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On-line duplex molecularly imprinted solid-phase extraction for analysis of low-abundant biomarkers in human serum by liquid chromatography-tandem mass spectrometry

Nicholas McKitterick[a], Tugrul Cem Bicak[b], Magdalena A. Switnicka-Plak[b], Peter A.G. Cormack[b,*], Léon Reubsaet[a], Trine Grønhaug Halvorsen[a,*]

[a] Section for Pharmaceutical Chemistry, Department of Pharmacy, University of Oslo, PO Box 1068 Blindern, 0316 Oslo, Norway
[b] WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, Thomas Graham Building, 295 Cathedral Street, Glasgow G1 1XL, Scotland, UK

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ABSTRACT

In the present work, a pair of molecularly imprinted polymers (MIPs) targeting distinct peptide targets were packed into trap columns and combined for automated duplex analysis of two low abundant small cell lung cancer biomarkers (neuron-specific enolase [NSE] and progastrin-releasing peptide [ProGRP]). Optimization of the on-line molecularly imprinted solid-phase extraction (MISPE) protocol ensured that the MIPs had the necessary affinity and selectivity towards their respective signature peptide targets – NLLGLIEAK (ProGRP) and ELPLYR (NSE) – in serum. Two duplex formats were evaluated: a physical mixture of the two MIPs (1:1 w/w ratio) inside a single trap column, and two separate MIP trap columns connected in series. Both duplex formats enabled the extraction of the peptides from serum. However, the trap columns in series gave superior extraction efficiency (85.8±3.8% and 49.1±6.7% for NLLGLIEAK and ELPLYR, respectively). The optimized protocol showed satisfactory intraday (RSD≤23.4 %) and interday (RSD≤14.6%) precision. Duplex analysis of NSE and ProGRP spiked into digested human serum was linear ($R^2 \geq 0.98$) over the disease range (0.3-30 nM). The estimated limit of detection (LOD) and limit of quantification (LOQ) were 0.11 nM and 0.37 nM, respectively, for NSE, and 0.06 nM and 0.2 nM, respectively, for ProGRP. Both biomarkers were determined at clinically relevant levels. To the best of our knowledge, the present work is the first report of an automated MIP duplex biomarker analysis. It represents a proof of concept for clinically viable duplex analysis of low abundant biomarkers present in human serum or other biofluids.

1. Introduction

The analysis of clinically relevant protein biomarkers has played an indispensable role in medicine for decades. To date, the most widely available and viable technologies available to clinicians are singleplex immunoassays [1]. Some diseases can be monitored adequately by the quantification of a single analyte (e.g., HIV-1 p24) [2], however myriad diseases require the determination of two or more biomarkers for improved diagnosis and treatments. Small cell lung cancer (SCLC) is one such disease, with two key protein biomarkers requiring determination for optimal clinical management: progastrin-releasing peptide (ProGRP) and neuron-specific enolase (NSE) [3]. Presently, these biomarkers are analyzed *via* two singleplex assays; however, this approach can be laborious, expensive, and require larger quantities of patient sample compared to a potential multiplex assay [4]. Over the last 20 years, there have been considerable developments in multiplex immunoassays, including assays brought to the market [5]. This is due to the considerable advantages of multiplex assays, including reduced labour and consumable costs, lower sample volumes, lower turnaround times, and improved disease monitoring [5]. More detailed and in-depth biomarker information allows for a greater focus on patient-specific treatments [2]. Therefore, the development of novel multiplex technologies will contribute to improvements in patient care and clinical outcomes.

The key limiting factors associated with multiplex immunoassays are reduced selectivity and increased cross-reactivity [6]. Due to the quantification methods used in many immunoassays (i.e.,

* Corresponding authors.
E-mail addresses: Peter.Cormack@strath.ac.uk (P.A.G. Cormack), trinegha@farmasi.uio.no (T.G. Halvorsen).

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the use of detector antibodies), cross-reactivity can potentially hamper assay performance, resulting in raised detection limits due to higher background noise and an increase in the frequency of false positives and false negatives [2,6]. The prevalence of cross-reactivity among antibodies is near-ubiquitous and presents a significant technical challenge to be overcome [7].

Mass spectrometry (MS), or more specific multiple reaction monitoring (MRM), allows for highly selective, sensitive and accurate analyses of multiple proteins and peptides without cross-reactivity. However, a selective sample clean-up step is necessary to determine low abundance proteins [8]. The use of immunoaffinity clean-up in combination with MS/MS has been shown to have greater differential power than conventional immunoassays while circumventing the limitations of cross-reactivity and improving the detection limits of mass spectrometry [9]. Single immobilised antibodies have been used to successfully quantify multiple protein isoforms from a single sample [10–12] as well as protein cleavage products [13,14]. Furthermore, combinations of immobilized antibodies have been used to quantify multiple protein biomarkers from a single sample, including multiplex analyses of the SCLC biomarkers ProGRP and NSE in patient samples [15–18].

Whereas the use of immobilised antibodies is effective in multiplex analyses, some limitations persist, such as the cost of novel functional antibody production and automation difficulties. On-line extraction of proteins and peptides offers significant benefits, namely: ease of use, limited manual sample preparation, and analyses with higher throughputs. Antibodies have been immobilised in columns for use in on-line immunoextraction and proteolytic digestion [19–21]. Immobilised antibody columns for use in bottom-up proteomics are limited by fewer applicable mobile phases and extraction conditions. Organic solvents (e.g., acetonitrile, MeCN) and acids (e.g., formic acid, FA), which are used routinely in ESI-MS, can perturb the paratope, or cleave the antibody itself [21].

Molecularly imprinted polymers (MIPs) are synthetic polymers with antibody-like molecular recognition properties. MIP synthesis involves the formation of a three-dimensional polymer network incorporating complementary functional monomers (FMs) around a template molecule. Template removal reveals cavities within the polymer network that are selective for the target molecule. MIPs are highly versatile and have myriad formats, including, but not limited to: monoliths, micro-, nano-, and magnetic particles, membranes, and films. The crosslinked, polymeric nature of MIPs imparts temporal stability, which is ideal for their use in on-line analyses. MIPs applied in an on-line mode of operation have been used to extract proteins and peptides from human serum, including ProGRP, NSE, and others [22–24]. MIPs targeting the signature peptides of ProGRP (i.e., NLLGLIEAK) [24] and NSE (i.e., ELPLYR) [23] were prepared, optimised and used for single-plex on-line extractions in human serum with detection limits in the low pM and low nM range, respectively. To date, multiplex analyses using single or dual/multiple template MIPs as clean-up media for small molecule analytes have been described for water analysis [25,26], the analysis of dairy products [27] and meat [28], and for urine analysis [29]. These reports are based on off-line MIP extraction, such as conventional SPE [26,27], magnetic SPE [25,29], and stir bar sorptive extraction [28]. To the best of our knowledge, on-line MIP analysis has not yet been described for multiplex MIP extraction and, in addition, there are very few studies that report MIP-multiplexing in biomarker analysis. A recently reported MIP-multiplex assay involved the addition of isotope-labelled mass tags into plasma and extraction by a single magnetic MIP imprinted against the mass tags [30]. Another study described the multiplex analysis of three protein isoforms where a peptide fragment common to three isoform-specific surrogate peptides was used as template in the MIP production [31].

The present study aimed to evaluate the potential for automated MIP-duplex extraction and MS/MS analysis of the signature peptides of protein biomarkers. Building upon previous disclosures, two MIPs optimised to capture one or other of the signature peptides of the SCLC biomarkers ProGRP (NLLGLIEAK) [24] and NSE (ELPLYR) [23] were combined for duplex analysis. The extraction conditions were optimised for extraction affinity and selectivity, and two different duplex formats were evaluated: a physical mixture of the two MIPs inside a single trap column, and two MIP trap columns connected in series. MIP-duplex extractions from digested human serum were performed to determine the viability of duplex biomarker analysis. This work represents a proof of concept for MIP duplex biomarker analysis. To our knowledge this is the first report of on-line analysis of more than one analyte using duplex MISPE, and the model system explored is highly relevant for the follow-up of SCLC treatments.

2. Methods and materials

2.1. Materials

Formic acid (FA, MS grade, ≥98%), dithiothreitol (DTT, ≥98%), and iodoacetic acid (IAA, ≥98%) were purchased from Sigma Aldrich, St Louis, MO, USA. Acetonitrile LC-MS grade (MeCN, 99.9%), was purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (BioUltra, ≥99.5%) was purchased from Fluka (Milwaukee, WI, USA). Water was filtered through a Merck Millipore Milli-Q Integral 3 water dispenser (resistivity: 18.2 MΩ cm). Human serum from consenting healthy individuals was obtained from Oslo University Hospital, Ullevål (Oslo, Norway). All serum samples were stored at -32°C.

2.2. Human serum

Serum from healthy donors was used in strict adherence to Norwegian Law (*Lov om medisinsk og helsefaglig forskning [helseforskningsloven]*). The present research project has been registered in the database for health-related research at the Department of Pharmacy, University of Oslo (Oslo, Norway). All serum samples were prepared and analyzed using methods following all relevant ethical guidelines and regulations.

2.3. Peptide and protein standards

Stable isotope labelled internal standard (IS) peptides ELPLY[R_$^{13}C_6{}^{15}N_2$] (>95%) and NLLGLIEA[K_$^{13}C_6{}^{15}N_2$] (>95%) were purchased from Innovagen (Lund, Sweden). Bovine serum albumin (BSA) and trypsin (TPCK-treated) from bovine pancreas (sequencing grade) were purchased from Sigma Aldrich. NSE was obtained from Scripps Laboratories (San Diego, CA, USA). Recombinant ProGRP isoform 1 was obtained from Radiumhospitalet, Oslo University Hospital, Oslo, Norway. ProGRP isoform 1 was cloned from human cDNA (Origene Technologies) and expressed in *Escherichia coli* (Promega) *via* pGEX-6P-3 constructs (GE Healthcare) and purified as described previously [32]. ProGRP and NSE concentrations were determined *via* UV absorbance (280 nm, A280), diluted to the desired concentrations with 50 mM ammonium bicarbonate (ABC), and stored at -20°C.

2.4. MIP Syntheses

The syntheses of the NLLGLIEAK and ELPLYR MIP and non-imprinted polymers (NIPs) have been reported previously [23,24]. They were prepared in the form of polymer microspheres using precipitation polymerization. A summary of the chemical make-up of the MIPs and NIPs is outlined in Table 1. The detailed synthetic information can be found in Table S1.

Table 1
Chemical make-up of the MIPs and NIPs.

Polymer	Template(s)	FM	CL	Mean particle diameter (µm)
NLLGLIEAK MIP	Z-NLLGLIEA[Nle]	EAMA.HCl	DVB-80	2-3
NLLGLIEAK NIP	-	EAMA.HCl	DVB-80	2-3
ELPLYR MIP	Z-ELPLY[Nle]	TPVU	DVB-80	2.8
ELPLYR NIP	-	TPVU	DVB-80	2.4

Z=Carboxybenzyl; EAMA.HCl= *N-(2-Aminoethyl)methacrylamide*; DVB-80= divinylbenzene-80; TPVU=*N*-3,5-*bis*(Trifluoromethyl)-phenyl-*N'*-4-vinylphenylurea; FM=functional monomer; CL=crosslinker.

Table 2
Composition of the MIP and NIP trap columns.

Column	Polymer	Mass of polymer (mg)
NLL-MIP	NLLGLIEAK MIP	6
NLL-NIP	NLLGLIEAK NIP	6
ELP-MIP	ELPLYR MIP	6
ELP-NIP	ELPLYR NIP	6
1:1 mix MIP	NLLGLIEAK MIP: ELPLYR MIP	3: 3
1:1 mix NIP	NLLGLIEAK NIP: ELPLYR NIP	3: 3

2.5. Column packing

The four polymers were wet packed, either alone or in combination, into 5 × 1.5 mm ID PEEK cartridges (G&T Septech AS, Kolbotn, Norway) to give six trap columns (Table 2). This was achieved by suspending each polymer (6 mg), or 3 mg of both polymers, in 380 µL MeCN prior to ultrasonication for five minutes and subsequent vacuum-packing using heptane at a fixed pressure of 100 bar.

2.6. Protein and serum digestion

Human serum was defrosted at 4°C for 1 h. Human serum (250 µL) was diluted to 905 µL with 100 mM ABC before the addition of 100 mM freshly prepared DTT (12.5 µL) in 50 mM ABC. The samples were incubated at 60°C for 1 h and allowed to cool to room temperature before the addition of 12.5 µL of freshly prepared 250 mM IAA in 50 mM ABC. The samples were incubated at room temperature and shaken (800 rpm) for 30 min in the dark prior to the addition of 70 µL of 10 mg/mL trypsin prepared freshly in 50 mM ABC. Digestion was initiated by the addition of trypsin at an enzyme to substrate ratio of 1:20. Digestion was performed overnight at 37°C and stopped after placing the samples at -20°C until required.

NSE digest standards were prepared as described for human serum digestion, with the following exceptions: NSE (53.2 µg/mL) was reduced and alkylated by the addition of DTT (2.5 mM) and IAA (10 mM). The resulting digest had a final concentration of 50 µg/mL (1.28 nM). The digest was divided into 50 µL aliquots and stored at -32°C until further use.

BSA digest standards were prepared as described for human serum digestion, with the following exceptions: reduction and alkylation were performed by 50 mM DTT and 200 mM IAA, respectively. The BSA standards were divided into 100 µL, 1 µM aliquots, and stored at 32°C.

ProGRP standard solutions were diluted in 50 mM ABC to a concentration of 51.0 µg/mL. Digestion was performed as described for human serum digestion but omitting the reduction and alkylation step (ProGRP does not exhibit disulfide bonds). The resulting digest had a final concentration of 50 µg/mL (3.57 nM). The digest was divided into 50 µL aliquots and stored at -32°C until further use.

2.7. On-line liquid chromatography-tandem mass spectrometry

The chromatographic system consisted of an ISO-3100 A loading pump, an LPG-3400 M pump with a degasser, a WPS-3000TRS autosampler, and an FLM3000 flow-manager (all Dionex, Sunnyvale, CA, USA). Extraction was performed by the NLL-MIP ELP-MIP series trap columns. The chromatographic separation was carried out using an Aquasil C_{18} analytical column (Thermo Scientific, 100 Å, 3 μm, 50 mm × 1 mm). The extraction was performed by injection of 10 µL of sample onto the MIP trap columns, and subsequent washing of the MIPs *via* an isocratic flow of 98:2 20 mM aq FA:MeCN at 20 µL/min for 5 min. Following extraction, the system was switched to forward-flush the sample through the MIP trap columns to the analytical column, and finally to the MS for analysis. The microflow pump was directed to waste and kept at an isocratic flow of 50 µL/min at 100% mobile phase A (0.1% FA and MeCN 95:5, v/v) during extraction (i.e., for the first 5 min). After 5 min of loading, the target peptides were eluted from the MIPs. The trap columns were connected to the microflow pump and washed for 3 min at 100% mobile phase A. The target peptides were then eluted from the MIPs using a 20 min linear gradient from 0 to 85% mobile phase B (0.1% FA and MeCN 5:95, v/v). Three min into the gradient (12.75% B), the 10-port valve was switched and the analytical column was coupled directly to the microflow pump. After the gradient reached 85% mobile phase B, the analytical column was washed for 3 min with 90% mobile phase B. The MIPs and the analytical column were re-equilibrated for 20 min with 98:2 20 mM aq FA:MeCN and 100% mobile phase A, respectively. The column oven temperature was set and kept constant at 25°C.

A triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Scientific, Waltham, MA, USA) was used to determine the signature peptides and their corresponding IS by MRM in positive mode (*see Table S2 for MS transitions*). The heated capillary temperature was set at 265°C, and the spray voltage was 4 kV. Auxiliary gas (N_2): 10 arbitrary units (arb). Sheath gas (N_2): 5 arb. TSQ data was processed by Xcalibur's QualBrowser (version 2.2 SP 1.48, Thermo Scientific), and MS responses based on the peak intensity, processed automatically by a Genesis peak detection algorithm, were used. Among them, the only peaks considered were those with a signal-to-noise (S/N)-ratio above 10 and with retention time and ion ratios corresponding to those of reference samples at high concentration.

2.8. Recovery determination

Recoveries were calculated by single-point calibration towards QC samples comprised of NLLGLIEAK IS (10 nM) and ELPLYR IS (10 nM) in 50 mM ABC containing 100 nM BSA by applying the following formula:

$$\text{Recovery} = \frac{\text{peak area (sample)}}{\text{peak area (QC)}} \text{ x } 100\%$$

2.9. Optimizing initial duplex extraction conditions

NLLGLIEAK IS (10 nM in 50 mM ABC) was injected onto the NLL-MIP and washed with 100:0, 99:1, 98:2 and 97:3 20 mM aq FA:MeCN for 10 min. The analysis was performed by eluting NLLGLIEAK *via* gradient elution over the C_{18} analytical column before determination by MS/MS. The selectivity of the ELP-MIP and NLL-MIP trap columns was evaluated by the extraction of both peptides (ELPLYR IS, 10 nM, and NLLGLIEAK IS, 10 nM, in 50 mM ABC) separately on each column. The compositions of the loading buffers tested were 100:0, 99:1, 98:2 20 mM aq FA:MeCN (MIPs were washed for 10 min).

2.10. Selectivity comparison of single and duplex formats

NLLGLIEAK IS (10 nM) and ELPLYR (10 nM) in 50 mM ABC containing 100 nM of digested BSA was extracted by all MIP (and NIP) single columns, columns in series and 1:1 mix columns (n = 3). Complex samples were prepared similarly; ELPLYR IS (10 nM) and NLLGLIEAK IS (10 nM) were added to pre-digested human serum (n = 3). Each sample (10 µL) was injected onto the MIP (and NIP) columns.

2.11. Method evaluation

Digested serum samples (50 µL) were spiked with a range of digested NSE and digested ProGRP levels to give final concentrations of 0.3, 1, 5, 15, and 30 nM of both proteins (n = 4). Each sample was also spiked with 2 nM of both ELPLYR IS and NLLGLIEAK IS to a final volume of 150 µL. Linear regression (1/x weighted) using IS correction was performed. Signal to noise (S/N) ratios were used to calculate the limit of quantification (LOQ; S/N = 10) and limit of detection (LOD; S/N = 3) from samples containing 0.3 nM digested ProGRP and NSE. The method accuracy and ability to handle samples with varying levels of the two biomarkers were carried out by preparing two standards: one containing digested human serum (50 µL) spiked with 30 nM digested NSE and 0.3 nM digested ProGRP, and another containing 30 nM digested ProGRP and 0.3 nM digested NSE (both samples containing 2 nM of each IS, total sample volume 150 µL). Each sample was injected (10 µL) onto the NLL-MIP ELP-MIP series columns (n = 3). Method accuracy was determined using IS correction.

3. Results and discussion

3.1. Establishing extraction conditions

Optimized loading and elution conditions are essential for MIPs to function well as SPE sorbents for extractions from complex biological fluids. An important aspect of the loading mobile phase is optimal molecular recognition and binding site accessibility [33]. As the primary function of MIPs in SPE is the capture of the target analyte, considerations such as binding site heterogeneity and mass transfer kinetics are of less relevance in the optimisation of the loading mobile phase. The two MIPs used in the present work were optimised separately in two previous studies [23,24], and were found to require distinct optimal extraction conditions to establish affinity and selectivity: 100:0 20 mM aq FA and 97:3 20 mM aq FA:MeCN for the NLL-MIP and ELP-MIP, respectively. The MIPs differ primarily in terms of FM which leads to distinct binding properties. A duplex extraction loading mobile phase must bridge the differences between each polymer to limit compromises in affinity and selectivity. Previous work on the ELP-MIP found that 100:0 20 mM aq FA (i.e., the optimal conditions for the NLL-MIP) gave demonstrable losses in selectivity [23], while no information about NLL-MIP performance when using 97:3 20 mM aq FA:MeCN for loading was available. Therefore, to find the optimal extraction conditions for duplex extraction, the extraction efficiency (recoveries of NLLGLIEAK and ELPLYR) and selectivity of the NLL- and ELP-MIPs were evaluated *via* extractions with the following loading mobile phases: 100:0, 99:1, 98:2, and 97:3 v/v 20 mM aq FA:MeCN. The first key finding was poor retention of NLLGLIEAK by the NLL-MIP at 97:3 20 mM aq FA:MeCN (i.e., the optimal loading mobile phase for the ELP-MIP) with an extraction recovery of 27.9±4.4%, while the other evaluated combinations resulted in 100% recovery of the target analyte (Fig. 1A, blue bars).

NLL-MIP-based extractions at 100:0, 99:1 and 98:2 v/v 20 mM aq FA:MeCN (Fig. 1A) demonstrated increasing selectivity (i.e., less binding of non-specific peptide) with increasing MeCN. While the recovery of NLLGLIEAK stays rather constant, the recovery of ELPLYR drops dramatically to less than 5% for 98:2 FA:MeCN. Conversely, the ELP-MIP did not show significant improvements in selectivity with increasing MeCN in the loading mobile phase. The recovery of NLLGLIEAK was in the same range as the recovery of ELPLYR throughout the 100:0, 99:1, 98:2 v/v 20 mM aq FA:MeCN compositions (Fig. 1B). The presence of MeCN is likely to lower the level of non-specific hydrophobic interactions. For the NLL-MIP, low selectivity (defined as high recovery of a non-specific peptide) is seen at 100:0 v/v 20 mM aq FA; under these conditions, high recovery of the non-specific peptide ELPLYR (90.9±7.5%) was observed. However, when the organic component was increased to 98:2 v/v 20 mM aq FA:MeCN, the ELPLYR recovery dropped to 3.8±0.7%. The increased level of MeCN most likely reduces the level of non-specific hydrophobic interactions.

Another way of evaluating MIP selectivity is by comparison of the MIP with its non-imprinted counterpart: The selectivity of both MIPs was determined by measuring the recoveries of the target peptide in MISPE and comparing these values to the corresponding recoveries in NISPE (non-imprinted solid-phase extraction) at 98:2 20 mM aq FA:MeCN (Fig. 1C). The NLL-MIP had demonstrably poor selectivity. The recovery of NLLGLIEAK following extraction by the NLL-MIP was 107±4.4% compared to 109.2±7.8% for the NLL-NIP extractions. NLLGLIEAK has a strong affinity toward both the ELP-MIP and NLL-NIP columns. This behaviour can be attributed to non-specific interactions with the polymers under the loading conditions chosen, where the hydrophobic amino acids in NLLGLIEAK can interact with the non-polar component of the polymers (i.e., DVB-80). This does not appear to be the case for ELPLYR, most probably because it is less hydrophobic than NLLGLIEAK. This can be seen in the grand average of hydropathicity (GRAVY) values of ELPLYR (-0.550) and NLLGLIEAK (0.711); positive GRAVY values indicate hydrophobic peptides whereas negative values indicate hydrophilic peptides [34].

The ELP-MIP demonstrated excellent selectivity with respect to its corresponding NIP. The extraction recovery of the target peptide (ELPLYR) was 108.0±4.4. Conversely, the ELP-NIP demonstrated poor affinity towards ELPLYR; the recovery following NISPE was 10.5±0.7%.

3.2. Selectivity of the MIPs in single-plex analysis in human serum

Although information about MIP selectivity for simple matrices is interesting, it is the selectivity of the MIPs in the presence of the actual matrix where it is intended to be used that is most important for efficient clean-up. In complex matrices such as human serum, and especially for extractions of low-abundant targets, there is an overwhelming number of matrix components that need to be removed from the sample and which may perturb the MIP-analyte interactions. High selectivity is essential to achieve this goal. The single column set-up was therefore used to determine the selectivity of the optimized extraction conditions (82:2 20 mM aq FA:MeCN) from a complex matrix (digested

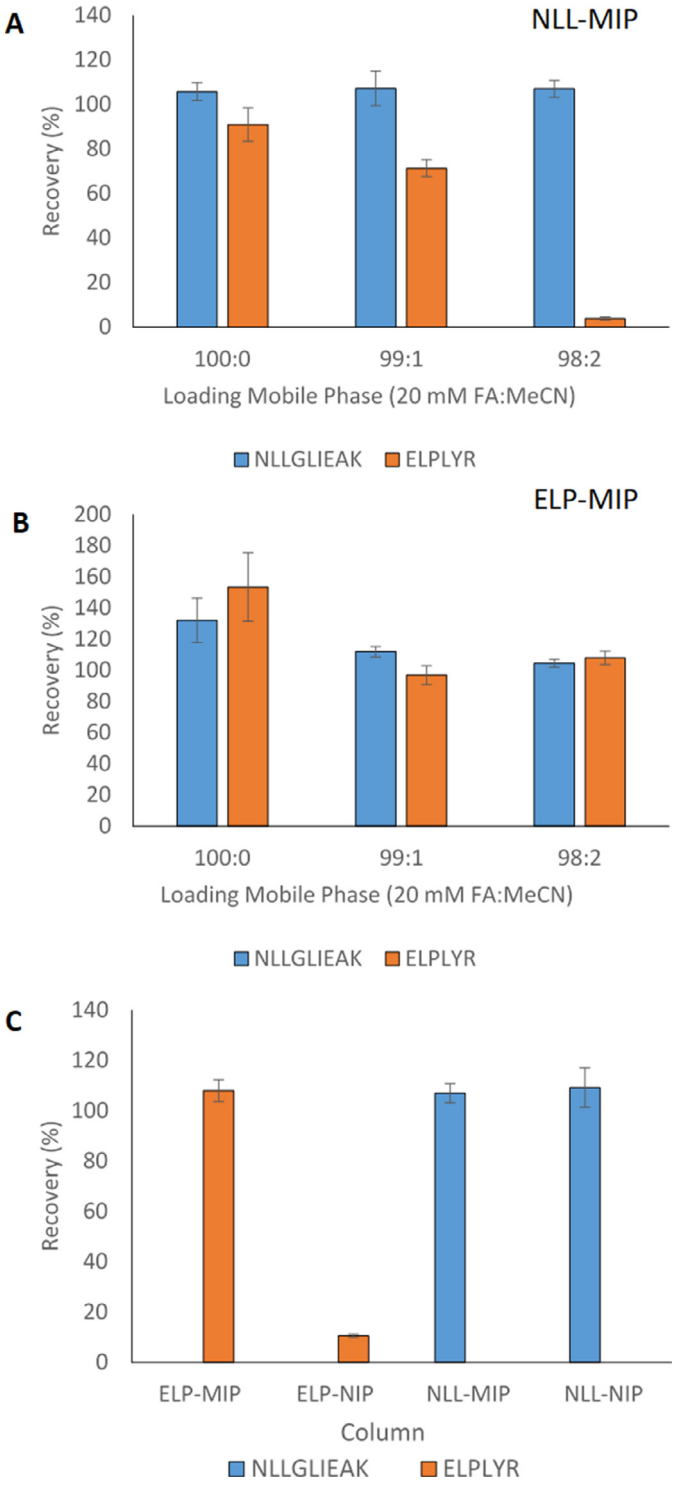

Fig. 1. Single column optimisation. (A) The recoveries of NLLGLIEAK (10 nM in 50 mM ABC) and the non-specific ELPLYR (10 nM in 50 mM ABC) after extraction by the NLL-MIP at loading mobile phases 100:0. 99:1, 98:2 v/v 20 mM aq FA:MeCN (n = 3). (B) The recoveries of ELPLYR (10 nM in 50 mM ABC) and the non-specific NLLGLIEAK (10 nM in 50 mM ABC) after extraction by the ELP-MIP at loading mobile phases 100:0. 99:1, 98:2 v/v 20 mM aq FA:MeCN (n = 3). (C) The recoveries of ELPLYR (10 nM) and NLLGLIEAK (10 nM) in 50 mM ABC after extraction by single columns (NLL-MIP, ELP-MIP, NLL-NIP and ELP-NIP (n = 3). The target peptides were eluted from the MIPs/NIPs using a 20 min linear-gradient from 0 to 85% mobile phase B (0.1% FA and MeCN 5:95, v/v).

human serum; Fig. 2). From this matrix the individual NLL-MIP still demonstrated selectivity between the specific NLLGLIEAK peptide and the non-specific ELPLYR peptide with extraction recoveries of 24.7±5.7 and 1.8±0.3% for NLLGLIEAK IS and the non-specific ELPLYR IS, respectively. Conversely, the ELP-MIP which had not been able to discriminate between the specific ELPLYR peptide and the non-specific NLLGLIAK peptide from a simple matrix, efficiently discriminated between the two peptides from the complex matrix. This is shown by the extraction recoveries of 8.5±1.9% and 31.3±2.3% for the non-specific NLLGLIEAK IS and ELPLYR IS, respectively. Representative MS chromatograms of the target pepides using either the ELP-MIP or the NLL-MIP can be found in Fig. S1B-C. The chromatograms demonstrate a clear preferance for each MIP towards its target peptide (compared to directly injected standard, Fig. S1A).

MIP-NIP selectivity was also seen for both MIPs from a complex matrix. Conversly to from the simple matrix, both individual NIPs showed very poor extraction recoveries from the complex matrix: the NLL-NIP had extraction recoveries of 0.5±0.1% and 0.2±0.3% for NLLGLIEAK IS and ELPLYR IS, respectively, while the ELP-NIP had extraction recoveries of 3.2±0.9% and 1.1±0.5% for NLLGLIEAK IS and ELPLYR IS, respectively. Hence, the MIPs have far greater selectivity in the complex matrix, albeit with an expected drop in extraction recovery. The ELP-MIP had no selectivity within a simple matrix with 1:1 recovery of ELPLYR and NLLGLIEAK; however, from a complex matrix the ELP-MIP has a nearly 4:1 recovery of ELPLYR and NLLGLIEAK. Furthermore, the ELP-NIP recovered only 1.1±0.5% of ELPLYR from the complex matrix, giving a 28-fold increased recovery (i.e., an imprinting factor, IF of 28). Similarly, the NLL-MIP had demonstrable selectivity with an approximately 14-fold improved extraction recovery of NLLGLIEAK compared to ELPLYR respectively from the complex matrix. Furthermore, from the same matrix the NLL-MIP had a 49-fold improved extraction recovery of NLLGLIEAK with respect to the NLL-NIP (i.e., IF=49). The high selectivity can be attributed to the imprinting effect. The MIPs are saturated with myriad non-specific serum tryptic peptides, leaving predominantly specific sites for the capture of the target peptides. The recovery of NLLGLIEAK by the NLL-MIP from digested human serum is approximately the same as reported previously [24]. However, the recovery of ELPLYR by the ELP-MIP is approximately half that reported previously [23]. This can probably be attributed to less organic content in the loading mobile phase. While the selectivity is excellent for the individual MIPs, the rather low recoveries may present challenges; namely, the detection limits needed for clinical analyses.

3.3. Duplex extraction and analysis

The duplex analysis of NLLGLIEAK and ELPLYR by MIPs was explored using two formats: NLL- and ELP-MIP trap columns connected in series and a single trap column packed with a 1:1 w/w mixture of the two MIPs (Fig. 3). Initially, the viability of both duplex formats was determined by extractions of both target peptides (ELPLYR IS [10 nM] and NLLGLIEAK IS [10 nM]) in 50 mM ABC containing 100 nM of digested BSA. The results of these analyses were in concordance with the results of single column extraction using a simple matrix and demonstrates that the use of two MIPs in conjunction or series can enable the retention of two targets, however the same selectivity issues were seen as for single MIP extraction from a simple matrix (Fig. S2). The results of these experiments are described and discussed in the supplementary information.

Based on the results of the experiments using single columns, as well as the duplex set-up and a simple sample matrix, it was decided that further evaluation of the MIP performance should be done using a complex sample matrix (digested human serum). Both the 1:1 w/w mix MIP and the MIPs in series were evalu-

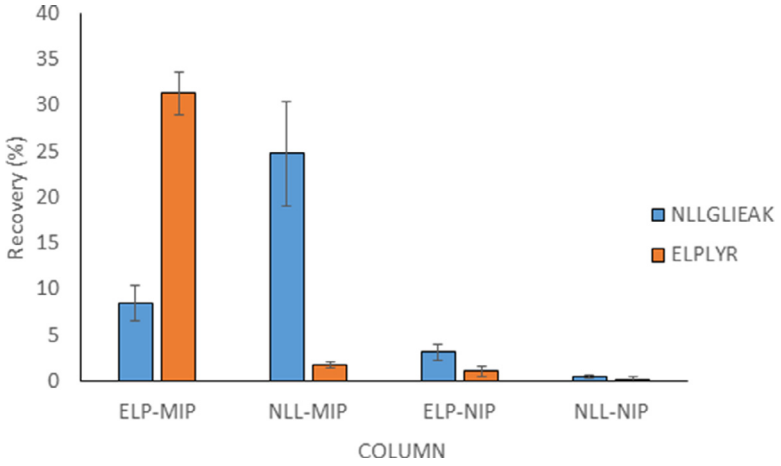

Fig. 2. The recoveries of ELPLYR (10 nM) and NLLGLIEAK (10 nM) in digested human serum after extraction by single columns (NLL-MIP, ELP-MIP, NLL-NIP and ELP-NIP) (n = 3).

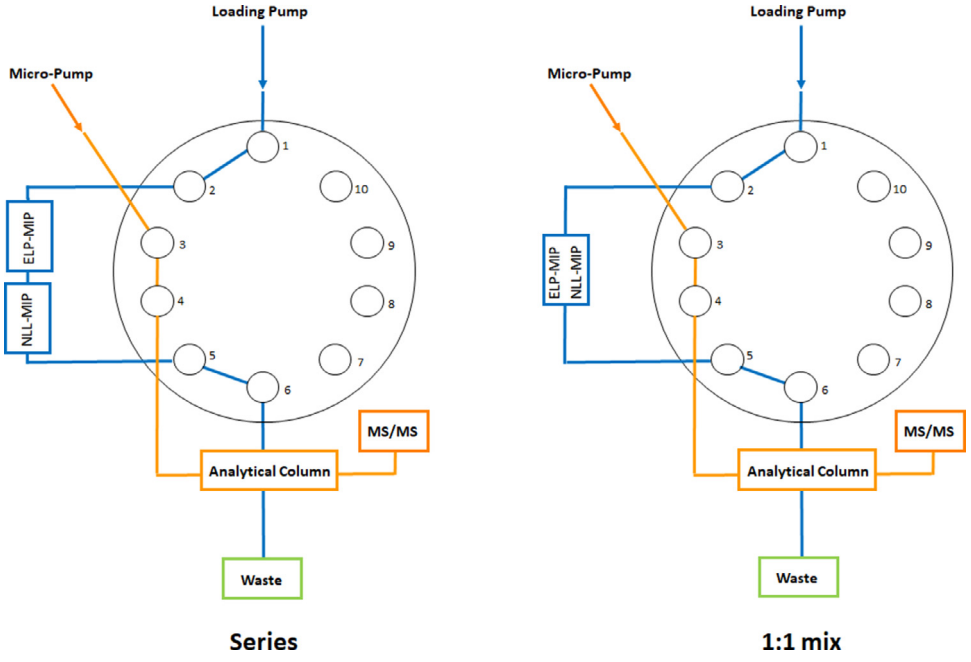

Fig. 3. Schematic representation of the MISPE duplex formats. The MIPs can be attached either in series (ELP-MIP and NLL-MIP columns (5 mm x 1.5 mm ID) connected by HPLC tubing) or as a 1:1 mixture of the two MIPs in a single column (5 mm x 1.5 mm ID).

ated. The 1:1 w/w mix MIP enabled the capture of both peptides (Fig. 4A). The recovery of NLLGLIEAK and ELPLYR was 15.7±2.3 and 19.2±8.4%, respectively. This represents an approximately 40% drop in recovery with respect to the individual MIPs in complex matrices (Fig. 2). This was to be expected since the 1:1 w/w mix MIP column contains 3 mg of each MIP whereas a single column contains 6 mg of each MIP. The co-packing of the MIPs is challenging due to the poly-disperse nature of the particles. It is difficult to ensure the even distribution of particles throughout a single column. Having said this, and the low recoveries notwithstanding, the 1:1 w/w mix MIP demonstrates that two MIPs can be combined successfully to capture two peptides in a single assay from a complex biological matrix.

The MIPs in series demonstrate far better performance with increased recoveries of both peptides compared to the 1:1 mix MIP (Fig. 4B). The order of the MIPs in series does not have any significant effect on the extraction recovery. The NLL-ELP MIP series had recoveries of 85.8±3.8% and 49.1±6.7% for NLLGLIEAK and ELPLYR, respectively, whereas the ELP-NLL MIP series had recoveries of 86.5±0.9% and 50.8±%11.6 for NLLGLIEAK and ELPLYR, respectively. This represents an approximately 5.5-fold increase in recovery for NLLGLIEAK and a 2.6-fold increase in recovery for ELPLYR compared to the 1:1 mix MIP. The selectivity of the MIPs is clearly demonstrated in the serum duplex extractions of NLLGLIEAK and ELPLYR, and is comparable to the single column extraction using a complex matrix. In the serum extractions by the series NIPs, the retention of both peptides were significantly poorer compared to the MIPs. The NLL-ELP NIP series had recoveries of 18.2±7.4% and 7.6±1.1% for NLLGLIEAK and ELPLYR, respectively, whereas the ELP-NLL NIP series had recoveries of 19.0±7.4% and 11.0±1.1% for NLLGLIEAK and ELPLYR, respectively. The difference in recovery between MIP and NIP is somewhat lower than that seen for the single-plex analysis of complex matrices probably due to the addition of a second column with increased possibilities for non-specific interactions of the target peptides. The use of MIPs in series is a more productive duplex format compared to the 1:1 mix MIP format due to a 2-fold increase in the mass of MIP (i.e., enhanced capacity) and column volume. The use of two columns

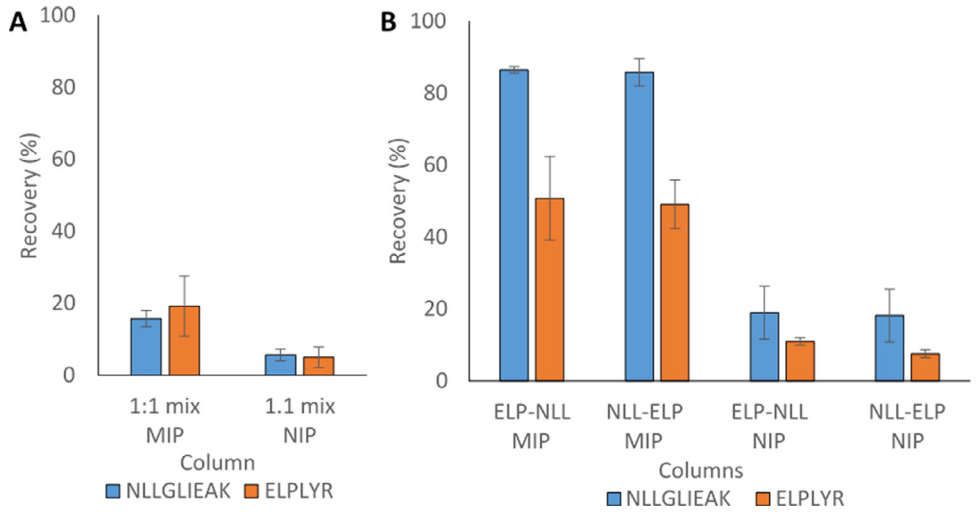

Fig. 4.. Duplex analysis of NLLGLIEAK and ELPLYR in digested human serum. (A) The recoveries of ELPLYR (10 nM) and NLLGLIEAK (10 nM) in digested human serum after extraction by a 1:1 mixture of both MIPs and NIPs (n = 3). (C) The recoveries of ELPLYR (10 nM) and NLLGLIEAK (10 nM) in digested human serum after extraction by MIPs (and NIPs) connected in series (both orders, n = 3).

also allows for a more simplified and controlled combination of the two poly-disperse sorbents. The two columns act in combination to enhance the recoveries of the target peptides, as exemplified by comparisons to single MIP extractions in serum (Fig. 2). The recoveries of both peptides are higher following extractions in series compared to their extraction by their corresponding MIP alone. This is most likely a result of the series format, where peptides that may have washed off a single column can be retained on the second column in a series extraction. This could account for the lower recovery of ELPLYR compared to NLLGLIEAK in the series MIPs. In the simple matrix the NLL-MIP has very poor retention of ELPLYR (due to its high selectivity), therefore there is potentially less capture of ELPLYR by the NLL-MIP in either column position, hence a lower recovery of ELPLYR compared to NLLGLIEAK. Conversely, NLLGLIEAK has non-specific interactions with the ELP-MIP from a simple matrix. The interactions with both MIPs in series most likely enhances NLLGLIEAK recovery. MIPs in series are an ideal format for combining two poly-disperse materials as separate columns since this ensures sufficient quantities of both materials while avoiding any packing irregularities.

The series MIPs perform well in comparison to the duplex capture of ProGRP and NSE by immunoextraction; where recoveries of 72% and 93%, respectively, have been reported for the latter [18]. While the recoveries of each peptide are marginally lower than the recoveries achieved by immunoextractions, the automation of the analysis is a key advantage, with ease of use and potentially higher throughput being other attractive features.

The series MIPs demonstrate higher recovery compared to extractions by single MIPs and mix MIPs and a 2-fold faster analysis time with respect to single-plex analysis. The order of the columns affects neither the extraction recoveries nor the selectivity. In Figure S1D-E, representative chromatograms using both column orders of the MIPs is included. The chromatograms also show that the column order does not affect the chromatography or the signal intensity of the target analytes. Therefore, either order of columns could be used but, for consistency, the NLL-ELP MIP series was selected for use in all experiments moving forward.

3.4. Intra-and interday repeatability

MIPs are often considered to be readily reusable materials owing to their robust crosslinked structures [22]. Optimization of the on-line MISPE protocols to ensure good repeatability is essential as the materials are routinely re-used. The intra- and interday repeatability were assessed over eight days of extractions by the NLL-ELP MIP series (Table 3).

Table 3
The intra- and interday recoveries of NLLGLIEAK and ELPLYR after extraction by the NLL-ELP MIP series over 8 days.

	NLLGLIEAK		ELPLYR	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Day 1	86.5	1.0	50.8	22.9
Day 2	95.2	19.5	34.5	32.4
Day 3	60.1	16.2	23.5	6.7
Day 4	81.0	20.6	49.3	22.6
Day 5	54.9	11.5	49.1	11.5
Day 6	37.0	0.6	33.1	14.4
Day 7	41.8	16.9	16.1	23.4
Day 8	69.2	5.1	43.7	6.7
Interday	65.7	32.0	37.5	34.4

The intra- and interday variability of both peptides were high following eight days of serum extractions (n = 3). The intraday variability is similarly high for both peptides. The intraday variation of NLLGLIEAK recovery ranges from 0.6-20.6% RSD with a median RSD of 13.85%. ELPLYR has similar intraday RSDs ranging from 6.7-32.4% with a median RSD of 18.5% (no IS correction). The interday recoveries were 65.7±21.0% (RSD = 32.0%) and 37.5±12.9% (RSD = 34.4%) for NLLGLIEAK and ELPLYR, respectively. The high variation (i.e., RSD > 30%) may affect the detection limits and represents a significant problem concerning the precision of the analysis unless IS correction is performed in the quantitative step. There is an observable drop in extraction recovery after several extractions; the MIPs may become saturated due to the high volume of serum tryptic peptides. This is seen as early as the second extraction for the ELP-MIP (32% drop in recovery and RSD > 30%) and the third extraction for NLLGLIEAK (37% drop in recovery and RSD > 15%). Continuous extractions in complex matrices (e.g., serum) are likely to block access to and/or saturate the imprinted binding sites. The NLL-MIP and ELP-MIP have been shown previously to have a high affinity for serum albumin peptides and tryptic peptides of other high abundant proteins [23,35]. Binding of other peptides may result in an apparent loss of binding sites and subsequently reduced extraction recoveries. Extraction time may be a contributing factor to the loss of the peptides. As the MIPs become more contaminated, the average strength of the interaction

Table 4
The intra-and interday recoveries of NLLGLIEAK and ELPLYR after extraction by the NLL-ELP MIP series using the optimised extraction method over 5 days.

	NLLGLIEAK		ELPLYR	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Day 1	94.1	5.3	60.4	9.9
Day 2	86.3	23.4	54.3	22.9
Day 3	67.8	23.6	61.2	16.8
Day 4	76.7	21.1	70.5	8.5
Day 5	84.9	6.9	78.4	11.8
Interday	82.0	12.2	64.9	14.6

between the target peptide and the MIP may be reduced and peptides lost more easily during loading. As these competing peptides are retained on the MIPs, they may also be co-transferred onto the analytical column along with the target peptides. Potentially, this can lead to ion suppression. Therefore, the MISPE needs further optimization to minimise the effects of tryptic peptides of high abundant proteins.

3.5. Optimization of extraction conditions for complex matrices

The extraction method was optimised by focusing on two key changes: a reduction in the extraction time from 10 min to 5 min, and changing the valve switching protocol to limit the amount of non-specific peptides transferred from the MIPs to the analytical column. To ascertain if these changes to the extraction method improved the robustness of the method, the intra- and interday precision of extraction recovery was measured for five consecutive days (Table 4). The intraday variation of NLLGLIEAK recovery ranges from 5.3-23.6% RSD (median = 21.1%). ELPLYR has similar intraday RSDs ranging from 8.5-22.9% (median = 11.8%). The interday recovery of both peptides was increased to 82.0±10.0% and 64.9±9.5% for NLLGLIEAK and ELPLYR, respectively. The extraction optimisation improves the interday variability of both NLLGLIEAK (Interday RSD = 12.2%) and ELPLYR (Interday RSD = 14.6%). Reducing the extraction time introduces the possibility of more non-specific peptides being retained on the MIP and not being washing off during the extraction. This could lead to ion-suppression, a reduction in signal and, ultimately, lower recoveries. However, by ensuring the MIP is connected to the analytical column only at the beginning of the gradient, the more strongly retained non-specific peptides will not be transferred to the analytical column. This potentially reduces ion suppression and improves the precision of the analysis.

The present MIPs demonstrate comparable performance to other imprinted materials. An NSE-imprinted electrochemical sensor had reported recoveries between 96-100% in a similar matrix (i.e., 1:100 diluted serum) [36]. Previously reported studies on the present ELPLYR imprinted on-line MIP demonstrated an approximately 60% recovery of ELPLYR [23]. The NLL-MIP in the present study was previously reported to have an approximately 25% recovery in human serum after single-plex extractions [24]. A magnetic NLLGLIEAK imprinted MIP had a similar recovery of approximately 25% [37]. This suggests the duplex format for NLLGLIEAK gives greater performance than the single-plex systems. Other single-plex peptide imprinted MIPs have shown comparable efficacy in complex matrix extractions [38–40].

3.6. Method evaluation

The analytical performance was evaluated by determining the linearity, LOD, LOQ, and accuracy of ProGRP and NSE in digested human serum (n=4) after on-line MISPE by the NLL-ELP MIP series. The method is linear in the disease range for both proteins

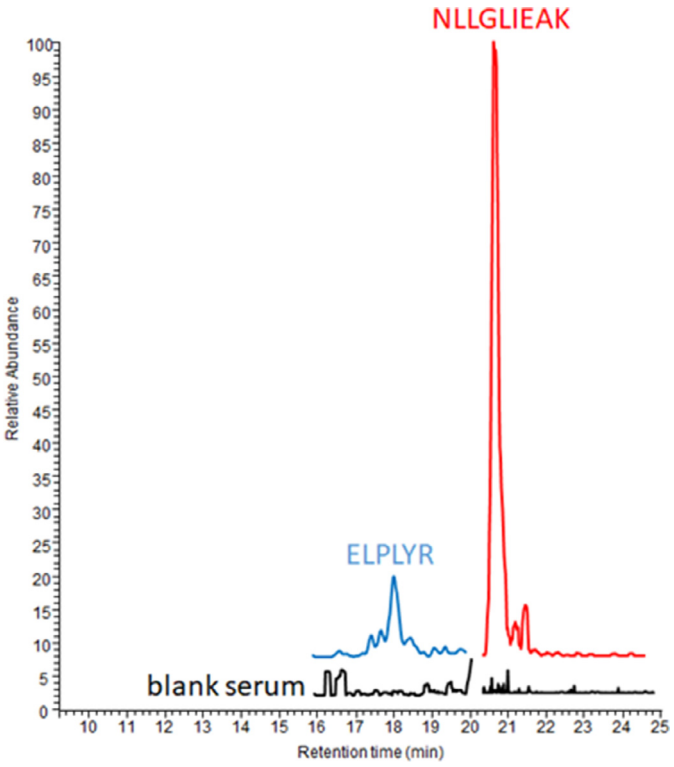

Fig. 5. The overlaid chromatograms of on-line MISPE extracted digested blank serum (black) and on-line MISPE extractions of 0.3 nM digested ProGRP (red) and NSE (blue) in digested serum used to calculate the LOD and LOQ values. Scale=1.1 × 10^3.

(0.3–30 nM) (Table 5), and the linear regression was within acceptable limits: R^2= 0.99 for NSE and R^2= 0.98 for ProGRP. The chromatograms used to calculate the LOD and LOQ can be seen in Fig. 5. The LOD and LOQ for NSE were estimated to be 0.11 nM and 0.37 nM, respectively. Hence the LOD for NSE is below the median reference levels in humans (0.22 nM) [23]. The LOD and LOQ for ProGRP were estimated to be 0.06 nM and 0.2 nM, respectively. The levels for ProGRP are in the disease range but approximately eight times higher than the reference level in humans (7.6 pM) [35]. These LOD and LOQ values are comparable to values reported in previous studies involving on-line MIPs [23,24].

The accuracy was determined at the high (30 nM) and low (0.3 nM) ends of the calibration curve using standards containing either high levels of ProGRP and low levels of NSE or *vice versa*. The accuracy at 0.3 nM was 87.3±41.4% and 85.8±50.2% for NSE and ProGRP, respectively. The accuracy is within the suggested limit for bioanalytical methods [41], but with concurrent high variability (RSDs >45%) despite IS correction. At the high end of the calibration curve (30 nM), the accuracy was 97.3±12.8% and 108.1±7.8% for NSE and ProGRP, respectively. Here, both the accuracy and precision is satisfactory according to established bioanalytical method validation guidelines [41]. The poor repeatability at low levels might be due to the low analyte levels resulting in low analyte signals as the concentration is below the estimated LOQ for NSE and only slightly above the LOQ for ProGRP.

Refinements in MIP synthesis to enhance the selectivity, affinity, and capacity may aid in lowering detection limits yet further. The use of larger trap columns, as well as the use of a state-of-the-art mass spectrometer, is also likely to improve detection limits and hence improve method precision at low analyte levels. The present work represents a proof of concept and, the potential for further refinements notwithstanding, the on-line MISPE duplex protocol developed and presented here still can detect and quantify both

Table 5
Duplex on-line MISPE method evaluation using spiked serum samples.

Protein	Linearity (r^2)	Slope	Intercept	Accuracy (%, ±STD)		Repeatability (RSD, %)	
				0.3 nM	30 nM	0.3 nM	30 nM
NSE	0.99	0.2406	0.0041	87.3±41.4	97.3±12.8	47.4	13.2
ProGRP	0.98	0.4413	0.0165	85.8±50.2	108.1±7.8	58.5	3.4

ProGRP and NSE in human serum from the same sample with good detection limits.

4. Conclusions

The present work demonstrates a successful proof of concept for automated duplex analysis *via* the use of two MIP trap columns connected in series targeting the signature peptides of the SCLC biomarkers NSE and ProGRP. An optimized MISPE protocol enabled the efficient extraction of both peptide targets from complex biological samples; the on-line duplex extraction method had acceptable recoveries, good linearity, and accuracy. The LODs and LOQs for NSE and ProGRP were in the pM range and, very significantly, were below the human reference level in serum for NSE (0.2 nM). The optimized on-line duplex MISPE represents one of the first steps towards MIP multiplexing, and the approach has promising potential in the future of SCLC diagnosis. Further improvements in MIP synthesis, such as for instance using a dual template approach, may yield materials that have enhanced selectivity that may improve extraction performance. With these improvements, quantification of NLLGLIEAK and ELPLYR below the reference levels may be achievable via MIP duplex extractions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Nicholas McKitterick: Methodology, Investigation, Writing – original draft, Visualization. **Tugrul Cem Bicak:** Methodology, Investigation, Writing – review & editing. **Magdalena A. Switnicka-Plak:** Methodology, Investigation. **Peter A.G. Cormack:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Léon Reubsaet:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Trine Grønhaug Halvorsen:** Conceptualization, Writing – review & editing, Supervision, Visualization, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462490.

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