The Flexible Microtube Plasma as Post-Ionization Source for Cholesterol in nano-Electrospray Mass Spectrometry

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Abstract: Cholesterol serves as a biomarker in clinical- and life-sciences. The determination of abnormal levels can indicate several types of human diseases. However, the low polarity of free cholesterol makes it hardly accessible by (nano) electrospray ionization mass spectrometry (nESI-MS). As novel approach, the flexible microtube plasma (FµTP) for post-ionization allows the determination of low-polar compounds like cholesterol in combination with nESI-MS. Focusing on the analytical performance, the activated post-ionization leads to an increased cholesterol signal by a factor of 22. The repeatability and long-term stability could be successful evaluated by using a complex liver extract. Via the method of standard addition, a linear dynamic range of 1.7 orders of magnitude, a minimum detectability of 3.71 mg/L and a high accuracy (deviation: −8.11 %) is demonstrated proofing the FµTP-nESI-MS as an excellent approach for a derivatization-free determination of cholesterol without the necessity of high-resolution Orbitrap devices or enhanced MS acquisition-methods.

Introduction

The development of the electrospray ionization (ESI) is a milestone in mass spectrometry (MS) history and provides new opportunities in the analysis of large biomolecules. Nowadays, ESI is widely available and has been integrated as an important ionization technique for liquid samples in MS with biological and medical background. Nevertheless, the ionization efficiency decreases with decreasing polarity of the target molecules. Low-polar and non-polar compounds are not accessible using electrospray ionization. In the past decades, plasma based ionization techniques like the Low-Temperature Plasma (LTP), Direct Analysis in Real Time (DART) or Dielectric Barrier Discharge Ionization (DBDI) were developed and became suitable ionization sources for a wide range of applications. In contrast to ESI, plasma based ionization techniques enable the ionization of non- and low-polar molecules like phenanthrene, anthracene or cholesterol if the analyte was converted into the gas phase.

Cholesterol is an essential sterol lipid, which plays a crucial role in the human body and covers a wide range of different intra- and extracellular functions. It serves as precursor of different bioactive molecules like Vitamin D, bile acid, estradiol and testosterone.

14 It is a key component of cell membranes, regulates the fluidity of the membrane and participates in transmembrane signaling processes. In addition, cholesterol plays an essential role in the transport processes of hydrophobic molecules in hydrophilic media via lipoproteins. Classified in different lipoproteins like low-density lipoproteins (LDL) and high-density lipoproteins (HDL), several diseases including non-insulin-dependent diabetes mellitus, Smith-Lemli-Opitz syndrome, brain diseases and prostate cancer correlate with atypical cholesterol, LDL and HDL levels. In addition, Gofman et al. found a correlation between high LDL and low HDL levels and cardiovascular diseases.

The antiatherogenic property of HDL is directly related to the cholesterol reverse transport, which includes the transport process of free and esterified cholesterol from peripheral tissue to liver tissue. In this process the esterification of free cholesterol to cholesteryl ester (CE) is an important step. Therefore a challenging task in clinical- and life-science is the analytical determination of esterified cholesterol as well as free cholesterol levels within a complex sample. In this context, Lüthjohann et al. shows in 2019 very high inter-laboratory variations in the determination of cholesterol concentrations. A range of several analyzing methods to determine cholesterol levels exist, but MS methods are more accurate compared to classical approaches. For the analysis of liquid, biological and complex samples, electrospray is the ionization technique of choice. By adding ammonium acetate to the sample, Cholesteryl esters (CE) form ammonium adducts, which are efficiently ionized by electrospray. In contrast, the ionization of free cholesterol is challenging due to its very low-polar nature. In 2006 Liebisch et al. published a derivatization method to detect free cholesterol in form of cholesterol acetate. A disadvantage of this technique is the occurring non-specificity between the fragment ion of cholesterol esters and free cholesterol as depicted in Fig. 1.

Furthermore, chemical derivatization steps in the sample preparation are necessary. Furthermore, in 2018 Gallego et al. published a comparison of three MS techniques on a highly modern Orbitrap Fusion™ Tribrid™ mass spectrometer to determine cholesterol concentrations. In this study, the best results are given by the MSX (multiplexed MS/MS) technique followed by PRM (parallel reaction monitoring), and high-resolution FTMS (Fourier Transform MS) in full-scan acquisition.

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Figure 1. Mechanism of a) cholesterol derivatization and fragmentation according to Liebisch et al. and b) cholesteryl ester fragmentation. Both mechanisms lead to a cholestadiene ion signal at the same m/z 369.35.25,26

However, these techniques require high-resolution Orbitrap-enhanced Orbitrap Tribrid™ mass spectrometers, or use the pathway of fragmentation technologies as insource fragmentation, CID (collision induced dissociation) or HCD (higher-energy collisional dissociation).28,29 In contrast to these techniques, a very different approach is to solve the described challenge in the region of the ionization source by increasing the ionization efficiency of the electrospray in relation to cholesterol. Therefore, a modification of the nESI source is necessary. This work demonstrates the combination of a nano-electrospray ionization (nESI) and the flexible microtube plasma (FµTP). In this sense, the FµTP serves as additional post-ionization source to increase the ionization yield for compounds like cholesterol, that are not efficiently ionized by nESI.

Results and Discussion

The present publication focuses on the determination of the analytical performance of the combination of nESI and FµTP by investigating cholesterol in a complex liver sample. The here shown FµTP post-ionization process for nESI-MS is electrically switchable. Therefore, the FµTP-nESI-MS facilitates two operation modes by the use of only one combined ionization source. If the plasma is deactivated, the nESI generates typical electrospray mass spectra of polar compounds. If the plasma is activated, the nESI serves mainly as miniaturized nebulizer and the plasma ionization process dominates, resulting in significantly changed mass spectra.

Application to Liver Extract. Due to the additional plasma source, the distance between the nESI emitter of the nESI-FµTP setup and MS inlet is increased compared to a classical nESI. Therefore, a classical nESI measurement in optimized geometry was used as reference.

The nESI reference measurement was tuned to the m/z 369.42 signal (LTQ analyzer) to yield the highest signal-to-noise ratio. Here, a tube lens voltage of 135 V and an inlet capillary voltage of 80 V was carried out. The signal-to-noise ratios (SNR) are determined by dividing the absolute signal intensity through the background noise. Latter is determined by the 0.5 quantile (median) of the related mass spectrum. The liver sample was prepared as described in the methods and analyzed by each of the three modes.

Fig. 2a shows the nESI-reference spectrum (green) which is an average of 200 mass spectra, collected with a microscan value of three. In the m/z-range 720-850 different protonated species of polar lipids like phosphatidylcholines (PC) and phosphatidylethanolamines (PE) dominate the spectrum. The most abundant signal is represented by m/z 786.58, which is used for ongoing interpretation and calculation. A signal, which is generated by the protonation and dehydration of cholesterol is detectable, but low abundant in the nESI-reference.30 This cholestadiene signal at m/z 369.42 reaches a relative signal abundance of 0.9 %, which corresponds to a SNR of 7.6. A signal of the cholesterol ammonium adduct at m/z 404.35 is not detectable.

In contrast, Fig. 2 b shows an average of 100 mass spectra (single microscan) of the nESI-FµTP setup with deactivated plasma (nESI-mode). Here, a tube lens voltage of 65 V and an inlet capillary voltage of 15 V was used to minimize cholesterol ester interferences. The relative abundance of the m/z 786.58 is slightly decreased compared to the nESI-reference measurement. This fact is based primarily on the increased distance of the nESI emitter in the nESI-FµTP setup compared to the nESI-reference setup. Again, the cholestadiene signal at m/z 369.42 is missing or very low abundant, respectively. Consequently, a SNR of 1.7 is reached showing that the m/z 369.42 nearly disappears in the background noise.

With additional activated plasma (nESI-FµTP-mode) the mass spectrum changes significantly as shown in Fig. 2c. Here, the dominating peak in the mass spectrum is the cholestadiene signal at m/z 369.42. The SNR is calculated to 368.1, which highlights the efficiency of the excitation by the plasma post-ionization source.
Figure 2. Mass spectra of the liver extract by using the LTQ analyzer in positive ion detection as an average of 200 mass spectra (a) and 100 mass spectra (b,c) with a microscan value of three (a) and one (b,c). a) classical nESI measurement as reference, averaged out over 200 mass spectra with three microscans. b) nESI-mode of the nESI-FµTP. Instead of cholestadiene \([M+H-H_2O]^+\) at \(m/z\) 369.42 only highly intensive signals of protonated polar lipid species can be detected. The most abundant peak is the \(m/z\) 786.58 signal. c) nESI-FµTP-mode. Significantly increased signal of the cholestadiene species. Intensities in spectra a) – c) are scaled to the most abundant signal, marked with an asterisk. d) Comparative view of nESI-mode (b) and the nESI-FµTP-mode (c). Here, both spectra are normalized to the most abundant signal in nESI-mode (*).

Compared to the nESI-mode, the signals of the polar lipids in the \(m/z\)-range 720-850 disappear or are very low abundant. This could be explained by slight electrical field disturbances in between both ion sources. As a consequence a specific amount of ions produced by the nESI is lost. By help of an overlay of both mass spectra, the absolute intensities of the nESI- and nESI-FµTP-mode can be compared shown in Fig. 2d. In this case, all signal intensities are normalized to the most abundant signal of the polar lipids (\(m/z\) 786.58) of the nESI-mode. The cholestadiene ion at \(m/z\) 369.42 shows a significantly increased intensity and reaches a relative signal abundance of 39 %. This high abundancy is based on the high amount of cholesterol (7 % wt/wt) inside the analyzed liver sample. In Tab. 1 the weight fraction of all included components of the liver extract is given.

Figure 3. Ion current signals of a) total ion current (TIC), b) extracted ion current (XIC) of the lipid signal at \(m/z\) 786.58, and c) cholestadiene signal at \(m/z\) 369.42. Signals are depicted as a function of time and are evaluated with the LTQ analyzer set to three microscans per acquisition. Five switching processes between the nESI-mode (white area) and the nESI-FµTP-mode (grey area) demonstrate the high repeatability and stability of this technique. With including post-ionization (nESI-FµTP-mode) signals of the TIC and XIC of the polar lipid decrease by -76(4) % and -100(7) % whereas the XIC of the cholestadiene increase significantly by +8776(2059) %.

Table 1. Composition of the total liver extract of a bovine according to Avanti lipids.31

<table>
<thead>
<tr>
<th>Component</th>
<th>Fraction (wt/wt) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylycholine*</td>
<td>42</td>
</tr>
<tr>
<td>Phosphatidylethanolamine*</td>
<td>22</td>
</tr>
<tr>
<td>Phosphatidylinositol*</td>
<td>8</td>
</tr>
<tr>
<td>LysoPhosphatidylinositol**</td>
<td>1</td>
</tr>
<tr>
<td>Cholesterol**</td>
<td>7</td>
</tr>
<tr>
<td>Neutral Lipids / Other*</td>
<td>20</td>
</tr>
</tbody>
</table>

*class or group of compounds
**single compounds
With activated plasma post-ionization (nESI-FµTP-mode) the TIC decreases by -76(4) % as shown in Fig. 3a. This is based on the suppression of the most abundant protonated polar lipid signal at m/z 786.58 by -100(7) % as shown in Fig. 3b. The cholestadiene signal, generated by cholesterol is only detectable in the nESI-FµTP-mode, which is marked by the grey areas. Here, the cholestadiene signal increases by 8776(2059) % respectively a factor of 88 and demonstrate that this is induced solely by the post-ionization mechanism of the FµTP as shown in Fig. 3c. The high dynamic range of the cholestadiene signal intensity at m/z 369.42 evaluates the fast switching capability and the high repeatability of the here shown approach. However, the XIC of m/z 369.42 (cholestadien) in nESI-FµTP-mode shows a higher temporal signal fluctuation compared to the XIC of m/z 786.58 (most abundant polar lipid) in the nESI-mode.

To express the stability and repeatability of the signals referring to Fig. 3, Tab. 2 shows the relative standard deviation (RSD) and the relative error of the repeatability (EREP) of the TIC and both XICs (m/z 786.58, m/z 369.42).

Table 2. Signal quality, stability and repeatability of the total ion current (TIC) and extracted ion current (XIC) of the most abundant polar lipid (m/z 786.58) and cholestadiene (m/z 369.42). The signal quality is given by the signal-to-noise ratio (SNR) and the stability by the relative standard deviation (RSD). The error of repeatability (EREP) shows the precision of the switching process. The calculation of all values are described in the text. Due to the missing mode-switching capability of the commercial nESI reference, an EREP is not available (n.a.).

<table>
<thead>
<tr>
<th>Operation-Mode</th>
<th>Ion current</th>
<th>SNR</th>
<th>RSD [%]</th>
<th>EREP [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>nESI reference</td>
<td>TIC</td>
<td>4.6</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>nESI</td>
<td>TIC</td>
<td>2.2</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>nESI-FµTP</td>
<td>TIC</td>
<td>8.1</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>nESI reference</td>
<td>XIC (369.42)</td>
<td>7.6</td>
<td>60.2</td>
<td>n.a.</td>
</tr>
<tr>
<td>nESI</td>
<td>XIC (369.42)</td>
<td>*1.7</td>
<td>*16.4</td>
<td>*21.1</td>
</tr>
<tr>
<td>nESI-FµTP</td>
<td>XIC (369.42)</td>
<td>368.1</td>
<td>16.4</td>
<td>13.3</td>
</tr>
<tr>
<td>nESI reference</td>
<td>XIC (786.58)</td>
<td>831.6</td>
<td>6.4</td>
<td>n.a.</td>
</tr>
<tr>
<td>nESI</td>
<td>XIC (786.58)</td>
<td>408.2</td>
<td>4.7</td>
<td>3.4</td>
</tr>
<tr>
<td>nESI-FµTP</td>
<td>XIC (786.58)</td>
<td>*0.04</td>
<td>*198.6</td>
<td>*19.7</td>
</tr>
</tbody>
</table>

*A low signal intensity within the range of the noise induce high a RSD, EREP and low SNR value.

In order to calculate the RSD and EREP values, the acquisition is set to three microscans and 50 scans are performed. For the RSD the best value out of five mode-switching processes is taken into account. For the EREP the deviation of the switching processes in relation to the average of all five switching processes is calculated. As already visualized in Fig. 3, the grey marked areas of Tab. 2 represent the post-ionization (nESI-FµTP-mode) whereas the white marked areas represent the nESI-mode as well as the nESI-reference. Comparing the TIC RSD of the nESI-FµTP-mode (8.1 %) to the TIC RSD of the nESI-mode (2.2 %) an increase by a factor of 3.7 can be observed. The reason for this phenomenon might originate from the additional electrical field disturbances, which are generated by the active plasma post-ionization. The TIC EREP of the switching process is 4.6 % for the nESI-FµTP-mode and not that worse compared to the nESI-mode (3.6 %).

Without plasma post-ionization (nESI-mode) a low XIC RSD of only 4.7 % and an EREP of 3.4 % can be observed for the most abundant lipid signal at m/z 786.58. By using the nESI-FµTP mode an increased cholestadiene intensity of +8776(2059) % can be shown. Compared with the nESI reference of Fig. 2, an increase of 2169(509)% respectively a factor of 22 can be found. Besides, a repeatability or a method precision in respective with a relative error below 15-20% argue for a suitable chromatographic analytical method. Even if the nESI-FµTP mode shows an inferior RSD (16.4 %) and EREP (3.3 %) (in relation to m/z 369.42) value compared to those of the nESI-mode (in relation to m/z 786.58), the values are acceptable for an analytical method as stated before. In addition, the RSD (in relation to m/z 369.42) of the nESI-FµTP setup (16.4 %) is 3.7 times better compared to the nESI-reference (60.2 %).

A long-term observation of the XIC (in relation to the cholestadiene at m/z 369.42) over 30 min was performed to evaluate a possible long-term signal drifting. Referring to this data, the slope of a linear fit shows no significant deviation to the constant average as shown in Fig. S1 of the supporting information. Furthermore, a residual analysis of the long-term observation shows a Gaussian distribution of the signal intensity (see Fig. S2). Both indicates an expected distribution and long-term stability over a period of 30 minutes without any significant signal intensity drifting (<0.061 %/min).

**Cholesterol ester interferences.** Cholesterol via a dehydration process as well as cholesteryl esters via a fragmentation process at the ester position produce the same ion species at m/z 369.35 (measured at m/z 369.42 with the LTQ analyzer). To evaluate the influence of the post-ionization process, cholesteryl ester CE(17:0) was analyzed with this setup in nESI-mode and in nESI-FµTP-mode as well. The mass spectra for both modes are depicted in Fig. 4. Here, the signal of the CE(17:0) ammonium adduct [M+NH₄]⁺ at m/z 656.08 is shown on the right and the corresponding fragment ion at m/z 369.42 on the left.

With activated post-ionization (nESI-FµTP-mode) the [M+NH₄]⁺ signal of CE(17:0) at m/z 656.08 increases by a factor of 1.52, whereas the CE fragment signal at m/z 369.42 increases by a factor of 1.59. As both signals are increased by nearly the same factor, the ratio between the CE fragment and CE ammonium adduct signal does no change by switching the operational modes. Therefore no significant additional fragmentation of CE(17:0) by using the post-ionization can be observed. This shows that the signal increase at m/z 369.42 during the total liver extract measurement is not influenced by a possible fragmentation of cholesteryl esters and is solely induced by free cholesterol.
Interferences of cholesterol esters. To reduce the tube lens and capillary voltage and minimize insource fragmentation and thus the cholesterol signal, it is possible to reduce the tube lens and capillary voltage and minimize insource fragmentation and thus the interferences produced by the nESI are not removable by the nESI-FµTP setup, but due to the very high SNR of the free cholesterol signal, it is possible to reduce the tube lens and capillary voltage and minimize insource fragmentation and thus the interferences of cholesterol esters.

**Voltage Variation.** In preliminary experiments, a high dependency of signal intensity on the plasma voltage amplitude could be observed. To determine the optimum voltage setting for the cholestadiene species a voltage variation between 1.6 and 2.4 kV was evaluated. In Fig. 5 the averaged signal intensity of 20 spectra acquired with the Orbitrap analyzer in dependency of the applied FµTP voltage amplitude is shown. Four specific post-ionization states of the nESI-FµTP mode are highlighted in Fig. 5 (top, a-d and shown as experimental top view (bottom, a-d).

In the nESI-FµTP mode, at voltage amplitudes below 1.85 kV (Fig. 5, post-ionization state a) no significant cholesterol signal can be observed and is therefore comparable with the nESI-mode. Here, the plasma is ignited, but the plasma front does not exceed the front tip position of the nESI nor reach the end of the FµTP capillary. With increased FµTP voltage amplitudes, the plasma front spatially exceeds the front position of the nESI towards the inlet of the MS device (Fig. 5, post-ionization state b). A significant increased cholesterol signal at m/z 369.42 can be detected. At optimal voltage amplitudes (2.00-2.10 kV) a maximum signal intensity is reached whereas the plasma front surpasses the FµTP capillary end and slightly crosses the nESI spray trajectory (Fig. 5, post-ionization state c). At voltage amplitudes ≥2.20 kV a decreased signal intensity occurs as the plasma touches the inlet surface of the MS device (Fig. 5, post-ionization state d). Nevertheless, no electric circuit shortcuts from the plasma to the surface of the MS device (Fig. 5, post-ionization state d).

**Performance via Method of Standard Addition.** For testing the performance of the nESI-FµTP setup the accuracy capability and the linear dynamic range (LDR) are determined by two concentration series. By utilizing the method of standard addition, matrix effects can be considered and demonstrate the nESI-FµTP setup in real life application. Explicit dilution steps, added volumes and resulting cholesterol concentrations of the calibration series 1 are summarized in Tab. S1 of the supporting information. By using the LTQ analyzer each data point contains 250 scans acquired in fullscan-acquisition (m/z-range 365-375) with a single microscan. For interpretation, the XIC cholestadiene species (m/z 369.42) with a mass tolerance of 200 mmu was used. To determine the LDR, calibration series 1 is measured over a wider range of concentration and plotted in a double logarithmic scale as shown in Fig. 6. Here, the slope (b) of the linear fit (y = log a + x) is used to estimate the LDR. The optimum voltage amplitudes (Fig. 5, top, grey marked area) are used for the following characterization of the analytical performance.
The LDR expressed in dB is calculated by using the lower and the upper limit of linearity. Both are determined by the interception of the confidence interval hyperbolas (blue) and a two data point connection line (dotted). The coefficient of determination \( R^2 = 0.9945 \) of the regression depicted in Fig. 6, indicates a high correlation between acquired data and the linear fit. The slope of 0.9901 is very close to the expected value of one for an ideal linearity. Therefore, the nESI-FµTP setup shows a high linearity over the concentration range of 4.37-163.95 mg/L. The lower limit can be determined to 3.71 mg/L and represents the minimum detectability (MD). With the upper limit of 189.37 mg/L, the calculated LDR is 17.1 dB or 1.7 orders of magnitude (OOM), respectively. This represent a high performance of the nESI-FµTP setup by solely using a simplified direct infusion.

The MSX technique of Gallego et al. as gold-standard reaches a LDR of ~2.7 OOM, but requires a highly modern robotic TriVersa NanoMate® system combined with a state-of-the-art and high-end Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. Compared to that, the here shown nESI-FµTP technique indeed underlie negligibly, but an expensive high-resolution mass spectrometer nor the use of enhanced MS acquisition-methods are not necessary. Furthermore, a better LOD should be achievable by using a chromatographic separation technique instead of the direct infusion by a syringe.

In Fig. 6 the intensity of the highest added concentration of 217.51 mg/L shows an underperforming intensity, which cannot be explained by a saturation or an exceeded filling of the ion trap. The reason for this might be droplet forming at the tip of the electrospray emitter, which can be observed at frequencies of 0.33-0.5 Hz. A periodical ejection of droplets towards the FµTP and the MS inlet indicates a not stable ion generation. The result is an underperforming intensity averaged over time. To show this limitation, in Fig. S3 of the supporting information a typical XIC of cholestadiene injected in low concentrations (Fig. S3a) is compared to those of a high concentration injection (Fig. S3b).

For the determination of accuracy, the cholesterol response is used and compared to the distributor’s literature value of the liver extract. To determine the cholesterol concentration in the liver sample a second calibration line using the method of standard addition is analyzed. Explicit dilution steps, added amounts of substance and resulting cholesterol concentrations of the calibration series 2 are summarized in Tab. S2 of the supporting information. In Fig. 7 the acquired data, the calculated linear regression and the 95 % confidence interval hyperbolas are plotted in a linear scale.
Here, an unknown concentration of analyte is given by the interpolation of the linear regression line fitted for positive concentrations (first quadrant, Q I) and the abscissa (second quadrant, Q II). The target concentration at 8.75 mg/L in Q II is marked with a crossed cyclic symbol. The acquisition is performed with the LTQ analyzer. For interpretation, the XIC of cholestadiene signal (m/z 369.42) with a mass tolerance of 200 mmu was used. To yield a high coefficient of determination (R²) of the linear regression and the highest method accuracy, only four out of five acquired concentrations are taken into account as shown in Tab. S2 of the supporting information (marked with asterisks).

The coefficient of determination (R²=0.9994) of the regression in Fig. 7 indicates a very feasible linear fit. An experimental determined concentration value of 8.04(19) mg/L can be calculated. With a cholesterol fraction of 7 % (wt/wt) inside the Avanti liver extract stock solution as already shown in Tab. 1, the literature concentration level can be calculated to 1.75 mg/ml. By diluting this stock solution (1:200) for the method of standard addition by the target concentration is 8.75 mg/ml (pipetting error not included). Comparing the literature value and the experimental result shows a deviation of only - 8.11 %. All values and parameters for the determination of the accuracy are summarized in Tab. 3.

### Table 3. Results of the determination of the accuracy by using the method of standard addition. The table shows the calculated coefficient of determination (R²) of the linear regression, the experimental evaluated concentration, the target concentration value according to the distributor’s and the relative deviation from the literature value in percent.

<table>
<thead>
<tr>
<th>Coefficient of determination (R²)</th>
<th>Experimental concentration [mg/L]</th>
<th>Literature/Target concentration [mg/L]</th>
<th>Deviation to literature [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9994</td>
<td>8.04(19)</td>
<td>8.75</td>
<td>- 8.11</td>
</tr>
</tbody>
</table>

An error of the distributor’s literature value is not available. In consideration of the immense diversity of compounds inside the very complex liver sample and the omitted separation technique, the deviation of - 8.11 % is an absolute acceptable value. An increased accuracy should be achievable by using a chromatographic separation technique instead of the direct infusion by a syringe.

### Conclusion

The flexible microtube plasma (FµTP) has been demonstrated as post-ionization source for nano-electrospray (nESI) mass spectrometry. To show the excellent performance, a challenging complex liquid liver extract containing cholesterol was used in a direct infusion experiment. The post-ionization process is quickly, electrically switchable thus enabling two operation modes of the nESI-FµTP setup. Firstly, the nESI-mode produces a typical electrospray mass spectrum. Secondly, by activating the plasma post-ionization (nESI-FµTP-mode) the resulting mass spectrum changes significantly. Signals of polar lipids in the m/z-range of 720-850 decrease, but an intense signal increase of the cholestadiene ion [M+H-H2O]⁺, which is generated by protonation and dehydration of cholesterol, is observable. Facilitated by the nESI-FµTP-mode, the cholesterol signal increases by a factor of 88 within the here shown nESI-FµTP setup and reaches a relative intensity of 39 % compared to the most intensive signals in the nESI-mode. Compared with the commercial nESI setup in optimized geometry as reference, an increase by a factor of 22 can be seen.

A high repeatability for the electrical switching process could be shown as well. The relative error of repeatability concerning the total ion current (TIC) is 4.6 % and 13.3 % for the extracted ion current (XIC) of cholestadiene in respective. The relative standard deviation concerning the TIC over time increases from 2.2 % (nESI-mode) to 8.1 % in the nESI-FµTP-mode but still shows an excellent stability. Additionally, a long-term measurement indicates no significant drifting of signal intensities (0.061 %/min) demonstrating the great possibility of this setup to be used in combination with a high-performance liquid chromatography.

Furthermore, via the method of standard addition, a high linearity of 0.9901, a linear dynamic range (LDR) of 1.7 orders of magnitude, a minimum detectability of 3.71 mg/L and a highly accurate concentration determination with a deviation of 8.11 % could be demonstrated.

Indeed, already produced interferences of cholesteryl ester fragments to the cholestadiene signal of cholesterol during the electrospray process (nESI) are not removable. However, experimentally it could be shown that the cholestadiene ions are not excessively produced by switching from the nESI to the nESI-FµTP-mode. Therefore, signals of the cholestadiene are solely generated by free cholesterol.

Additionally, due to the high signal-to-noise ratio of the free cholesterol signal in the nESI-FµTP-mode, it is possible to reduce the tube lens and capillary voltage to soften the ion transfer conditions inside the MS. By this, the insource fragmentation is reduced and so the interferences of cholesterol esters and free cholesterol.

In summary, it can be stated that the FµTP used as post-ionization source for nano-electrospray ionization mass spectrometry (nESI-MS) provides an excellent novel approach for the direct determination of cholesterol inside a complex liquid sample. The here shown combination of nESI and FµTP is a very practicable, fast and efficient approach. Moreover, there is no explicit requirement of a high-resolution mass spectrometer (FTMS), a derivatization technique or enhanced mass spectrometric acquisition-methods (PRM, SRM/MRM, MSX). As there is logical communication with the mass spectrometer, the nESI-FµTP setup is even not limited to the stated hybrid mass spectrometer. With comparable interfaces, the nESI-FµTP setup will work for Triple- and Orbitrap Elite instruments of the same distributor out of the box. As the nESI-FµTP setup changes already the ionization process, a combination with the previous mentioned enhanced methods concerning the sample preparation or the mass spectrometric acquisition, seems of great interest for shotgun lipidomics or other fields in clinical-research and life-science.
**Methods**

**Chemicals and sample preparation.** For the experiments ethanol in analytical grade (purity >99.5 %, Sigma-Aldrich, St. Louis, USA) was used. A liver total lipid extract (181104) in a concentration of 25 mg/mL and the cholesteryl ester CE(17:0) – cholest-5-en-3β-yl heptadecanoate (700186, Avanti Polar Lipids, Alabama, USA) – in a concentration of 1 mg/mL serving as stock solutions. Deionized water was generated by using a Millipore system. The basic electrospray solution contains water/ethanol in a ratio of 1:1 (v/v). For conductivity ammonium acetate (purity >98 %, Merck, Darmstadt, Germany) in water (1 mol/L) was added to this solution by one percent of the total volume.

For investigation of the liver extract in the first step a solution of 10 µL liver extract and 90 µL ethanol was mixed and stocked up to 1000 µL by using 900 µL of the basic electrospray solution. With a concentration of 0.25 mg/mL this solution was also used for the experiment of FµTP voltage variation. To determine the signal interferences between cholesteryl esters and cholesterol, a solution of 0.01 mg/mL CE 17:0 was utilized. Therefore, 10 µl of the CE (17:0) stock solution was mixed with 980 µL ethanol. Finally, 10 µL of ammonium acetate solution (1 mol/L) was added. All samples were supplied via direct infusion into the nESI emitter.

Finally, two calibration series using the method of standard addition were prepared. The concentration series 1 was used for the determination of the linear dynamic range (LDR). Therefore, a wide range of concentration was selected. The concentration series 2 was used for the determination of the method accuracy. Here, the concentrations focuses in the area of the target cholesterol concentration. For both calibration series a cholesterol stock solution of 1.093 mg/mL was prepared. Different amounts of the cholesterol stock solution were added to a dilution of the liver extract. In calibration series 1, a dilution of 1:1000 of the liver extract stock solution was prepared resulting in a concentration of 0.025 mg/mL. In calibration series 2, a dilution of 1:200 of the liver extract leads to a concentration of 0.125 mg/mL. Explicit dilution steps, added amounts of cholesterol, and resulting cholesterol concentrations of the calibration series are summarized in the Tab. S1 and Tab. S2 of the supporting information.

**Mass Spectrometer.** High-resolution Orbitrap mass-analyzers are prerequisite for enhanced MS acquisition methods like PRM (parallel reaction monitoring) or MSX (multiplexed MS/MS). Unless stated otherwise, the low resolving LTQ analyzer of a LTQ Orbitrap XL (Thermo Scientific™) hybrid mass spectrometer was used in this study. This shows that the nESI-FµTP approach enable the detection of cholesterol without the explicit need of using a high resolving mass spectrometer in enhanced MS detection methods (FTMS, PRM, MSX) nor SRM/MRM (multiple reaction monitoring) used on Tribrid™- or triple quadrupole mass spectrometers.

Throughout all nESI-FµTP measurements (activated and deactivated plasma), the inlet capillary temperature was set to 280 °C. If not otherwise stated, the tube lens voltage was set to 65 V and the inlet capillary voltage to 15 V, to minimize cholesterol ester and cholesterol interferences.

For comparison a reference measurement by using a classical nESI source was performed. For the nESI reference, the best signal-to-noise ratio (m/z 369.42) was carried out by using a tube lens voltage of 135 V and an inlet capillary voltage of 80 V. If not stated otherwise, a full-scan from m/z 300 to 900 was recorded in positive ion detection mode. To prevent an overloading of the linear ion trap, the automated gain control (AGC) was activated (target value 1×10^4 counts, maximum injection time 10 ms). The mass spectrometer was tuned to the cholestadiene signal (m/z 369.42).

**Ionization source.** The FµTP has been frequently used as highly efficient ionization source for the determination of perfluorinated compounds (PFCs), potential biomarker for oral cancer in GC-MS approaches, or in a laser desorption setup for the determination of cholesterol and propazine.

In this study, a nESI was used as first stage ionization source for polar compounds and as well as a nebulizer and sample introduction system for the activated FµTP (second stage). In the following, the first is named as nESI-mode, the latter one as nESI-FµTP-mode. In Fig. 8 the experimental setup with all geometrical parameters is shown.

The patent pending FµTP-design consists of a fused silica capillary (od. 360 µm; id. 250 µm) with a polyimide coating. A tungsten wire (o.d. 100 µm) serves as internal electrode and ends in a distance of 25 mm to the capillary’s orifice. From the capillary’s orifice, the polyimide coating was removed on a distance of 8 mm. As plasma operating gas, helium of 99.999% purity (helium 5.0) was applied with a volumetric gas flow rate of 50 mL/min. An in-house made square-wave generator supplies the FµTP operating high voltage. By this, high voltage amplitudes up to 3.5 kV at a frequency of 20 kHz with slopes of 8.75 V/ns can
be generated. Unless stated otherwise, the applied FµTP voltage amplitude is set to 2.05 kV. Additional information can be found in the introducing publication by Brandt et al.\textsuperscript{36}

The FµTP capillary is placed in a distance of 1 mm to the mass spectrometer's inlet capillary and in an angle of 30° to the inlet axis. The distance between the nESI emitter and the inlet capillary is 3 mm. As emitter for the nESI a selfmade emitter (i.d. 50 µm, o.d. 360 µm, sharpened to an opening angle of 60°) was prepared. However, commercially available emitters can used as well delivering smaller inner tip diameters and a more stable electrospray using lower flow rates of the liquid analyte solution.

In this setup the analyte flow rate passing the nESI emitter was set to 0.75-1.00 µL/min. The electrospray voltage was supplied by the mass spectrometer and is dependent on the nESI position, the FµTP position, and the applied plasma voltage. Finally, it was set to 2.6 kV. As shown in Fig. 9 the capillaries of the nESI and the FµTP are embedded in a polyether ether ketone (PEEK) interface, covered with a glass plate and secured by magnets serving as mechanical support of the experimental setup.

In this study, the nESI-FµTP setup is compared with a classical nESI as reference. Therefore, the same already mentioned nESI emitter was used with a flow rate of 1 µL/min. The distance between the nESI emitter's tip and the inlet capillary was 1 mm and the applied voltage was decreased to 1.7 kV.

![Figure 9. Photograph of the nESI-FµTP setup in front of the mass spectrometer (top). Both ionization sources (nESI: center, FµTP: right) are embedded in a PEEK interface which is covered by a glass plate. The analyte (nESI), gas (FµTP) and voltage supply (nESI, FµTP) is outside the photograph (bottom).](image)

**Safety Considerations.** The chemicals and solvents used in this study necessitate protective equipment (i.e. nitrile gloves, safety goggles, laboratory coats) in compliance with the material safety data sheets as well as trained personnel. The nESI and the FµTP source are operated with high voltages. Additionally, precautions such as warning signage and adequate isolation of cables and connectors are recommended. To prevent the operator and the mass spectrometer from electrical short circuits, a safety interlock is installed inside the in-house made square-wave high voltage generator.
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Competing Interests

The authors declare no competing financial interest.