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# Quantification of the sensitivity increase of a micro-high-performance liquid chromatography–electrospray ionization mass spectrometry system with decreasing column diameter

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**Abstract**

This study details the sensitivity achieved with capillary columns when used with a micro-HPLC–electrospray ionization MS system. It is comprised of two sections, the first is the comparative study of three columns, one of narrow-bore diameter and two of capillary diameter. The second section compares three columns of decreasing diameter in the capillary scale. All the experiments achieved enhanced sensitivity using capillary columns. The increase in the experimental MS response ranged from −20% to +20% compared to the UV experimental response when decreasing the internal diameter of the columns used. When comparing the experimental MS response to the maximum theoretical UV response achievable, the increase in response ranged from 40 to 50%.



*Keywords:* Column diameter; Sensitivity; Liquid chromatography

## 1. Introduction

Twenty years ago, Scott and Kucera were the first to study micro (μ)-flow columns [1–4]. Microbore liquid chromatography was the term already used by Scott in the 1980s to name separations conducted in columns whose internal diameter was equal to or less than 1 mm [5].

There were several problems associated with the running of these columns. First, these microcolumns had to be operated at very high pressure in order for the mobile liquid phase to flow along them. Second, only a small flow-rate had to be delivered continuously. Thirdly, small amounts of analyte should be injected. Furthermore, the internal dead volume had to be greatly minimised as the analyses experienced significant band broadening effects [6].

With the development of μ-HPLC instruments, these adversities have been resolved. Pumps in these instruments deliver flows in the μl/min range with very little fluctuation. These pumps usually split the flow and provide the selected microflow to the column while diverting a surplus to waste [7].

The system used in the Experimental section is an Agilent 1100 Capillary LC system. This system also works by splitting the flow-rate into waste and column. In addition, this system incorporates a novel flow-monitoring device which adjusts the flow whenever it senses it is too high or too low. This device works by assessing the velocity of flow through a set distance considering temperature changes at two different points.

In addition to these instruments, there are systems

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on the market where the engineered pump, normally a syringe-based system, is capable of delivering small flows without having to split the flow. It is difficult to determine which mode performs better. The direct pumping of μ-flows is very good for saving solvent costs and disposal, especially when using deuterated solvents. However, these systems might be limited in their ability to accurately mix low volumes [7].

The advantages of using microflows instead of normal flows are significant. One of these advantages is the use of less mobile phase and reduced waste. While the first saves cost, the latter diminishes the environmental impact of the toxic solvents.

Nevertheless, the most important advantage of using microflow through capillary columns, which is going to be commented upon throughout this paper, is the higher sensitivity one can achieve. When the internal diameter of the column diminishes, the dilution of the analyte is lowered in the separation column resulting in an increased sensitivity of detection [8].

In 1995, Ryan proposed a model to adapt normal flow in chromatography to micro flow chromatography [9]. An approximation of the amount of sensitivity gain that could be expected by diminishing the column diameter was proposed. This theory states that when going from a column of diameter $X$ to a smaller diameter $Y$, the gain in sensitivity would be equivalent to the partition of the squares of the internal diameters $[X^2/Y^2]$.

Thus, in practical terms, when adapting a method from a 2.1 mm I.D. column to a 0.5 mm I.D. column, there would be a sensitivity gain of $2.1^2/0.5^2 = 17$ i.e. 17 times more counts would be observed. From a 2.1 mm column to a 0.3 mm column, the sensitivity increase could be as large as 49 times.

Yet again, another important direct consequence of the small flow-rates delivered is the excellent conditions for coupling a μ-HPLC system to a mass spectrometer.

Relative sensitivity of UV and MS detection for most compounds differs by many orders of magnitude, MS relative detection being much higher than UV. This represents a problem because often calibration curves exhibit non-linear behaviour and curve downward at higher concentrations for MS [10]. The different concentrations used in the experiments were chosen so as to have a good UV response but not to overload the MS detector.

In this study, a comparison between the sensitivity gained by decreasing the column diameter in UV and MS is revealed.

## 2. Experimental

### 2.1. Chemicals

The compounds used for analysis were salmeterol, 415.3 u, and fluticasone propionate 500.3 u, both provided by GlaxoSmithKline (Ware, UK).

#### 2.1.1. Solvents

Acetonitrile (190 far UV grade), water (HPLC grade) and triethylamine (TEA) were purchased from Fisher. Acetic acid, ammonium formate and formic acid were purchased from Sigma.

### 2.2. Instrumentation

The μ-HPLC equipment used in this work comprised an Agilent 1100 Series system (Agilent Technologies, Germany). The Mass Chromatographic Analyser used was an API 150 MCA from Perkin-Elmer Sciex Instruments. The infusion pump was from Harvard Apparatus. The gas pneumatically assisted electrospray was provided and modified by Glaxo Welcome. The data were collected with MacDad Perkin-Elmer Sciex software for MS results, and with Chemstation software for the UV results.

The HPLC system was hyphenated to the MS system by an electrospray ionization (ESI) interface. The interface has been modified to perform best at flow-rates from 10 to 30 μl/min. The μ-HPLC instrument was linked to the MS system using a silica capillary of 100 μm I.D., which is passed through an ESI interface. The interface consisted of a Valco stainless steel tee to which the nebulizer gas is connected and the electrical contact is made by a connection on top of it (Fig. 1). The capillary is burned at the end allowing 1.5 mm of pure silica out of the concentric spray tip. When the voltage (4.5 kV) is applied a fine spray is produced directly from the connecting capillary.

Three Zorbax SB-$C_{18}$ chromatographic columns

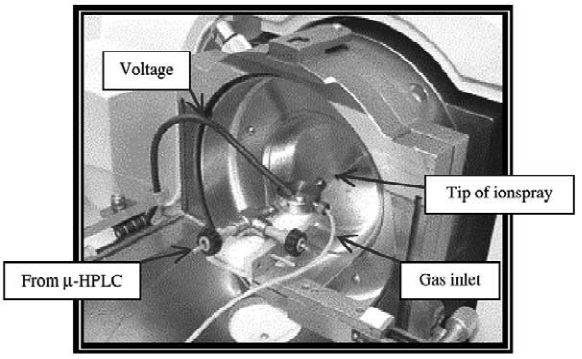


Fig. 1. ESI system used and its connections.

of internal diameter 2.1, 0.5 and 0.3 mm, all of 15 cm length were provided by Agilent Technologies. Three Zorbax SB-C(18) chromatographic columns of 500, 320 and 200 μm I.D., all 10 cm length were used. The 15 cm×0.5 mm I.D. column was provided by Agilent Technologies, whilst the 15 cm×0.32 mm I.D. and 15 cm×0.2 mm I.D. columns were made in the laboratory.

### 2.3. Procedure

#### 2.3.1. Pump and ESI-MS optimisation

First, a thorough optimisation of the flows, which would later be used in the hyphenated system, was made. The infusion pump was set at a flows of 4.2, 12 and 100 μl/min. The tip of the ionspray was set at 1.2, 0.8, 0.6, 0.4 and 0.2 cm (*x*-axis) from the entrance of the MS and moved on the *y*-axis to −3, −2, −1, 0, 1, 2 and 3 mm positions. The measurements were made with three different gas pressures (also called nebulizer pressure) 0, 3, and 6 on the instrument settings which are equivalent to flows of 0.03, 0.41 and 0.92 l/min. In total, 252 measurements were made for salmeterol. The same procedure was used for fluticasone at flows of 2, 4.2 and 12 μl/min. A schematic diagram is shown in Fig. 2.

#### 2.3.2. μ-HPLC–ESI-MS experiments

Once the optimisation was completed, the hyphenation of the μ-HPLC–ESI-MS system proceeded.

The experiments involving the analyte salmeterol were carried out in the columns of 2.1, 0.5 and 0.3 mm I.D. Two sets of concentrations were used with salmeterol as the analyte. The first set was in a proportion of *X*, *X*/4 and *X*/7 for the 2.1/0.5/0.3 mm I.D. columns, that was 227.5, 56.8 and 32.5 μg/ml.

The second set of concentrations was in a proportion of *X*, *X*/17 and *X*/49 for the 2.1/0.5/0.3 mm I.D. that was 227.5, 13.4 and 4.64 μg/ml. Both proportions were chosen so as not to overload the MS detector. The latter is also the same proportion of maximum theoretical increase in sensitivity using UV detection when decreasing I.D. from 2.1 to 0.5 to 0.3 mm, that is an increase of 1 to 17 to 49 times.

For the analyte fluticasone propionate, the chromatographic columns of 0.5, 0.32 and 0.2 mm I.D. were used. The concentrations were in a proportion of *X*, *X*/2.4 and *X*/6.25 for the 0.5/0.32/0.2 mm I.D. that was 30, 12.3 and 4.8 μg/ml.

For salmeterol, the mobile phase used throughout all the experiments performed in an isocratic manner was 80% acetonitrile–20 m*M* $NH_4OAc$–0.1% TEA and 20% water–20 m*M* $NH_4OAc$–0.1% TEA at pH 5.

For fluticasone propionate, the mobile phase was 80% acetonitrile–20 m*M* $NH_4OAc$–0.1% TEA and 20% water–20 m*M* $NH_4OAc$–0.1% TEA. Temperature 30 °C.

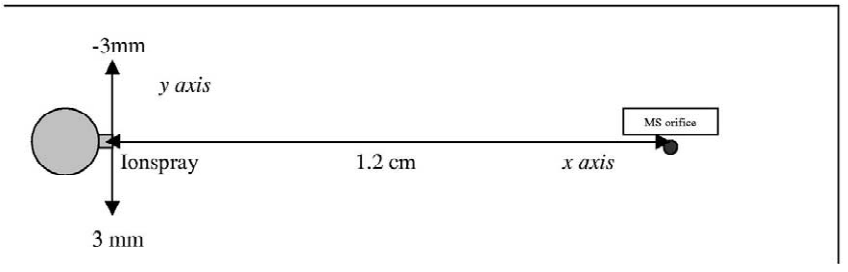


Fig. 2. *x*- and *y*-Axes from the ESI to the MS orifice.

The μ-HPLC instrument flow-rate was set at 2 μl/min for the 0.2 mm column, and 4.2 μl/min for the 0.3 and 0.32 mm columns, 12 μl/min for the 0.5 mm column and 200 μl/min for the 2.1 mm column. Injection volume remained constant at 0.1 μl. Column temperature was set at 30 °C. The mass spectrometer conditions were set on a method of characteristics as follows:

Mass range: 50.0 to 500.0 by 0.1 u; dwell: 0.2 ms; pause: 5.0 ms. The curtain gas was set at 10 which is equivalent to 1.25 l/min. The voltages were as follow, ion spray voltage was of 4.500 V, focusing ring voltage was 300 V, the orifice voltage was 30 V; offset voltages for lenses were Q0: −10 V, IQ1: −11 V, ST: −15 V, RO1: −11 V. The deflector potential was set to 0 and the multiplier CEM was set at 1800 V. All of these conditions remained constant for all the experiments in this section.

The chromatographic runs and the mass chromatographic runs were started at the same time. When the instruments had a stable response, a 10 run sequence was performed and the data recorded for later analysis.

## 3. Results and discussion

### 3.1. Optimisation

Average height, maximum and minimum detection were recorded from the spectra. The stability of the signal was assessed. Data refer to TIC (total ion count) and the maximum XIC for salmeterol and fluticasone propionate.

In Fig. 3, an example of data for TIC of salmeterol is represented on a diagram that considers all the highest responses for a flow of 12 μl/min.

In the salmeterol experiments, it was observed that for the lowest flow, 4 μl/min, the gas assisted evaporation and prevented formation of sparks. For the 12 μl/min flow, moderate gas pressure was the best choice. For the very high 100 μl/min also moderate gas pressure assisted best with the exception of pure flow spraying directly into the orifice.

All of the rates performed best at the shortest distance from the sampling orifice. Best evaporation was also achieved at the edges of the electrospray for all of the cases.

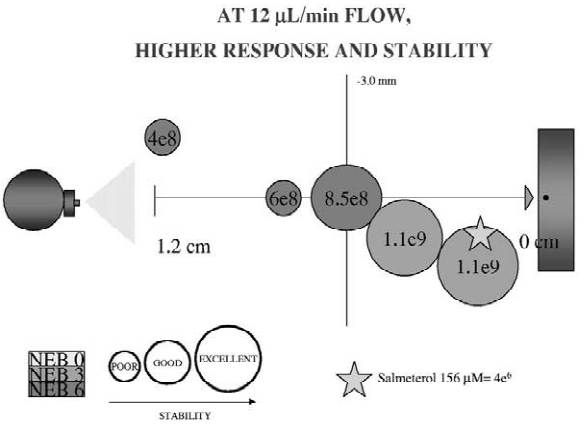


Fig. 3. Example of the data acquired for optimisation of flow-rates. Final diagram for 12 μl/min flow. Three different shades of grey illustrate which gas pressure was preferred for successful analysis. Data are for total ion count and the maximum response for salmeterol is also shown. A star identifies the spot where the tip would later be placed when coupling to the μ-HPLC instrument.

As a general rule, using positions where a stable total ion count of at least $1\times10^9$ was achieved would report the highest sensitivity.

For fluticasone experiments, the optimization of the set of flow-rates (2, 4.2, and 12 μl/min) was carried out.

All of the flow-rates performed best at the short distances. Some sparking was observed when there was some fluctuation in the flow. Best evaporation was also achieved at the edges of the electrospray for all of the flow-rates.

As a general rule and as predicted for this modified interface, for the very low flow-rate of 2 μl/min, no nebulizer was needed and pure spray was ionized successfully. For larger flows, it was better to aid the evaporation process with low gas at 3 μl/min.

### 3.2. μ-HPLC–ESI-MS experiments

Data were acquired for UV chromatograms and MS spectra. For UV data, retention time, efficiency of peak, peak height, and peak area were recorded. For MS data, gas pressure used, peak height raw and peak height once the peak has been smoothed were recorded. Spectra, which refer to TIC and the XIC,

the peaks for salmeterol and fluticasone propionate were recorded.

Next in Fig. 4, the more representative chromatograms of salmeterol are displayed. From top to bottom corresponds to the concentrations in order $X$ to $X/4$ to $X/7$ for diameters 2.1, 0.5 and 0.3 mm I.D., respectively.

### 3.2.1. Reason for discrepancy

In this experiment, there were great efficiency differences between the chromatographic columns. The 0.5 and 0.3 mm I.D. columns have lower plates counts. The cause of this is due to the fact that the Agilent μ-HPLC instrument was re-arranged in order to run at the higher flow of 200 μl/min. This rearrangement included changing the detection cell, bypassing the 20 μl/min flow sensor and changing the capillary from the pump to microvalve from 50 to 100 μm I.D.

Capillaries which could have been changed, like the one from the microvalve to the column, were not changed and remained at 50 μm I.D. The consequence of this was the exceptionally high efficiencies for the 2.1 mm I.D. column, at the expense of high backpressure. This backpressure did not affect the analysis as it was of a stable nature at 230 bar. This re-arrangement, although producing very high plates for this column, was not ideal. At this pressure, all

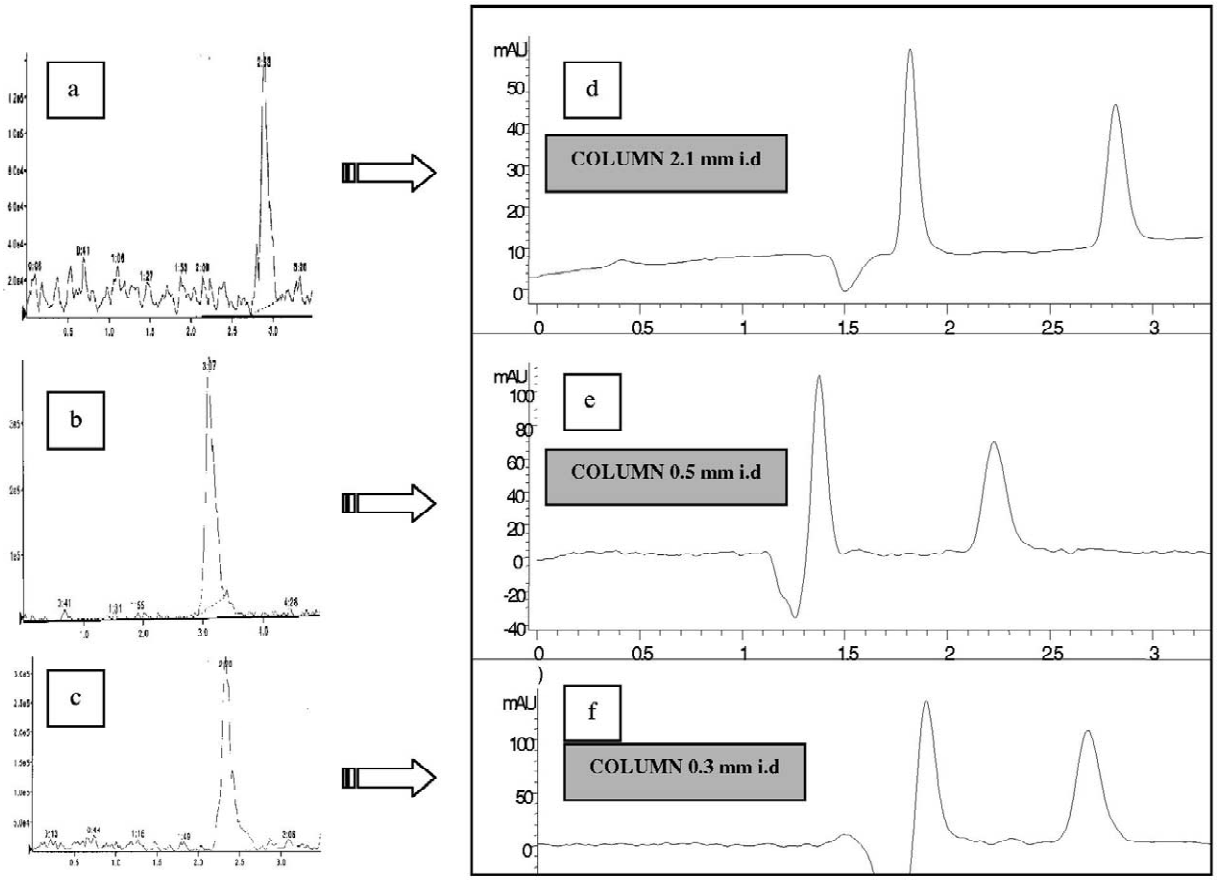


Fig. 4. (a, b, c) MS spectra for decreasing concentrations of 227.5, 56.8 and 32.5 μg/ml; (d, e, and f) UV chromatograms for the same decreasing concentrations. All are run under the same conditions. For MS, mass range 50 to 500 by 0.1 u; dwell: 0.2 ms; pause: 5.0 ms; XIC of 416.3 u; total TIC~$10^9$ u. For UV chromatograms: flow-rate was set at 4.2, 12 and 200 μl/min for Figs. 4, 5 and 6, respectively; injection, 0.1 μl. Mobile phase 80% acetonitrile–20 m$M$ $NH_4OAc$–0.1% TEA and 20% $H_2O$–20 m$M$ $NH_4OAc$–0.1% TEA. Temperature 30 °C.

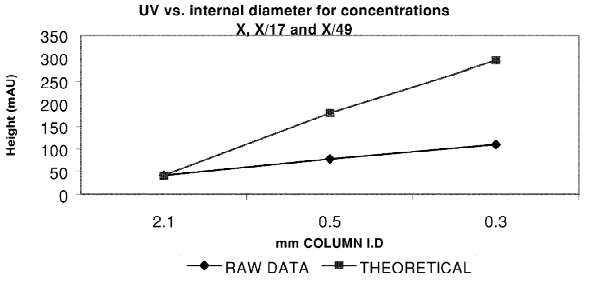


Fig. 5. Line for raw data and the theoretical line. The theoretical line shows what the results should have been for the expected increase in sensitivity following the ''partition of the squares rule''.

hand-fitted capillaries could easily start leaking or burst.

The UV graph shown in Fig. 5 accounts for this discrepancy. The theoretical line is the expected increase in sensitivity while the experimental outcome shows what was achieved.

The MS output versus column diameter graph (Fig. 6) shows the amount of times that sensitivity increases with decreasing column internal diameter for MS in both of the sets of concentrations used with salmeterol.

It is important to note that the final graph already accounts for the variation in concentration but does not account for the variation of efficiency between columns. This variation of efficiency has a very important direct effect on the outcome of the study. In the case of the present study the column of 2.1 mm I.D. is much more efficient (more than double) than the 0.3 and 0.5 mm columns, which display similar efficiencies. The consequence of this is that the increase that is observed in the final graph would be higher if the efficiencies were all similar.

For the first set of concentrations depicted in Fig. 6, there was an increase of 10.25 times from the 2.1 mm I.D. column to the 0.5 mm I.D. column, and an increase of 1.79 from the 0.5 mm I.D. column to the 0.3 mm I.D. column. The total from the 2.1 mm I.D. column to the 0.3 mm I.D. column is 18.44 times increased sensitivity. These results were calculated from the raw data.

For the second set of concentrations, $X$, $X/17$ and $X/49$, also shown in Fig. 6, the increase was 10.44 times from the 2.1 mm I.D. column to the 0.5 mm I.D. column, and 1.75 times from the 0.5 mm I.D. column to the 0.3 mm I.D. column.

The total from the 2.1 mm I.D. column to 0.3 mm I.D. column was 18.25 times increased sensitivity. The results were identical for both sets of concentrations attempted which confirms the reproducibility of the data. From the 2.1 mm I.D. column to the 0.3 mm I.D. column, the increase in MS was 59% if compared to that expected in the maximum UV theoretical increase. From the 2.1 mm I.D. column to the 0.3 mm I.D. column, this increase was 37% as the maximum theoretical UV was 49 times and the experimental outcome was 18.25 times.

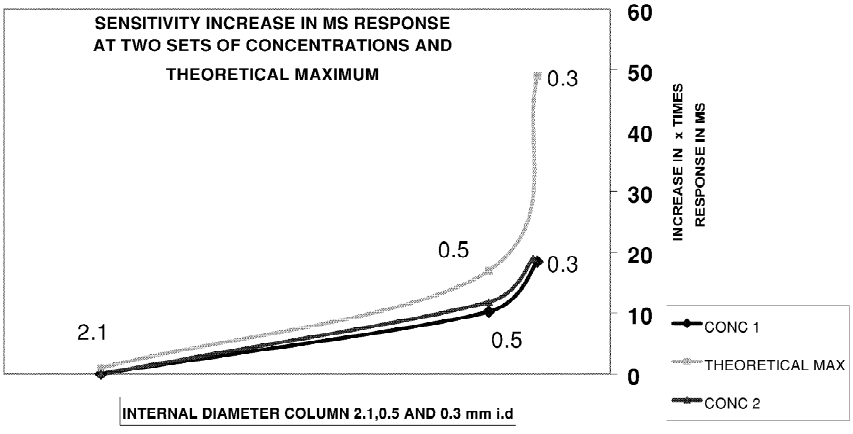


Fig. 6. This graph shows the final outcome for the salmeterol experiments. Concentration 1 is the first set at 227.5, 56.8 and 32.5 μg/ml, and concentration 2 accounts for the second set at 227.5, 13.4 and 4.64 μg/ml. The theoretical maximum is in times increase for UV detection.

When compared with the actual experimental outcome for UV, the MS response was of +23% from 2.1 mm I.D. to 0.5 mm I.D. and of +9% increase in sensitivity from 2.1 mm I.D. to 0.3 mm I.D.

For fluticasone experiments, the results are shown in Fig. 7 where the most relevant chromatograms for MS and UV are displayed.

The columns used also differed in efficiency. In this case, the efficiencies of the 320 and 200 μm I.D. columns were much lower than the efficiency of the 500 μm I.D. column. Similarly to the previous experiments, the UV response would have been much greater if all the efficiencies were the same.

Fig. 8 refers to the times increases that were obtained in MS response with fluticasone. The amount differs for raw data and theoretical. From the total expected, which was the maximum response for UV, that is 2.44 times increase from the 500 μm I.D. column to the 320 μm I.D. one and 6.25 times increase from the 500 μm I.D. column to the 200 μm I.D. one.

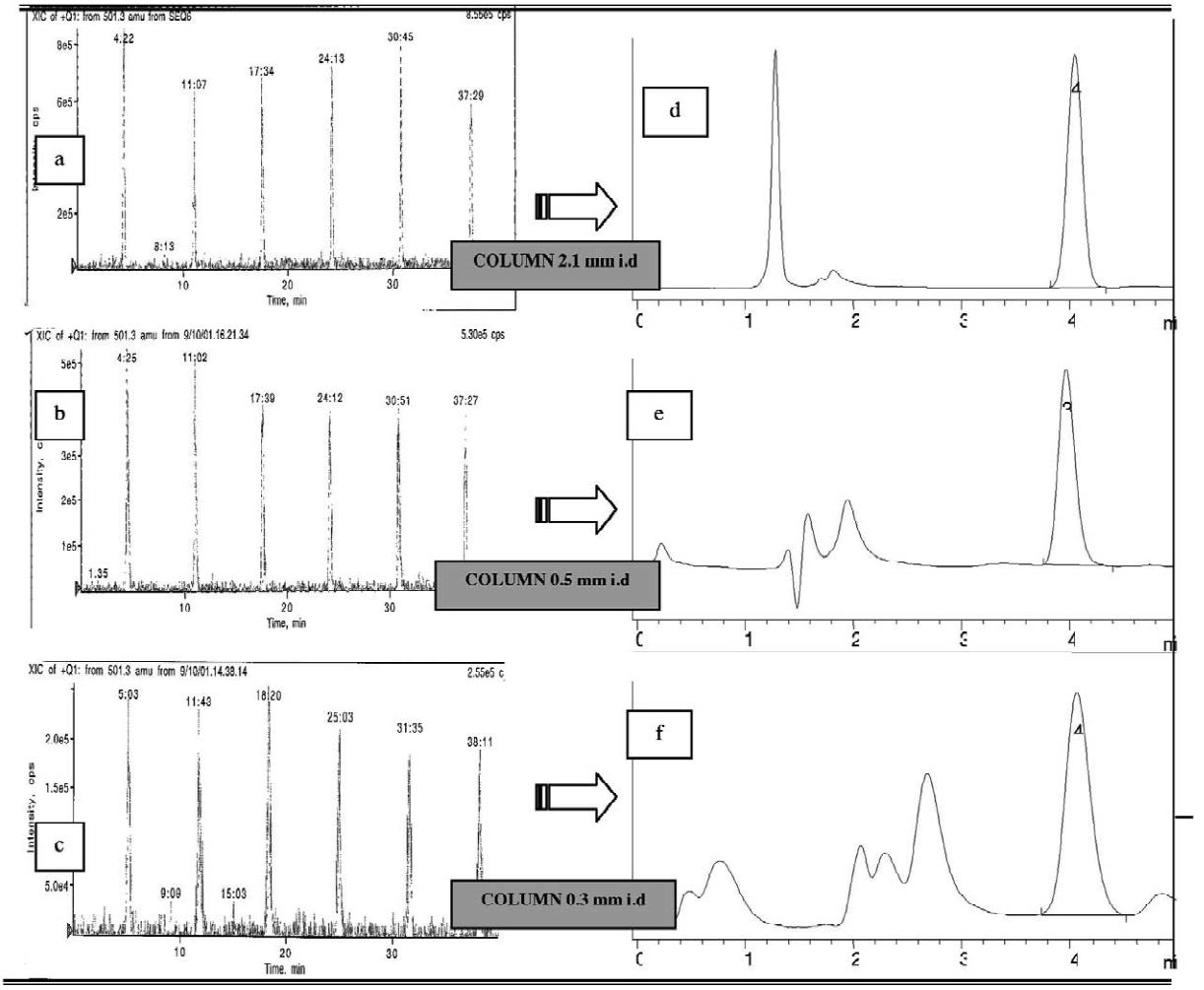


Fig. 7. (a, b, c) MS response of six consecutive runs of fluticasone propionate for decreasing concentrations of 30, 12.3 and 4.8 μg/ml; (d, e and f) UV response for the same decreasing concentrations. All are run under the same conditions. For MS, mass range 50 to 500 by 0.1 u.; dwell: 0.2 ms; pause: 5.0 ms; XIC of 501.3 u. For UV chromatograms. flow-rate was set at 12, 4.2 and 2 μl/min for d, e and f, respectively. Injection 4 μl. Mobile phase acetonitrile–water (75:25) with 20 m*M* ammonium formate, pH 5. Temperature 30 °C.

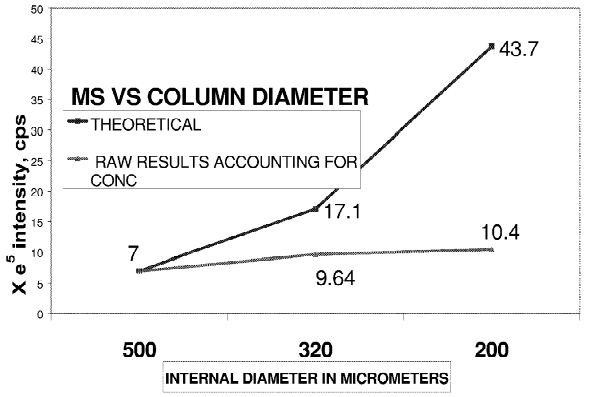


Fig. 8. Increase in sensitivity with the decreasing column diameters for the fluticasone experiments.

A total increase of 48% for the 500 μm I.D. column to the 320 μm I.D. one and a 38% increase was obtained for the MS data from the 500 μm I.D. column to the 200 μm I.D. one.

In comparison with the actual outcome for UV, the MS response was −19% for the 500 μm I.D. column to the 320 μm I.D. one and −3% from the 500 μm I.D. column to the 200 μm I.D. column.

## 4. Conclusions

In the first set of experiments, the investigation was carried out with salmeterol, a basic compound, and all the spectra were set to a maximum total ion count. Comparing the achieved increase in sensitivity in MS with what is expected in the UV response:

1. From 2.1 to 0.5 mm I.D., there was a 59% increase in sensitivity.
2. From 2.1 to 0.3 mm I.D., there was a 37% increase in sensitivity.

In the second set of comparative experiments, a neutral compound, fluticasone propionate, was used. The results were the following:

1. From 0.5 to 0.32 mm I.D., there was a 48% increase in sensitivity.
2. From 0.5 to 0.2 mm I.D., there was a 38% increase in sensitivity.

In general, it is possible to conclude that for these compounds, the increase in sensitivity using the capillary scale columns in ESI-MS is 40–50% of that expected for maximum UV detection. The reason for this result was that the efficiency of the capillary columns was lower and the theoretical maximum was not achieved, which shows that even in this case, the sensitivity is greatly increased.

Comparing with what was actually the UV response, the MS response was ±20%; this result shows a fluctuation derived from the different nature of the UV detection and MS ionisation processes.

Most importantly, it was shown that the capillary columns achieved increased sensitivity and reproducible results in all cases.

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