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A review of nanoscale LC-ESI for metabolomics and its potential to enhance the metabolome coverage

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Abstract

Liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) platforms are widely used to perform high throughput untargeted profiling of biological samples for metabolomics-based approaches. However, these LC-ESI platforms usually favour the detection of metabolites present at relatively high concentrations because of analytical limitations such as ion suppression, thus reducing overall sensitivity. To counter this issue of sensitivity, the latest in terms of analytical platforms can be adopted to enable a greater portion of the metabolome to be analysed in a single analytical run. Here, nanoflow liquid chromatography-nanoelectrospray ionisation (nLC-nESI), which has previously been utilised successfully in proteomics, is explored for use in metabolomic and exposomic research. As a discovery based field, the markedly increased sensitivity of these nLC-nESI platforms offer the potential to uncover the roles played by low abundant signalling metabolites (e.g. steroids, eicosanoids) in health and disease studies, and would also enable an improvement in the detection of xenobiotics present at trace levels in biological matrices to better characterise the chemical exposome. This review aims to give an insight into the advantages associated with nLC-nESI for metabolomics-based approaches. Initially we detail the source of improved sensitivity prior to reviewing the available approaches to achieving nanoflow rates and nanospray ionisation for metabolomics. The robustness of nLC-nESI platforms was then assessed using the literature available from a metabolomic viewpoint. We also discuss the challenging point of sample preparation which needs to be addressed to fully enjoy the benefits of these nLC-nESI platforms. Finally, we assess metabolomic analysis utilising nano scale platforms and look ahead to the future of metabolomics using these new highly sensitive platforms.

Key words: nanoflow, nanoESI, metabolomics, exposomics, ion suppression, sample preparation

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1. Introduction

The aim of untargeted metabolomics is the comprehensive analysis of all known and unknow metabolites in a biological sample such as cells, biofluids or tissues at any one time [1]. Quantitative strategy can also be used where dozens to hundreds of metabolites are targeted and this is referred to as targeted metabolomics [2]. The metabolites in question are typically <1000 Da [3, 4], and are reactants, intermediates or by-products of enzymatic activity [1]. In some cases, these metabolites may be exogenous in source (e.g., xenobiotics), such as food additives from the diet [5], pharmaceutical intake (e.g. anti-inflammatory drugs and mild analgesics, antidepressants) [6, 7] or use of cosmetics (e.g., parabens, UV filters) [8]. The combination of the metabolome (including microbial metabolism) and xenobiotics as well as their products from phase I and/or II metabolism in biological samples is known as the (xeno)metabolome [6, 9-12]. Profiling biological samples from different populations such as diseased and healthy subjects or exposed and non-exposed subjects using an untargeted metabolomics-based approach combined with chemometric pattern analysis allows for the potential to simultaneously uncover biomarkers of effect (i.e. disruption of endogenous metabolite profiles) as well as biomarkers of exposure (i.e., xenobiotic mixtures). Metabolomics-based approaches has thus wide applicability in medicine [13, 14], toxicology [6, 15, 16], food sciences [5, 17], exposomics and human health studies [12, 18, 19], plant sciences [20, 21], and environmental sciences [9, 22, 23].

To undertake untargeted analysis, analytical platforms capable of analysing thousands of metabolites simultaneously are required. To date, the most widely used analytical platform is liquid chromatography-mass spectrometry (LC-MS), typically utilising an electrospray ionisation source (ESI) [24, 25]. These LC-ESI platforms can offer highly reproducible and very high throughput methods to perform untargeted profiling of biological samples for metabolomics-based approaches. Furthermore, the soft ionisation process of LC-ESI platforms allows structural elucidation and the analysis of both volatile and non-volatile metabolites. However, these LC-ESI platforms usually favour the detection of metabolites present at relatively high concentrations because of analytical

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issues such as poor ionisation efficiency and ion suppression [26, 27]. This is a major limitation since the idea behind the use of a metabolomics-based approach is to discriminate new biomarkers of effect or exposure that have so far remained unidentified. It is therefore essential for these untargeted methods to be sensitive enough to detect signalling metabolites or xenobiotics present at trace levels and that could be important in health effect outcomes. There is therefore a need for innovative strategies which allow to overcome sensitivity issues caused by ion suppression for metabolomics-based approaches.

As a result of these deficiencies, LC and ESI technologies have slowly been miniaturised and the latest developments in LC-MS have taken miniaturisation a step further with the development of nanoLC-nanoESI (nLC-nESI) [28, 29]. As the name suggests, flow rates are on the nL/min scale and has been defined as LC platforms that deliver flow rates of 10-1000 nL/min with ESI emitters internal diameters (ID) of between 10 and 50 µm [30, 31]. The increased sensitivity of the nLC-nESI can be attributed to factors derived from both the nLC and nESI source, the latter being the main source of the increased sensitivity. The use of nESI emitters means that the droplets formed in the ESI plume are 100-1000 fold smaller than the typical droplets emitted from conventional ESI emitters [32]. The process of ESI is an evaporative one, and therefore the generation of significantly smaller plume droplets with lower volumes considerably increases the rate of desolvation, resulting in up to 500 times more ions being formed and entering the mass analyser [32-35]. Another advantage of nESI lies in the fact that the emitter is closer to the MS inlet compared to conventional ESI, allowing a more efficient introduction of the ions formed into the inlet [36]. In addition, due to the much lower flow rate, chromatographic dilution is significantly decreased allowing more concentrated peaks to elute from the analytical column [37-39]. Further advantages of the nLC-nESI include reduced desolvation temperature [40] and the decrease of the consumption of both mobile and stationary phases [28].

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Hence, these nano scale platforms offer a significant improvement in terms of sensitivity due to increased ionisation efficiency and reduced chromatographic dilution. These characteristics offer great potential to metabolomic researchers looking to encompass as much of the metabolome as possible. This review aims to give an insight to the advantages associated with nLC-nESI for metabolomics. To do this, we first describe the different techniques that have been used to achieve nLC and nESI for metabolomics. Here, we will focus on nanoscale instrumentation that have been successfully applied to metabolomic applications since comprehensive reviews on miniaturised liquid chromatography instrumentation can be found elsewhere, e.g.,[28]. We then discuss the robustness of these platforms in targeted analysis and global metabolomic studies. We also discuss a challenging point, the sample preparation, which needs to be addressed to get the most out of the platforms sensitivity while not adversely impacting upon the metabolomic analysis. Subsequently, we look ahead to the potential nLC-nESI-MS has in metabolomics for elucidating the roles played by very low abundance metabolites in health, disease and the potential nLC-nESI-MS has for the identification of mixtures of xenobiotics in exposure assessment.

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2. Overview of nLC-nESI platforms and advantages for metabolomics

The aim of this section is to present instrumentations which can be utilised for metabolomics to generate nanoflow rates, the different nanocolumns and nESI emitters and to discuss their advantages and drawbacks. An overview of instrumentation, nanocolumns and methods used for metabolomics can be seen in Table 1.

2.1 Nanoflow generation

The method by which nanoflow rates are produced can have a dramatic effect on the quality of the chromatography and repeatability of the analysis, either in terms of peak shape or retention time. Here the generation of nanoflow rates for direct infusion metabolomics will be discussed in addition to the formation of nanoflow rate by splitting higher flow rates and direct from pump

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generation for nLC-MS. These methods are known as self-fed/direct infusion, split flow, and direct flow, respectively.

2.1.1 Self-fed/ direct infusion nanoESI

While not a nLC-nESI method strictly speaking, direct infusion nESI-MS has proven popular for a number of metabolomic studies to analyse liver extracts [41-43], plasma [44], urine [45], zebrafish embryos [46], *Daphnia magna* [47]. These studies benefitted from the generation of nanoflow rates within a nESI emitter to achieve sensitive and high throughput analysis [30, 42]. Furthermore, direct infusion nESI reduces the volume of solvent used by 1000 fold compared to conventional LC-MS [48]. Self-fed systems load sample directly into an emitter to which a voltage is applied initiating a nanospray via capillary action and electrostatic force [33, 49, 50]. These systems were initially utilised in proteomics, however, they were known to offer poor reproducibility [51-53]. In addition, samples must be manually loaded individually into the tips and be installed for each run, making automated high-throughput analysis difficult [51-53].

New chip based methods automatically aspirate sample into a disposable tip which interfaces with a nESI emitter [30, 51-54]. Each tip and emitter is single use, eliminating carryover, reduces the impact of emitter blockage and significantly increases sample throughput [48, 52, 53, 55, 56]. A novel method known as SIM stitch, collects direct infusion Orbitrap MS data using a series of overlapping SIM windows. Once collected SIM windows are “stitched” together to create a full MS spectra covering the entire m/z range. This technique alleviates the effect of space charge effects in the Orbitrap mass analyser and reduces the impact of high abundance metabolites being preferentially analysed [42, 57].

The use of direct infusion nESI for high-throughput analyses seems to fit well with the application of metabolomics in studies which requires consecutive analysis of very large numbers of samples (e.g., epidemiological studies) by reducing dramatically the time and the cost of analysis. However, the lack of chromatographic separation in direct infusion methods means that the analysis

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still suffers from ion suppression [48, 55]. In addition, these methods are unable to differentiate between isobaric/isomeric molecules or provide definitive metabolite identification without the need for subsequent fragmentation and/or chromatographic experiments, such as nLC-nESI-MS [56].

2.1.2 Split flow

When nLC was first introduced commercial pumps capable of delivering nanoflow rates were not available [31]. To counter this, HPLC pumps provided high flow rates which were subsequently split to provide nano scale flow rates with surplus mobile phase going to waste [27]. Depending upon where the flow is split, up to 99% of the sample can be lost, thus, eliminating the benefit of low volume or precious samples [36]. In addition, the green chemistry benefits are lost as most of the solvent goes to waste in the splitting process [31, 58]. A further complication is evident when trying to perform a gradient separation. Due to differing mobile phase viscosities, variation in back pressure and surface tension is observed, making it more difficult to achieve stable retention characteristics when using non-commercial flow splitters [58, 59]. One large advantage to the use of split flow systems is that they have a dual use as a conventional LC and a nanoflow system effectively giving researchers two instruments in one. The lack of split flow use in metabolomics potentially reflects upon the aforementioned disadvantages.

2.1.3 Direct flow

In recent years, technological advances have enabled commercially available nano reciprocal or syringe pumps to become widely available [60]. These platforms have been called direct nanoflow, due to the nanoflow rates being provided directly from the LC pumps. Unlike most of their split flow counterparts, direct flow platforms make use of micro fluidic flow controllers which control flow rates much more precisely than flow split systems [61]. Furthermore, there are fewer connections in the system thus reducing the risk of leakages, zero dead volumes and blockages [61]. The use of direct nanoflow is of particular importance in analysis utilising a gradient system. This is due to its ability to allow for variation in mobile phase viscosities using sophisticated

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flow controllers [58, 59]. It is known that providing nanoflow rates directly from the pump improves retention and spray characteristics of the nano platform [37, 58]. In addition, the cost and environmental benefits of reduced solvent usage are also realised using direct nanoflow pumps [58]. To date direct nLC has been implemented in a small number of metabolomic analysis of urine [62-65], plasma [11], tissue [23], faeces [66], exhaled breath condensates [67], sweat [68], cerebrospinal fluids [69] and cell extracts [70].

2.2 Nanocolumns

According to the classification of Saito et al. [71], nanocolumns range from 0.01 to 0.1 mm internal diameter (ID). In this review, we have included metabolomic studies using nanocolumns with ID up to 0.2 mm but with flow rates within the nanoflow rate ranges (i.e., up to 1000 nL/min) (Table 1). The main advantages of columns with lower ID compared with conventional HPLC columns are reduced chromatographic dilution, lower consumption of mobile phases and lower consumption of sample mainly due to the lower flow rates [28, 72].

Due to the broad range of physico-chemical properties of small molecules (e.g., polarity, size) analysed in metabolomics, it is important that as many as possible can be retained on an analytical column with sufficient separation between each other. Nanocolumns used in metabolomic studies so far include BEH C18, HSS T3, Acclaim PepMax C18 and ZIC-HILIC for a wide range of matrices (Table 1). It must be noted that the majority of nanocolumns available utilise conventional reversed phased column chemistries. However, normal phase such as HILIC are now available from Merck (ZIC-HILIC 75-100 µm i.d), Sciex cHiPLC (75 µm x 15cm HALO HILIC), Tosoh (TSK-GEL Amide-80 HILIC, 50 µm i.d.) and NewObjective (Unison UK-Amino 75 µm -10 mm) which could then be used to improve the retention of very polar and polar metabolites for nanoscale platforms as seen in the sole nanoHILIC study to date [69].

A comparative study of three reversed phase nanocolumns with two column chemistries and three different pore sizes (BEH C18 300 and 130 Å, and HSS T3 100 Å) was performed in view of

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metabolomic applications for urine and plasma [73] (see Fig. 1). Increased retention, separation and increased peak area were observed for most of the metabolites tested with the low porosity columns (100 and 130 Å), due to a combination of decreased chromatographic dilution, increased stationary phase surface area and increased mass transfer rate [39, 73, 74]. Furthermore, the retention of many analytes was favoured by the HSS phase compared with the BEH, suggesting that the use of HSS T3 phase with porosity smaller pore size could increase the performance of the system by reducing co-elution and ion suppression for metabolomic applications.

Similar phases to these used for metabolomics with conventional HPLC columns are already available for nanocolumns and allow to achieve a comprehensive coverage of the metabolome of urine [64], plasma [10] and tissues samples [23]. However, more nanocolumns adapted in terms of column chemistry and pore sizes (i.e. lower pore size than for proteomics) would be welcome to gain optimal sensitivity and chromatographic separation of polar, mid-polar and apolar metabolites for metabolomic analysis.

2.3 Nano ESI sources

A nESI source is characterised by the narrow bore nanospray emitter. The sensitivity and reproducibility of nESI analysis is directly related to the quality of the emitter [75, 76]. Several factors such as the emitter material, tip geometry and the internal diameter influence the quality of nESI emitters [49, 75, 77]. For metabolomic applications, metal emitter may provide the most robust qualities and can be cleaned thus reducing costs associated with replacing damaged or blocked emitters [75, 78]. It is worth noting that homemade emitters can be constructed and these are generally less expensive that commercial ones. The geometry of the emitter is an important consideration for small molecule analysis as it impacts upon spray stability and emitter longevity. The use of tapered emitters has been demonstrated to have a shorter usable lifetime than non-tapered tips, mainly as a result of clogging [77]. In other studies, tapered emitters have been found to produce a more stable nanospray, meaning that more reproducible mass spectra can be produced

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[75, 76]. The internal diameter of the emitter is a further source of increased ionisation efficiency of the nESI source. A study by Lopes et al. [75] investigated 4 internal diameters; 5, 10, 20 and 30 µm and found that all emitters had similar sensitivity in terms of spectral features and total ion current. However, it was determined that the 30 µm was preferred as clogging is less frequent due to the larger orifice [75]. In other studies, the smaller the internal diameter, the greater the observed ionisation efficiency, however, emitter clogging became problematic at internal diameters below 10 µm [79].

With these factors considered, the use of non-tapered emitters with an internal diameter in the range of 10-30 µm offers an acceptable compromise between sensitivity, system longevity and potential loss of sample.

3. Robustness of nanoflow platforms for metabolomics

In metabolomics, the ability to generate highly repeatable and reliable data is a key factor to ensure high quality studies can be carried out [80, 81]. Two major sources of variation that limit the quality of metabolomic data sets are retention time drift and poor peak intensity reproducibility [80, 82]. The stability of retention time in particular is of great significance during the peak picking process prior to multivariate statistical analysis. To date, many studies have reported on retention time and mean peak area repeatability while analysing a broad range of compounds and matrices. While many of these have been in targeted approaches they provide a good indication as to the suitability for untargeted small molecule analysis.

3.1 Retention time stability

For metabolomic analysis, it has been suggested that the % coefficient of variation (%CV) for retention time should not exceed 2% [24]. The retention time variation reported in studies using both direct and split nanoflow systems for small molecule analysis in targeted and untargeted studies are detailed in Table 2. The range of CVs reported was lower with direct nanoflow systems

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(<0.20% to 2.2%) than for split flow ones (0.50-3.4%). Furthermore, all reported CVs were below the 2% threshold with direct nanoflow systems (with the exception of the 2D nLC system) and usually lower than 1% (in 12 out of 19 studies). On the other hand, 3 out 13 reported CVs were higher than the 2% threshold for the split flow and these systems typically have CVs greater than 1% (in 9 out of 13 studies). Overall, these studies reported highly reproducible results for retention times but seems to highlight the importance of pump fed direct nanoflow for retention time stability [24].

The number of metabolomic studies reporting retention time stability for nanoflow platforms is very limited. To date, only two have reported retention time stability of metabolites [11, 62]. In both studies, the CV was below 1.9%. A further metabolomics study has reported the retention time stability of 6 internal standards, and CVs below 0.2% were reported for these internal standards spiked in plasma and urine [73].

Comparing retention time repeatability reported in different studies is quite difficult because of differences in chromatographic methods (e.g. solvents, additives such as formic acid) and matrices used for the experiment. Nevertheless, the reported CVs for the split and direct nanoflow platforms suggest that reproducible retention characteristics are achievable on both nanoflow systems even if direct nanoflow provides slightly more retention time reproducibility.

3.2 NanoESI MS response stability

It has been suggested that a CV of up to 30% is acceptable for variation of mean peak area in metabolomics analysis [83]. The mean peak CV has been reported in a number of studies using nanoflow systems, covering a wide range of analytes and samples matrices (Table 3).

Overall, these targeted and untargeted studies indicate that a high degree of peak area reproducibility can be achieved since CV of peak area calculated for a wide range of metabolites were lower than 20% in all but one study. There appears to be little difference between emitter geometry and internal diameters for those studies, showing that no single diameter or geometry

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appeared preferential in terms of reproducibility for these studies. The low reproducibility reported by Kiefer et al. [84] may be due to the fact that ion pairing solvents were in use which can be less stable in the ESI source. Moreover, this was reported for only 1 compound (all others being below 14%). While these studies investigated only a limited number of compounds, they demonstrate that nLC-nESI is reproducible in terms of peak area.

As with retention time stability, the mean peak area reproducibility in metabolomic analysis has only been investigated in a small number of cases. In these cases the variation in mean peak areas for the whole metabolome were investigated using either the method or a modification of the method proposed by Want et al. [83]. In all these studies, >70% peaks of quality control samples returned CV of <30% for negative and positive nESI respectively [11, 62-64]. Furthermore, Jones et al. reported that >50% of all peaks measure in the metabolome of yeast cell extracts had a CV of < 14% [70]. Each of these studies indicates that the reproducibility of the nESI source is capable of supplying reliable results for metabolomics.

4. Sample preparation strategies for metabolomic studies using nLC-nESI

Sample preparation is an important aspect to consider for the use of nanoflow platforms for metabolomics [11, 73, 85]. The narrow bore columns, emitters and connections are prone to blockages and consequently, efficient sample clean-up are recommended to remove particulates, salts and proteins [11]. In the field of untargeted metabolomics, it has often been argued that selective sample preparation such as solid phase extraction (SPE) should be avoided in order to have the whole picture of the sample. However, several studies have shown that sample preparation based on SPE can increase the coverage of the metabolome compared with conventional protein precipitation (PPT) as well as the repeatability of the method [11, 86-88].

Sample preparation for metabolomic purposes that allow for the efficient removal of interfering matrix components other than PPT include, for instance, offline or online SPE, solid-phase

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microextraction (SPME), ultrafiltration, delipidation using commercially available lipid depletion plate and Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) methods [11, 25, 87, 89-92]. These techniques can be used on their own or in combination with other techniques such as LLE, with or without fractionation [93]. Among these different techniques, delipidation (mainly lyso- and phospholipids) of the sample has gained increased interest because it can reduce ion-suppression effects and can increase column lifetime [11, 90]. These recent innovations are effective for removing phospholipids and they have been shown, in combination to solvent deproteinisation, to enhance analyte detection of non-lipid species in comparison to extractions with organic solvents and a membrane-based solvent free technique [87]. It is also worth noting that miniaturised sample preparation techniques such as SPME [94], stir bar sorptive extraction (SBSE) [95]or microextraction by packed sorbent (MEPS) [96] may be applied to nanoscale LC, and applications in metabolomic studies are numerous in the case of SPME [94, 97].

Examples of applications of these sample preparation techniques in metabolomics-based approaches using a nLC-nESI system include a study where plasma samples were extracted using phospholipid filtration plates in combination with polymeric or mixed mode exchange SPE [11]. Another study described a SPE methodology to enable a high urine equivalence to be injected on a nLC-nESI-MS platform [62]. Additionally, lyophilisation and SPE has been used to prepare yeast samples for metabolomic analysis using a nLC-nESI-MS platform [70]. These studies showed that injections of more concentrated extracts onto nLC-nESI-MS platforms using more selective sample preparation can result in a wider coverage of the metabolome and can also extend the column life time.

In addition, column trapping has also been used in metabolomics-based approaches using a nLC-nESI system to load a larger volume of sample. While the metabolites are “trapped”, mobile phase continues to wash through the column thus removing any salts and any other unretained contaminants [68]. This method can be used to replace offline SPE and automate the entire sample preparation and analysis process [98].

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5. Current and potential use of nLC-nESI in metabolomics

Although still limited, several papers have already used nLC-nESI-MS platforms in metabolomics. These studies have highlighted that these systems may be up to 2000 fold more sensitive than conventional LC-ESI-MS [73] and with LOD and LOQs up to 300 fold lower using nLC-nESI-MS [85]. Two studies have also shown how nLC-nESI-MS can be combined with chemical isotope labelling to further increase the coverage of the metabolome and enhance the relative quantification of metabolites [68, 98]. In this section, we review the different studies which have used nLC-nESI-MS systems in metabolomics according to their applications.

5.1 Health and disease metabolomics

The prospect of very highly sensitive analysis of low abundance or difficult to ionise metabolite species provides an exciting prospect for metabolomic studies of health and disease. Metabolic pathways of particular interest are, for instance, conjugated and unconjugated sex steroids such as the estrogens and androgens which are implicated in several cancers, infertility and other related endocrine disruption problems [99]. These metabolites are typically found at low concentrations and usually difficult to ionise using conventional ESI-MS. Also of interest and found at low concentrations are metabolites involved in inflammation and several disease pathways such as eicosanoids [100].

A couple of early metabolomics-based studies investigated the effect of dietary compounds on health and disease using nLC-nESI-MS. In one study, the potential for phenol rich olive oil was investigated for its cell proliferation inhibition capacity in colon cancer [101]. The other nutraceutical investigation using nLC-nESI-MS was implemented to investigate the health benefits associated with the consumption of citrus juice [65]. In this study, significant changes in steroidogenesis pathways were detected following citrus juice consumption. Some of the detected steroids, in particular 17-hydroxyprogesterone, are typically found at low levels in urine, thus requiring a highly sensitive analysis to detect these changes.

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In human health and disease, four studies using nLC-nESI-MS have been carried out to date, one on HIV [64], one on drug resistant multiple myeloma [70], a third on paediatric spondylarthrosis [66] and the most recent investigating lung disease in new-born neonates [67]. The metabolomic analysis of anti-retroviral toxicity in the urine of HIV positive patients detected for the first time all the anti-retroviral drugs and a wide range of their metabolites in addition to several low abundance endogenous metabolites in an untargeted analysis (see Fig. 2 for example of chromatograms) [64]. The most recent study investigated exhaled breath condensates from two lung morphologies in intubated neonates (<20 days old). The ability to analyse samples such as exhaled breath condensates requires a highly sensitive technique due to the low volumes available. Concentrations of a number eicosanoids were found to differentiate between the control group and the diseased lung groups highlighting an inflammatory aspect of the conditions [67]. Furthermore, eicosanoids were detected in 60% of all samples underlying the sensitivity of the nLC-nESI-MS approach used as previous analysis has shown these compounds are present at a pg/mL level in exhaled breath condensates [67, 102].

An example of application using targeted metabolomics includes a study where a sensitive nLC-nESI-MS method was developed to monitor 184 phosphorus-related metabolic changes in small volumes of cancer cells treated with metabolic enzyme inhibitors [103].

These studies indicate that nLC-nESI-MS platforms are well suited for metabolomic analysis in health and disease studies. Using nLC-nESI-MS to uncover low abundance signalling metabolites, it may become possible to characterise unique metabolites and metabolic pathways affected in different cancers and diseases.

5.2 Exposure assessment and biomonitoring studies

With regards to the very high diversity of xenobiotics present in the environment, metabolomics-based approaches using highly sensitive nLC-nESI platforms offer exciting perspectives to detect xenobiotic mixtures (i.e., the chemical exposome), usually present at trace

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levels, in environmental and biological samples and simultaneously study their potential associated health effects [104, 105].

To date, the use of nLC-nESI-MS platforms in untargeted analysis for exposure assessment has been very limited. It includes a study in which a metabolomics-based approach using a nUPLC-nESI-TOFMS platform was used to investigate the chemical mixtures accumulating in fish exposed to a treated wastewater effluent and the associated changes in the tissues metabolome [23]. A wide range of contaminants including 31 pharmaceuticals from 11 different classes, endocrine disrupters, personal care products, pesticides, antibacterials and human dietary products were detected, sometimes at very low concentrations, in blood plasma and tissues of effluent-exposed fish (see Fig. 3 for example of chromatograms). Concurrently, metabolite profiling revealed for the first time a widespread reduction (between 50% and 90%) in prostaglandin (E2 and F2α) profiles in effluent-exposed fish tissues/plasma [23]. Another example of application using nLC platform for exposure assessment includes a study where an untargeted approach was used to investigate changes in a small benthic invertebrates exposed to a wastewater treatment plant effluent. Significant changes in lipid metabolism were observed and several xenobiotics were detected (e.g., ibuprofen and propranolol) in invertebrates exposed to the wastewater treatment plant effluent using very small invertebrate samples (i.e., 12 mg) [92].

These studies show that highly sensitive untargeted techniques based on nanoscale could be extremely useful to identify xenobiotics that need to be prioritised for future toxicological investigations.

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6. Conclusions and future perspectives

The current literature surrounding nLC-nESI clearly demonstrates that significant gains in sensitivity for metabolites can be achieved compared to conventional LC-ESI platforms (see Table 4 for a summary of advantages and drawbacks of nLC-nESI platforms). While in the past there has

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been concern about nLC retention time reproducibility, the review of existing data shows that the improvements made since the introduction of nanoflow, such as the implementation of direct nanoflow platforms, have greatly contributed to improve the reproducibility of these platforms since the majority of these studies fall within the guidelines proposed within the metabolomic community. As mentioned in this review, one key factor is sample preparation, which is required to extend column and emitter lifetimes by reducing blockages. While this process is indeed more time consuming and may be seen as selective, several studies have shown that more extensive sample preparation can increase the coverage of the metabolome as it allows injection of more concentrated extracts and, on the other hand, improve the repeatability of the method. Hence, more automated sample preparation methods should be developed in the future to implement metabolomic methods based on nLC-nESI platforms for high throughput applications. Taking the current nLC-nESI methods and potential future advances into account, new and exciting possibilities exist to expand impact of metabolomics on human and environmental health and disease. The increased sensitivity of these nLC-nESI platforms offer the potential to uncover the roles played by low abundant signalling metabolites in health and disease studies, and would also enable an improvement in the detection of xenobiotics present at trace levels in biological matrices to better characterise the chemical exposome and uncover potential health outcomes associated. While many aspects of metabolomics are still under progress (e.g., development of more automated annotation work-flows to overcome problems linked to unidentified features), the development of analytical methods of higher sensitivity is also a priority to be as comprehensive as possible.

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Table 1: Overview of existing nLC-nESI instrumentations and associated parameters (nanocolumns characteristics, flow rate, injection volume, run time and matrices) used in metabolomic studies.

nLC	MS	Flow rate (nL/min)	Column ID (µm)	Length (mm)	Particle size (µm)	Phase	Injection volume (nL)	Run time (min)	Matrice	Refs
Thermo Ultimate 3000 nano	Thermo LTQ-Orbitrap	1000	150	150	5	HILIC	Not defined	60	CSF	[69]
Eksigent nanoLC Ultra	Thermo LTQ-Orbitrap	500	100	100	5	C18	1000	45	*In vitro* cell lines	[84]
Bruker EASY-nLC	Bruker MicroTOF	300	75	100	3	C18	5000	47	*In vitro* cell lines	[106]
Agilent 1100 Series	Bruker MicroTOF	312	75	150	3	C18	6.25	23	Urine	[65]
Waters nanoAcquity	Orbitrap Elite	300	75	100	1.7	BEH C18	2000	60	*In vitro* cell lines	[70]
Waters nanoAcquity	Waters XEVO G2 QTOF	700	100	100	1.8	HSS-T3	500	50	Urine/Plasma	[73]
Waters nanoAcquity	Waters XEVO G2 QTOF	700	100	100	1.7	BEH C18	500	50	Plasma	[11]
Waters nanoAcquity	Waters XEVO G2 QTOF	700	100	100	1.8	HSS-T3	500	50	Urine	[62]
Waters nanoAcquity	Waters QTOF Premier	350	75	200	1.7	Acclaim Pepmax C18	5000	45	Urine and sweat	[68]
Waters nanoAcquity	Waters XEVO G2 QTOF	700	100	100	1.8	HSS-T3	500	50	Urine	[63]
Eksigent 415 - nanoflex cHiPLC	Sciex 5600 Triple TOF	300	200	150	Not defined	C18	5000	24	Faeces	[66]

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Waters nanoAcquity	Waters XEVO G2 QTOF	700	100	100	1.8	HSS-T3	500	50	Urine	[64]
Waters nanoAcquity	Waters XEVO G2 QTOF	700	100	100	1.8	HSS-T3	500	50	Plasma and tissues	[23]
Agilent 1100 Series	Thermo 7-T LTQ-FT Ultra	300	75	120	3	C18	2000	105	Exhaled breath condensate	[67]
Waters nanoAcquity	Bruker Impact HD QTOF	350	75	150	2	Acclaim Pepmax C18	11.4 pmol of sample	45	*In vitro* cell lines	[98]

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Table 2: Relative standard deviation (RSD) of retention time (RT) for small molecules (<1000 Da) analysed in different matrices using direct (D) and split (S) flow nLC platforms.

Analyte	Matrix	Flow rate (nL/ min)	RT (%RSD)	nLC system	Flow type	Ref
6 Deuterated standards	Plasma and urine	700	<0.20 %	Waters nanoAcquity UPLC	D	[73]
100 (Xeno)metabolites	Plasma	700	<1.9%	Waters nanoAcquity UPLC	D	[11]
100 Metabolites	Urine	700	<1.9%	Waters nanoAcquity UPLC	D	[62]
8 Amino acids	Urine and sweat	350	<1.1%	Waters nanoAcquity UPLC	D	[68]
Tryptic digests	Bovine serum albumin	325	0.73%	Proxeon EASY-nLC	D	[107]
Tryptic digests	Bovine serum albumin	325	0.66%	Waters nanoAcquity UPLC	D	[107]
Tryptic digests	Bovine serum albumin	325	0.87%	Eksigent nanoLC-Ultra	D	[107]
Tryptic digests	Bovine serum albumin	325	2.2%	Eksigent nanoLC-2D,	D	[107]
Pharmaceuticals	Standard solution	300	<0.50 %	Agilent 1200 HPLC	D	[108]
Biogenic amines	Wine	634	<1.2%	Dionex Ultimate Capillary HPLC	D	[109]
Nonglycosylated tryptic peptides	Plasma	450-500	<1.6%	Waters nanoAcquity UPLC	D	[110]
Phosphoproteins	Semen	300	2.0%	Waters nanoAcquity UPLC	D	[111]
Penicillin antibiotics	Standard solution	200	<0.35 %	Dionex Ultimate 3000 nano LC	D	[112]
Pharmaceuticals	Standard solution	300	<0.50 %	Dionex Ultimate	D	[113]
Phenolic compounds	Standard solution	300	≤0.80 %	Bruker Easy-nLC™	D	[114]
Peptides	HeLa protein standard	20-50	≤0.30 %	Thermo Ultimate 3000 RSLC nano LC	D	[115]
Glycans	Plasma	500	<0.50	Eksigent Technologies nanoLC 2D	D	[1

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			%			16]
Peptides	Mitochondrial extracts	200	≤0.65 %	Eksigent direct-flow nano-LC pumps	D	[117]
Parabens	Standard solution	200	<0.50 %	AT10PV nanoGR generator	D	[118]
Pharmaceuticals	Standard solution	800	<2.0%	Rheos 2000 micro-pump	S	[38]
Oligosaccharides	Standard solution	300	<0.50 %	Agilent 1000 HPLC	S	[119]
Plant hormones	Tobacco seeds	350	≤1.1%	LC Packings Ultimate	S	[120]
Hormones, pesticides and PAHs	Standard solution	250	<2.0%	Kontron instruments 420 dual-pump binary gradient HPLC	S	[121]
Tryptic digests	Bovine serum albumin	400	<2.5%	LC-10AD$_{VP}$ solvent delivery pump	S	[122]
Perfluorooctanoic acid/ sulfonate	River water	700	<1.0%	Agilent 1100 HPLC	S	[123]
Organophosphorous pesticides	Standard solution	360	<1.0%	Dionex Ultimate Capillary HPLC	S	[124]
Flavanones	Citrus juice	500	<2.4%	Spectra System P2000 HPLC pump	S	[125]
Polyphenols	Bee pollen	500	<1.8%	Spectra System P2000 HPLC pump	S	[126]
Anthocyanins	Fruit juices	400	<3.4%	Dionex Ultimate–LC Packings	S	[127]
Aloe-based phytotherapeutics	Standard solution and leaf	350	<1.5%	Rheos 2000 micro-pump	S	[128]
Synthetic cannabinoids	Herbal mixtures	500	<1.7%	Rheos 2000 micro-LC pump	S	[129]
Peptides	Serum	200	<0.80 %	Finnigan quaternary Surveyor pump	S	[130]

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Table 3: Relative standard deviation (RSD) of peak area for small molecules (<1000 Da) analysed in different matrices using nLC-nESI platforms

Analyte	Matrix	Peak area (%RSD)	Emitter material	Emitter ID	Ref
Deuterated and normal standards	Standard solution/ urine and plasma	<18%	Fused silica	10 µm	[73]
Cationic metabolites	Cerebrospinal fluid	<20%	Fused silica	8 µm	[69]
8 Amino acids	Urine and sweat	<7%	Fused silica	5 µm	Z. L[68]
Tryptic digests	Cellular protein extract	<15%	Fused silica	5 µm	[131]
Ceramides	Cerebral spinal fluid	<15%	Fused silica	10 µm	[132]
Pharmaceuticals	Standard solution	<11%	Fused silica	10 µm	[113]
Anthocyanins	Fruit juice	<16%	Fused silica	25 µm	[127]
Pharmaceuticals	Standard solution	10%	Fused silica	25 µm	[38]
Perfluorooctanoic acid/perfluorooctane sulfonate	River water	<20%	Fused silica	30 µm	[123]
Aloe-based phytotherapeutic products	Standard solution and leaves	<12.5%	Fused silica	25 µm	[128]
Ketamine and metabolites	Human hair	≤4%	Tapered fused silica	20 µm	[133]
Plant hormones	Tobacco seeds	<11%	Tapered fused silica	8-9 µm	[120]
Phenolics	Olive oil and cell culture	<4%	Tapered fused silica	Not defined	[106]
Phenolic compounds	Standard solution	<7%	Tapered fused silica	Not defined	[134]
Phenolic compounds	Standard solution	<15%	Tapered fused silica	Not defined	[114]
Metabolite standards	Cell extracts	<23%	Silica	10 µm	[84]
Pharmaceuticals	Standard solution and plasma	<10%	Not defined	75 µm	[108]
Phytohormones	Leaves	<12%	Not defined	8 µm	[135]
Peptides	Serum	≤25%	Not defined	5 µm	[130]
50 Glycans	Serum standard	<10%	Not defined	Not defined	[136]
Polyphenols	Urine	≤13%	Not defined	Not defined	[137]
Penicillin antibiotics	Milk, liver, kidney	<8%	Not defined	Not defined	[112]
Wax esters	Seed oil extracts	<5%	Not defined	Not defined	[138]
Oligosaccharides	Standard solution	<5%	Not defined	Not defined	[119]
Environmental contaminants	Benthic tissue extracts	<13%	Not defined	Not defined	[139]

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Pharmaceuticals	Benthic tissue extracts	<15%	Not defined	Not defined	[140]
Carbamazepine, testosterone and oxazepam	*Gammarus fossarum*	<12%	Not defined	Not defined	[141]
Peptides	Mitochondrial extracts	<12%	Not defined	Not defined	[117]

Table 4: Advantages and drawbacks of nLC-nESI compared to conventional LC-ESI platforms for metabolomic analysis

Advantages	**Drawbacks**
Increased ionisation efficiency in the source and transfer efficiency in the mass spectrometer	More care to be done for sample preparation
Reduced chromatographic dilution	Less sample throughput due to longer LC run time
More comprehensive analysis due to the detection of trace level metabolites	More prone to column/emitter blockages
Reduced cost and environmental impact by using less solvent	Less array of column chemistries readily available
Reduce need for desolvation gas and temperature in the source	More expertise needed
Possibility to convert existing proteomic platforms for metabolomic analysis	

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Figure captions

Figure 1: Base peak intensity (BPI) chromatograms of a standard mixture of compounds analysed by BEH C18 300 and 130 Å and HSS T3 100 Å nUHPLC columns. The base peak intensity peaks of selected standards are labelled to demonstrate the different retention characteristics of each column. TIC = total ion count. 1, unretained polar compounds, 2, metropolol; 3, venlafaxine; 4, propranolol; 5, carbamazepine; 6, testosterone; 7, androstenedione; 8, sphingosine; 9, diazinon; 10, tris(2-butoxyethyl) phosphate. Reproduced with permission from Ref [73].

Figure 2: Positive ESI mode base peak intensity (BPI) of pooled HIV patient urine run using nLC-nESI-TOFMS (A) and extracted ion chromatograms of protease inhibitors and their metabolites (B) as described in [64]. D= darunavir, DM1-3 = darunavir metabolites 1-3, A= atazanavir, AM1-5 = atazanavir metabolites 1-5, R=ritonavir, RM1-2 = ritonavir metabolites 1-2). Samples run on a Waters nanoAcquity-nanoESI-XEVO G2 TOFMS with a 100 mm x 100 µm x 2.8 µm x 100 Å HSS T3 column.

Figure 3. Example of base peak intensity (BPI) chromatograms (A) of testis extracts from fish exposed to wastewater effluent in positive (+ESI) and negative (-ESI) modes. Chromatograms B show overlaid signals of selected xenobiotics extracted using their exact masses. Chemical identity was confirmed from accurate mass, isotopic fit and fragmentation data obtained from high energy collisional induced dissociation and from comparison with standard compounds. Testis samples were extracted by Strata-X-C solid-phase extraction after phospholipid removal and profiled in –ESI or +ESI modes by nUHPLC-nESI-TOFMS as described in [23].1=10 Hydroxyamitriptyline;2=Norclozapine;3=citalopram;4=Amitriptyline;5=norsertraline;6=sertraline;7=clopidogrel

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Figure 1.

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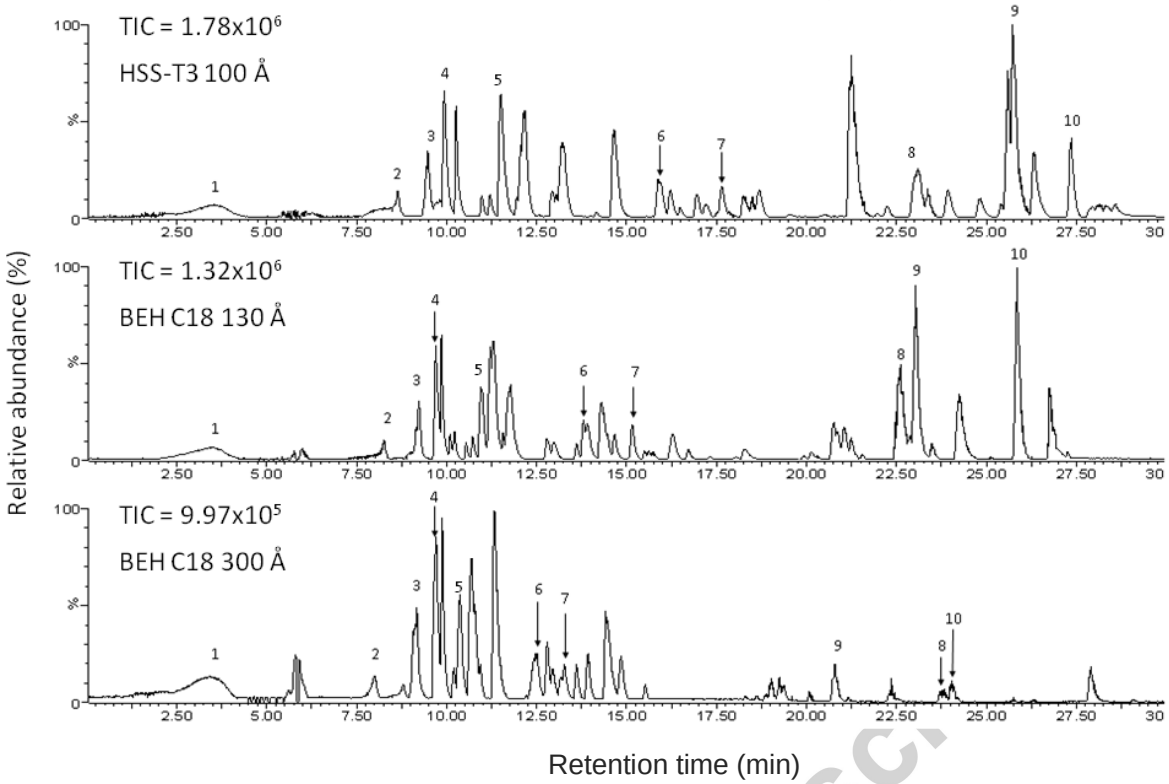

Figure 2.

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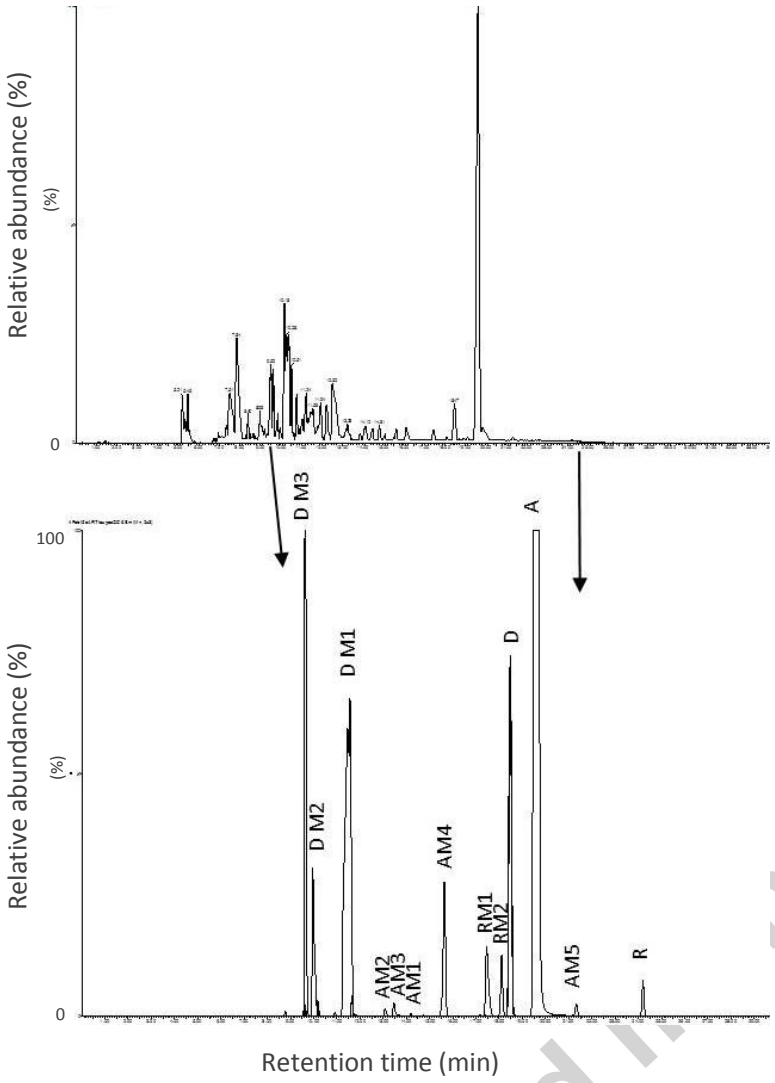
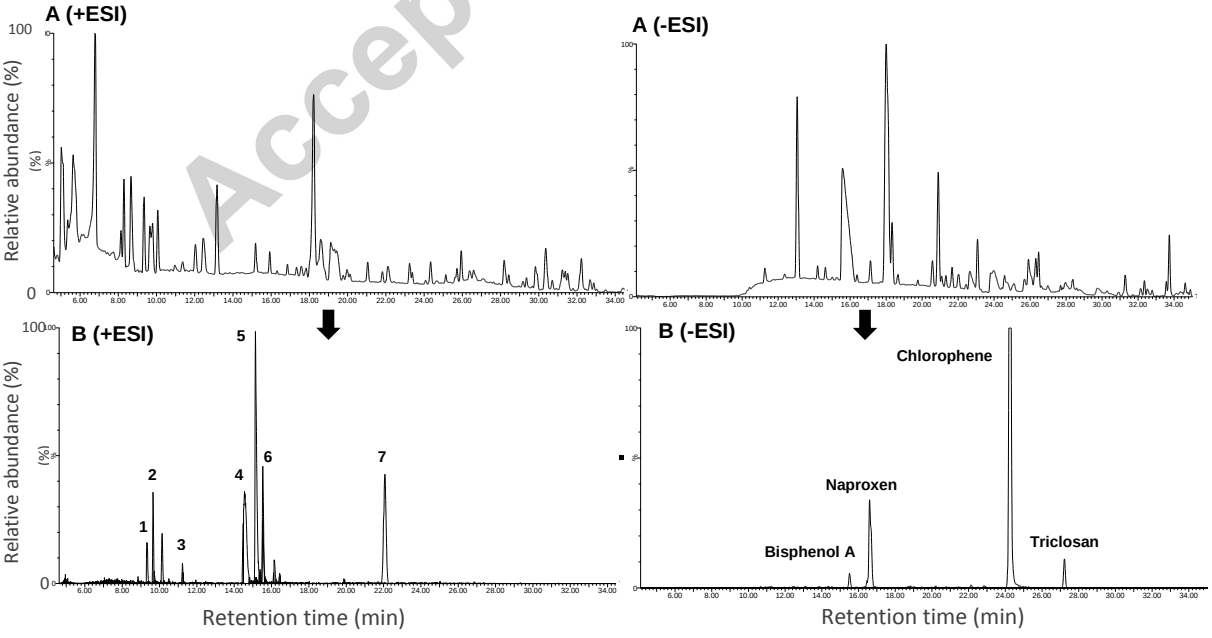


Figure 3.



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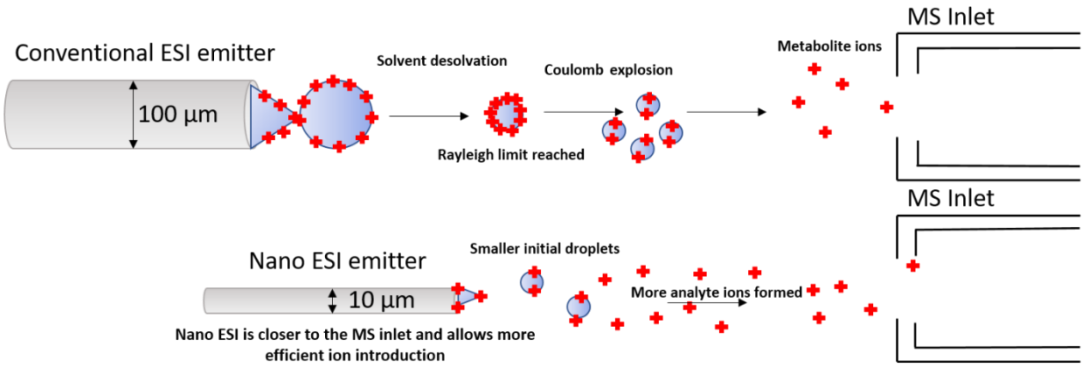
Graphical abstract



Highlights

- nanoLC-nanoESI-MS offers increased ionisation efficiency for small molecule analyses
- nanoLC-nanoESI-MS is reproducible making it suitable for metabolomics
- Sample preparation is a key step for nanoLC-nanoESI-MS
- Applications of nanoLC-nanoESI-MS in untargeted studies offer promising perspectives

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