of the technique, the instrumentation and packed column for some applications are still pretty expensive.

Other advantage of nanoLC over classical HPLC is the possibility to use low injected sample volumes (20–60 nL), fact which can be very useful for applications where sample availability is restricted, as for example in proteomic field. The analyst has to reach always a compromise between nanoliter injection and sensitivity determination. Another advantage that we can stand out is the better baseline noise, due to a reduced background, which is necessary to detect minor components.

Tabla 5 summarizes the advantages and disadvantages of the nanoLC methodologies versus HPLC.

4 Concluding remarks

In this work, a nanoLC-ESI-TOF MS method has been developed to demonstrate, for the first time, its application on the determination of phenolic compounds in olive oil. The use and analytical performance of nanoLC were compared with a HPLC method, and both of them were applied for the identification and quantification of different phenolic

compounds in olive oil. The most important analytical parameters of both methods (linearity, calibration range, detection and quantification limit, repeatability etc) were calculated to establish the comparison. The new nanoLC method provides comparable analysis time and offers better sensitivity with less consumption of mobile phases; however it presents worse inter-day repeatability and it can be a bit more difficult to operate by the analyst.

NanoLC-ESI-TOF MS showed the potential to become a very promising alternative, in particular, for studies where the determination of extremely low concentrations of analytes is required (biological samples, for instance). Further studies are already ongoing in our laboratory using nanoLC-ESI-TOF for the analysis of polyphenols in biological samples.

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Figure captions

Figure 1. Schematic figure of the nano-LC system, showing the moment when the sample is loading onto the pre column using pump A. Valve S is switched to positon 1-6 directing the flow through the loop carrying the sample onto the pre column. Valve W is set to position 1-6, because of the higher pressure drop on the analytical column the flow is directed to waste.

Figure 2. BPC (Base Peak Chromatogram) of an olive oil extract (variety Picual) using nanoLC-ESI- TOF MS (A) and comparison with the HPLC-ESI-TOF MS method (B). Chromatographic conditions are described in Materials and Methods.

Figure 3. Base peak chromatogram (BPC) achieved by nanoLC-ESI-TOF MS at the optima conditions for the variety Picual and extracted ions chromatogram (EICs) of the main phenolic compounds identified: **1**, Hydroxytyrosol; **2**, Tyrosol; **3**, Hydroxy elenolic acid; **4**, Elenolic acid; **5**, Hydroxy decarboxymethyl oleuropein aglycon; **6**, Decarboxymethyl oleuropein aglycon and hydroxy decarboxymethyl ligstroside aglycon; **7**, Syringaresinol; **8**, Luteolin; **9** Pinoresinol; **10**, Acetoxypinoresinol; **11**, Hydroxy oleuropein aglycon; **12**, Decarboxymethyl ligstroside aglycon; **13**, Apigenin; **14**, Methyl decarboxymethyl oleuropein aglycon, **15**, Oleuropein aglycon; **16**, Methyl oleuropein aglycon; **17**, Ligstroside aglycon.

Figure 4. Base peak chromatograms obtained by nanoLC-ESI-TOF at optima conditions for olive oils of three different varieties: a) Picual, b) Arbequina, c) Hojiblanca.

Table 1. Mass calibration matrix for internal calibration.

	Name	Formula $[M-H]^-$	Theoretical Mass	Charge
1	*Hydroxytyrosol*	$C_8H_9O_3$	153.05572	-1
2	*Tyrosol*	$C_8H_9O_2$	137.06080	-1
3	*Elenolic acid*	$C_{11}H_{13}O_6$	241.07176	-1
4	*Apigenin*	$C_{15}H_9O_5$	269.04555	-1
5	*Luteolin*	$C_{15}H_9O_6$	285.04046	-1
6	*Pinoresinol*	$C_{20}H_{21}O_6$	357.13436	-1
7	*Ligstroside aglycon*	$C_{19}H_{21}O_7$	361.12928	-1

Table 2. Phenolic compounds identified in an olive oil extract (Picual variety) by nanoLC-ESI-TOF including: mass/charge ratio, retention time, ISCID (Internal source collision induced dissociation) fragments, molecular formula, error (ppm) and Sigma value. Internal calibration was used by using 7 well-known phenolic compounds.

m/z	Retention time (min)	Fragments	Formula $[M-H]^-$	Error	Sigma	Identified Compounds
153.0559	11.3	123	$C_8H_9O_3$	-1.3	0.005	Hydroxytyrosol
137.0610	12.6		$C_8H_9O_2$	-1.6	0.029	Tyrosol
257.0660	15.3	181,137	$C_{11}H_{13}O_7$	2.7	0.007	Hydroxy elenolic acid
241.0712	16.2	139	$C_{11}H_{13}O_6$	2.5	0.014	Elenolic acid
335.1141	16.8	199	$C_{17}H_{19}O_7$	-1.5	0.014	Hydroxy decarboxymethyl oleuropein aglycon
319.1180	17.7	183	$C_{17}H_{19}O_6$	2.2	0.022	Decarboxymethyl oleuropein aglycon
417.1606	18.1		$C_{22}H_{25}O_8$	1.7	0.086	Syringaresinol
319.1185	18.6	199	$C_{17}H_{19}O_6$	0.7	0.003	Hydroxy decarboxymethyl ligstroside aglycon
285.0399	18.8		$C_{15}H_9O_6$	2.1	0.018	Luteolin
357.1348	18.9		$C_{20}H_{21}O_6$	-1.1	0.033	Pinoresinol
415.1404	19.2		$C_{22}H_{23}O_8$	1.5	0.026	Acetoxypinoresinol
393.1203	19.3		$C_{19}H_{21}O_9$	3.0	0.005	10-Hydroxy oleuropein aglycon
303.1244	19.6	183	$C_{17}H_{19}O_5$	-2.0	0.015	Decarboxymethyl ligstroside aglycon
269.0459	20.8		$C_{15}H_9O_5$	-1.2	0.079	Apigenin
333.1339	21.1		$C_{18}H_{21}O_6$	1.2	0.010	Methyl decarboxymethyl oleuropein aglycon
377.1256	21.5	345,307,275	$C_{19}H_{21}O_8$	-1.2	0.002	Oleuropein aglycon
391.1406	23.9	345,275	$C_{20}H_{23}O_8$	-2.0	0.016	Methyl oleuropein aglycon
361.1295	24.4	291,241	$C_{19}H_{21}O_7$	-0.5	0.012	Ligstroside aglycon

Table 3. Analytical parameters for the nanoLC and HPLC-ESI-TOF MS methods: relative standard deviation (RSD%), limit of detection (LOD) and quantitation (LOQ), linearity, calibration curves and r^2.

Analytes		RSD% Intra-day	RSD% Inter-day	LOD (ppb)	LOQ (ppb)	Linearity (ppm)	Calibration curves	r^2
Hyty	nanoLC	6.8	20.4	30	90	LOQ-4	y = 887868x - 31503	0.994
	HPLC	4.6	6.8	90	300	LOQ-50	y = 39934x + 42004	0.993
Ty	nanoLC	8.0	15.6	10	30	LOQ-2	y = 757872x + 4292	0.997
	HPLC	2.1	5.3	310	1030	LOQ-50	y = 12596x + 26635	0.991
EA	nanoLC	7.3	26.3	8	16	LOQ-4	y = 1057886x + 28193	0.982
	HPLC	3.4	7.5	1440	4800	LOQ-300	y = 6687.8x + 76261	0.991
Pin	nanoLC	8.1	16.2	1.2	3.6	LOQ-0.5	y = 1545997x + 25558	0.988
	HPLC	3.3	4.6	60	200	LOQ-50	y = 37578x + 53556	0.991
Lut	nanoLC	4.3	16.1	0.9	2.7	LOQ-1	y = 3854172x + 83079	0.980
	HPLC	2.8	5.8	20	60	LOQ-25	y = 114566x + 59826	0.994
Apig	nanoLC	9.8	18.2	0.7	2.1	LOQ-0.2	y = 6301191x - 28625	0.969
	HPLC	2.0	4.6	20	60	LOQ-25	y = 150131x + 118916	0.991
Lig Agl	nanoLC	9.5	22.0	2	6	LOQ-6	y = 314076x + 405488	0.964
	HPLC	3.0	6.7	430	1430	LOQ-300	y = 9018.9x + 59184	0.993

Hyty: Hydroxytyrosol; Ty: Tyrosol; EA: Elenolic acid; Pin: Pinoresinol; Lut: Luteolin; Apig: Apigenin; Lig Agl: Ligstroside aglycon.

In mass spectrometry detection limit was calculated considering S/N=3

Table 4. Quantitative results (mg/kg) achieved by HPLC and nanoLC-ESI-TOF MS for the three varieties of olive oil (Picual, Arbequina and Hojiblanca) included in the study.

Compounds	*PICUAL*		*ARBEQUINA*		*HOJIBLANCA*	
	nanoLC	**HPLC**	**nanoLC**	**HPLC**	**nanoLC**	**HPLC**
Hyty	30.15 ± 2.34 (b)	20.20 ± 0.77 (a)	6.15 ± 0.28 (b)	3.37 ± 0.11 (a)	21.93 ± 1.39 (b)	9.76 ± 0.34 (a)
Ty	15.19 ± 0.75 (a)	11.84 ± 0.56 (a)	4.65 ± 0.21 (b)	2.33 ± 0.10 (a)	13.61 ± 0.97 (b)	6.56 ± 0.17 (a)
EA	54.76 ± 2.54 (a)	58.18 ± 1.46 (a)	14.93 ± 1.86 (a)	10.47 ± 0.84 (a)	37.85 ± 2.33 (a)	33.00 ± 0.98 (a)
Lut	1.64 ± 0.12 (a)	1.84 ± 0.06 (a)	3.72 ± 0.23 (b)	4.41 ± 0.12 (a)	2.95 ± 0.28 (a)	3.29 ± 0.10 (a)
Pin	0.76 ± 0.10 (a)	0.77 ± 0.03 (a)	1.90 ± 0.45 (a)	2.24 ± 0.21 (a)	2.13 ± 0.31 (a)	1.86 ± 0.09 (a)
Apig	0.32 ± 0.06 (a)	0.43 ± 0.04 (a)	0.96 ± 0.23 (a)	1.22 ± 0.08 (a)	0.74 ± 0.06 (a)	0.99 ± 0.07 (a)
Lig Agl	65.5± 3.17 (a)	64.78 ± 1.19 (a)	20.09 ± 1.37 (a)	17.38 ± 0.32 (a)	34.77 ± 3.20 (a)	38.23 ± 0.71 (a)

Hyty: Hydroxytyrosol; Ty: Tyrosol; EA: Elenolic acid; Pin: Pinoresinol; Lut: Luteolin; Apig: Apigenin; Lig Agl: Ligstroside aglycon.

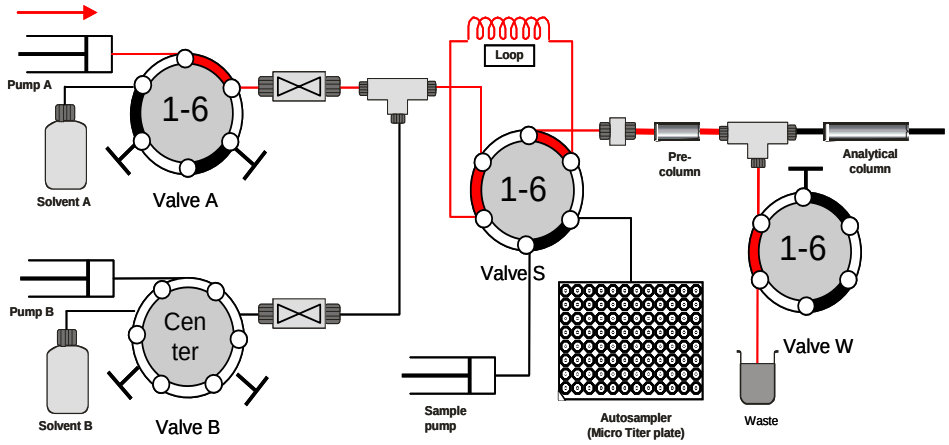
Values are given as Mean ± Standard deviation.

Means achieved by nanoLC-MS and HPLC-MS for the three varieties under study were compared. We indicated with different letters when means are significantly different ($p \leq 0.05$).

Table 5. Advantages and drawbacks of nanoLC (versus HPLC).

	nanoLC
Advantages	▪ Excellent sensitivity, very low LODs (ppb) when techniques to load large sample volumes are used ▪ Small amounts of stationary phase make, in most occasions, the columns cheaper ▪ Low flow rates (40-600 nL/min): lower cost and reduction of waste solvents ▪ Good coupling with MS ▪ Low sample consumption (20-60 nL) ▪ Better baseline noise due to the reduced background ▪ Very promising alternative for biological samples
Disadvantages	▪ Good intra-day repeatability but low inter-day repeatability ▪ Worse linearity ▪ Columns more easily blocked with real samples ▪ More technical problems because of the delicate plumbing and the use of fragile fused-silica ES emitters ▪ Mass spectrometry gets dirty early because of the use of lower flow rate.

Figure 1.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Valve S
Pre-
column
Analytical
column
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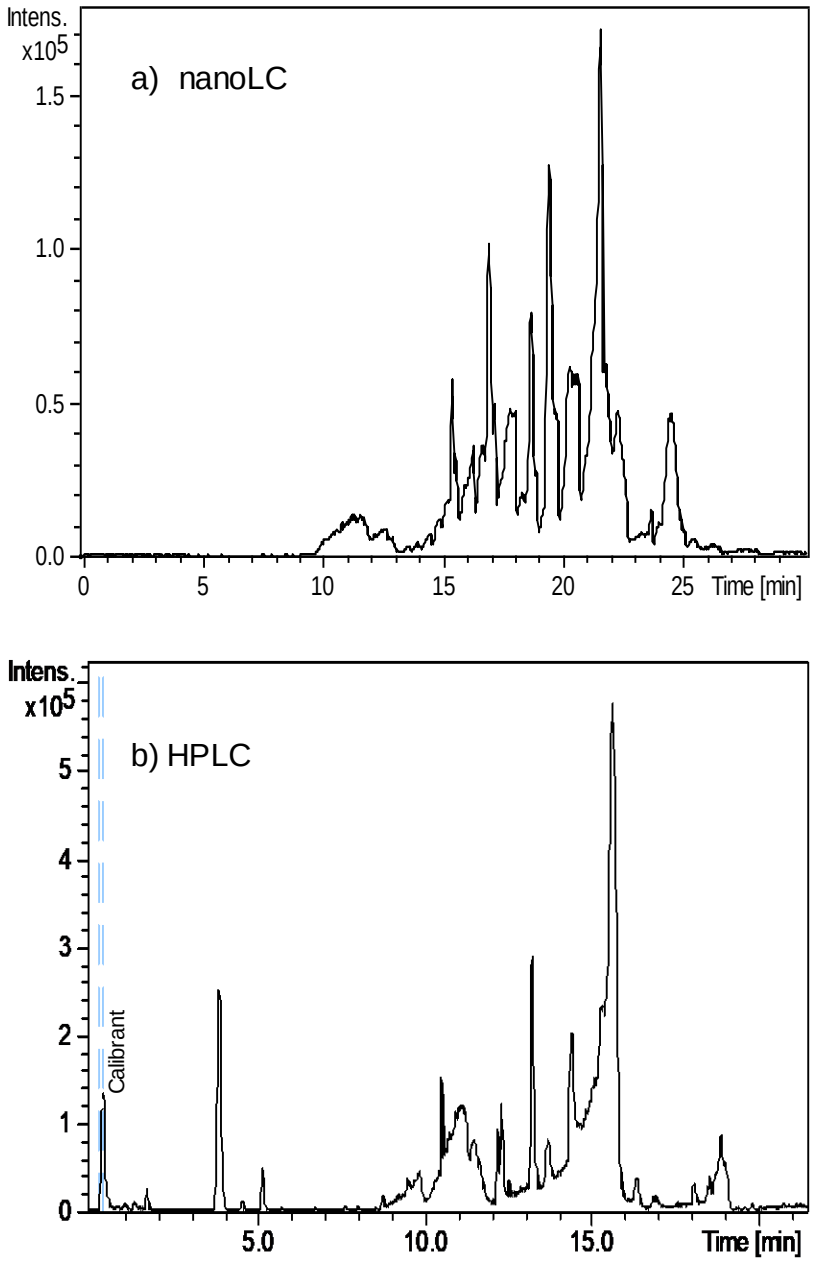
Cen
ter
Solvent B
Valve B
Sample
pump
Autosampler
(Micro Titer plate)
Waste

Figure 2.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15
20
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Intens.
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Time [min]

Figure 3.

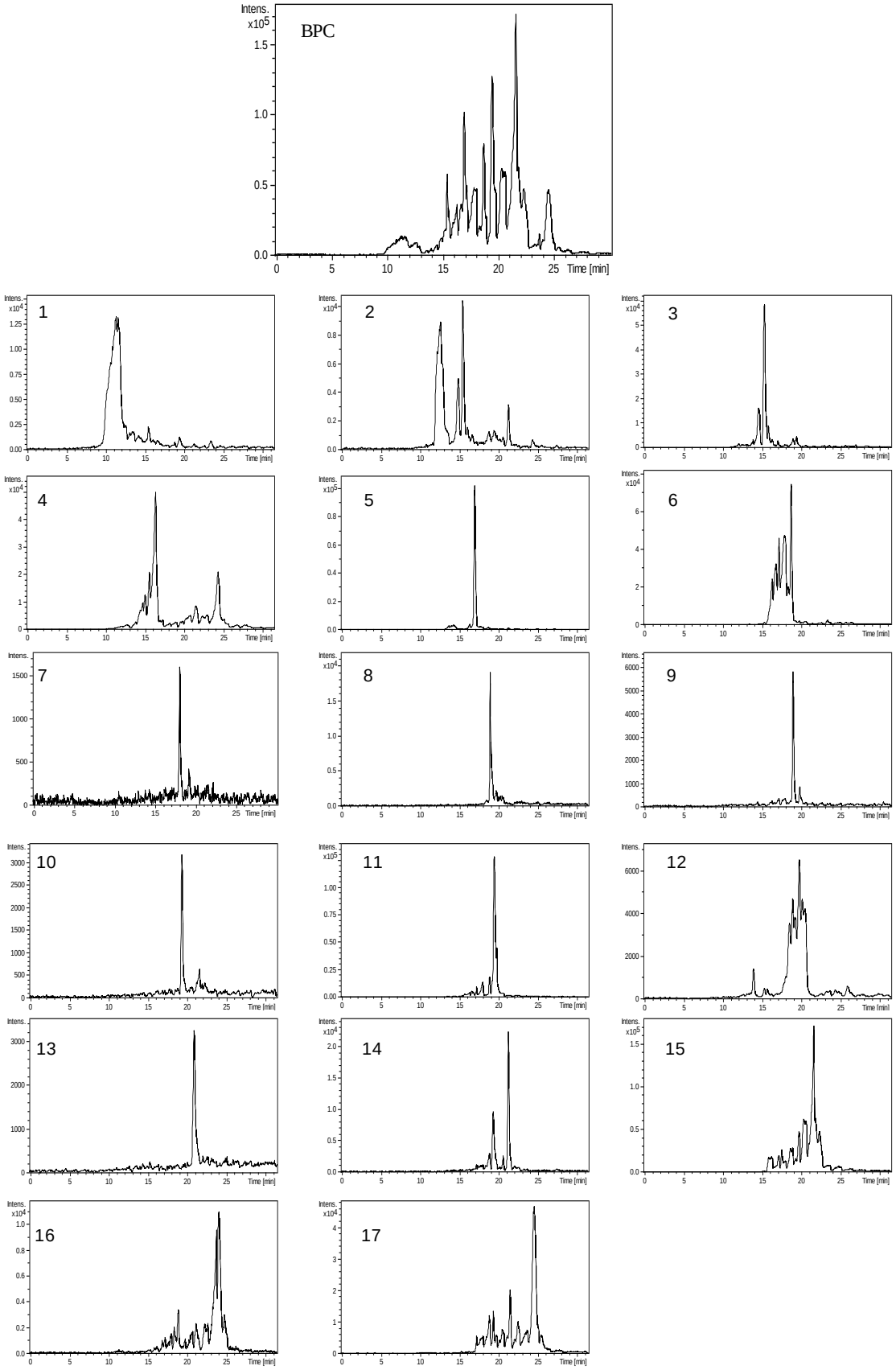
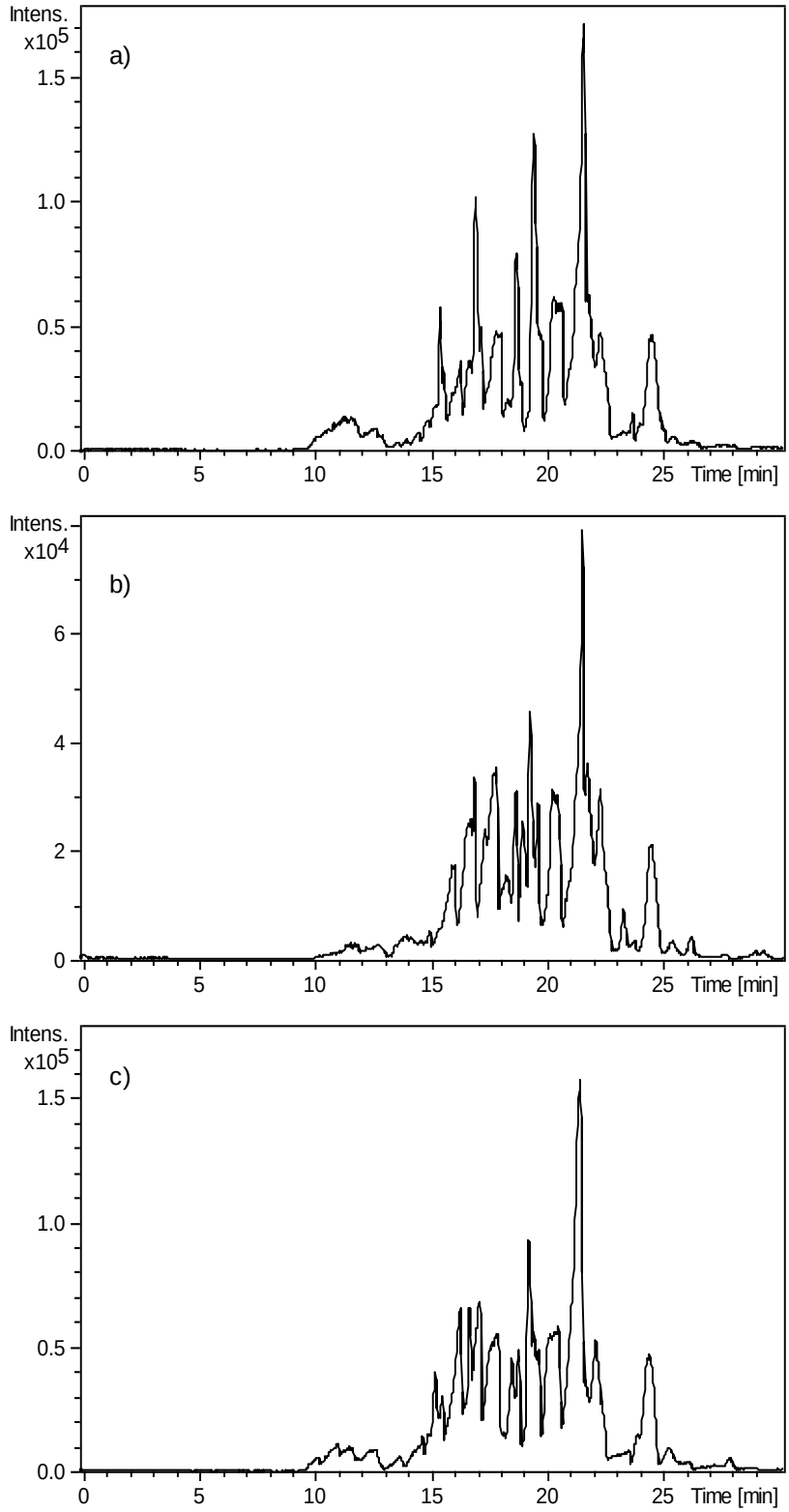


Figure 4.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x10^4
b)
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25
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x10^5
c)
1.5
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