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Surface-assisted laser desorption ionisation time-of-flight mass spectrometry with an activated carbon surface for the rapid detection of underivatised steroids

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ABSTRACT

This article reports the rapid detection of 10 underivatised steroids using surface-assisted laser desorption ionisation time-of-flight mass spectrometry (SALDI TOF-MS). Numerous carbon surfaces were investigated as possible substrates to promote underivatised steroid desorption and ionisation and compared to established matrix assisted laser desorption ionisation (MALDI) approaches. The inexpensive and readily accessible surface of activated carbon allowed detection of all 10 steroids of interest; estrone, β-estradiol, estriol, 17α-ethynylestradiol, progesterone, pregnenolone, 17α-hydroxy progesterone, corticosterone, cortisone and hydrocortisone with on plate limits of detection ranging from 1.57 to 18.1 ng. The activated carbon surface produced minimal background interference, simple mass spectral interpretation and significantly improved analyte distribution when compared to a more conventional MALDI approach.

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1. Introduction

Modern analytical protocols are required to provide not only excellent sensitivity and selectivity for the target analyte but also be time and cost efficient. However, often, meeting the first set of needs requires significant sample preparation, pre-treatment and lengthy analysis times. To overcome the disparity of these two requirements, laboratory efficiency can be dramatically improved by reducing the number of samples required to undergo full analysis by screening all samples prior to application of more time consuming and costly procedures [1]. Screening methods must be rapid, high throughput and require minimal sample pre-treatment. Laser desorption ionisation (LDI) is one such technique. This soft ionisation mass spectrometric approach has been of particular interest in recent years and enables the detection of non-volatile and thermally labile molecules [2]. The best known and most widely used LDI technique is matrix assisted laser desorption ionisation (MALDI) mass spectrometry, since the initial reports [3–5] MALDI has had wide success for the analysis of large molecules including proteins [6,7], peptides [8,9], oligonucleotides [10,11] and polymers [12,13]. However, the inherent ionisation of the matrix produces background interference in the low mass-to-charge (m/z) region complicating the detection of smaller molecules (<1000 Da). Additional processes are therefore required to overcome this complication [14,15].

LDI detection of steroids is typically achieved using MALDI after applying derivatisation with hydrazine compounds to produce a higher mass derivative with a readily ionisable moiety [16–21]. Whilst these approaches result in good sensitivity, these time-consuming reactions further complicate the resulting mass spectrum with the presence of peaks from unreacted derivatisation reagents and also have the potential for formation of multiple derivatisation products [22]. The use of MALDI for detection of underivatised steroids has been successfully achieved by Kosanam et al. who reported the detection of the five neutral steroids (nandrolone, boldenone, trenbolone, testosterone and betamethasone) at nanogram levels in human urine using a meso-tetrakis(pentafluorophenyl)porphyrin (F20TPP) matrix [23]. The large mass of this matrix resulted in reduced background interference in the low mass range (100–700 m/z). Kicha et al. have reported the detection of sulphated polyhydroxylated steroids extracted from various starfish populations without the need for derivatisation prior to analysis with MALDI [24–27].

Alternatively, surface-assisted laser desorption ionisation (SALDI) employs a surface (in place of a matrix) to facilitate analyte desorption and ionisation. The absence of the chemical matrix alleviates matrix ion interference, thus simplifying mass spectral interpretation and decreasing the sample preparation time because analyte/matrix mixing is unnecessary. Furthermore, as there is no requirement for matrix-analyte co-crystallisation, this also reduces the prevalence of sweet spots (commonly encountered in MALDI analyses) and improves analyte homogeneity. These combined

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benefits have, in recent times, been exploited for the detection of small molecules [15,28] and hold great promise for rapid analyte screening prior to more traditional laboratory analyses. Typical surfaces employed in SALDI are reported for both surfaces and particulates of carbon [29–34], silicon [35–40] and metals [41–46]. However, the detection of steroids using SALDI techniques is still in its infancy with a number of surfaces being investigated. In 2003, Compton and Siuzdak reported the use of a silica surface (DIOS) [47] for the detection of sulfated steroids in human urine. The inherent negative charge of the sulfated molecules resulted in relatively simple ionisation and clean spectra. Galesio et al. investigated the use of SALDI with TiO_2 and Al_2O_3 for the detection of derivatised androgenic anabolic steroids [21]. Chiu et al. reported the use of silver nanoparticles (AgNP) as a surface for the detection of estrogens [48]. Although this report does allow detection of estrogens without the necessity for derivatisation, the process is time consuming (>40 min extraction) and produces additional background ions as a result of the ammonium citrate buffer. Recently, Montsko et al. demonstrated the use of a C_{70} fullerene surface for the detection of a range of underivatised neutral steroids [49]. These reports illustrate the benefits of improved reproducibility, rapid analysis and sensitive detection associated with SALDI analyses; however the sample and surface preparation can be both costly and time consuming.

The research presented here investigates the use of SALDI with an activated carbon surface for the rapid detection of 10 structurally similar and predominantly endogenous steroids: progestogens, estrogens and corticosteroids. Of these steroids only progesterone [18,20,49], cortisone [50], and the estrogens (estrone, estradiol and estriol) [48,49] have previously been analysed with an LDI approach. The described SALDI method allows for rapid analysis from an inexpensive and simply prepared carbon surface making this approach ideal for steroid screening.

2. Experimental

Estrone (1,3,5(10)-estratrien-3-ol-17-one), 17β-estradiol (1,3,5-estratriene-3,17β-diol), estriol (1,3,5(10)-estratriene-3,16α,17β-triol), 17α-ethynyl estradiol (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol), progesterone (4-pregnene-3,20-dione), pregnenolone (5-pregnen-3β-ol-20-one), 17α-hydroxy progesterone (17α-hydroxy-4-pregnene-3,20-dione), corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione), hydrocortisone (11β,17α,21-trihydroxypregn-4-ene-3,20-dione), 20–60 mesh activated charcoal, 100–400 mesh activated charcoal, glassy carbon (2–12 μm) and carbon nanopowder (<50 nm) were all purchased from Sigma–Aldrich (Sydney, Australia). Steroid stock solutions were prepared to 1 mM in 1:1 (v/v) water:acetonitrile and diluted to the required concentration with water. In all cases the water used was Barnstead E-pure water (18 MΩ cm^{-1}). α-Cyano-4-hydroxycinammic acid (α-cyano) and 2,4-dihydroxybenzoic acid (DHB) matrix solutions were prepared to a concentration of 10 mg mL^{-1} in 1:1 (v/v) water:acetonitrile.

Samples analysed by MALDI were mixed 1:1 (v/v) with the prepared 10 mg mL^{-1} matrix solution and 0.5 μL of the mixture was spotted onto the MALDI target plate and allowed to dry at room temperature. The carbon surface for SALDI analysis was prepared by spotting 0.5 μL of a 10 wt% aqueous carbon suspension onto the target plate. Excess carbon was removed under vacuum after solvent evaporation with a modified water aspirator pump to reduce instrumental contamination. Samples analysed with SALDI were then directly deposited (0.5 μL) onto the carbon surface and allowed to dry at room temperature. Instrumental calibration was performed with a 20 point external mass calibration

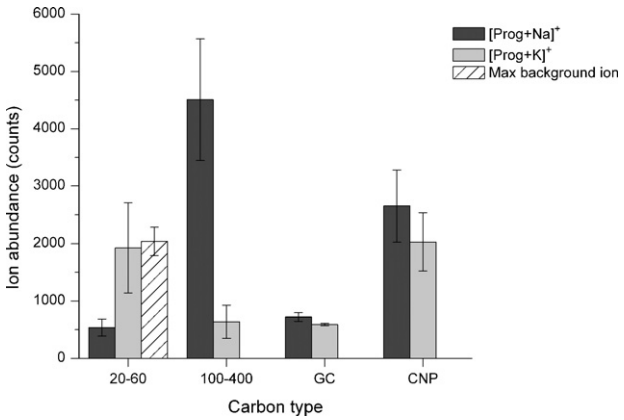

Fig. 1. Comparison of different SALDI carbon surfaces, 20–60 mesh, 100–400 mesh, glassy carbon and carbon nanopowder (denoted 20–60, 100–400, GC and CNP respectively) showing abundance of sodiated and potassiated progesterone adducts ([Prog+Na]+ and [Prog+K]+ respectively) for the analysis of 0.1 mM progesterone (constant laser fluence for all surfaces) and ion abundance of maximum background ion (S/N > 3) over the 200–400 *m*/*z* region of interest of blank carbon surface.

with 10 mg mL^{-1} PEG with NaI additive with samples for calibration prepared as described previously for MALDI or SALDI analyses.

Mass spectra were acquired with a Micromass M@LDI L/R (Waters, UK) operated in positive ion reflectron mode. Pulse voltage, 2500 V, delay extraction, 500 ns, accelerating voltage, 15,000 V, reflectron voltage, 2000 V. All mass spectra acquired are the sum of 5 spectra each of which is the summation of 10 laser shots.

3. Results and discussion

3.1. Preliminary studies

The use of carbon as a surface for SALDI has only recently been investigated for steroid detection by Montsko et al. who utilised a C_{70} fullerene surface [49]. Here we report an investigation into the use of an activated carbon surface. Our initial studies investigated a number of different commercially available carbons for the model steroid, progesterone: activated carbon (20–60 mesh and 100–400 mesh), glassy carbon (2–12 μm) and carbon nanopowder (<50 nm) as the surface for SALDI. As can be seen in Fig. 1 optimum steroid ionisation with minimal background interference was achieved with the 100–400 mesh activated carbon. This carbon surface resulted in maximised ionisation of the model steroid (progesterone) with formation of the sodiated adduct, $[Prog+Na]^+$, dominating the spectrum, and the potassiated adduct, $[Prog+K]^+$, in much lower abundance (Fig. 2B). Additionally there were no high abundance ions in the background spectrum in the 200–400 *m*/*z* region of interest (Fig. 2A). Conversely the 20–60 mesh carbon resulted in a greater presence of high abundance background ions in this region, and lower progesterone ionisation than that achieved with the 100–400 mesh carbon. It was also evident that the glassy carbon and carbon nanopowder did not facilitate steroid ionisation as readily as the 100–400 mesh carbon surface. The mass spectra for the two MALDI matrices investigated show significant ionisation in the low mass region (100–500 *m*/*z*) for both matrices: α-cyano and DHB. The resulting ions in this low mass-to-charge region from the matrix α-cyano are attributed to the protonated and sodiated α-cyano adducts ($[M+H]^+$, m/z = 190.05 and $[M+Na]^+$, m/z = 212.03 respectively) along with the protonated adduct of α-cyano after loss of neutral water ($[MH–H_2O]^+$, m/z = 172.04). Additionally α-cyano dimer ions are also evident with the protonated dimer ($[2M+H]^+$) at m/z = 379.09 and protonated dimer after loss of neutral CO_2 ($[2M+H–CO_2]^+$) at m/z = 335.10. The mass spectrum of the DHB matrix also resulted in a number of low *m*/*z* ions attributed

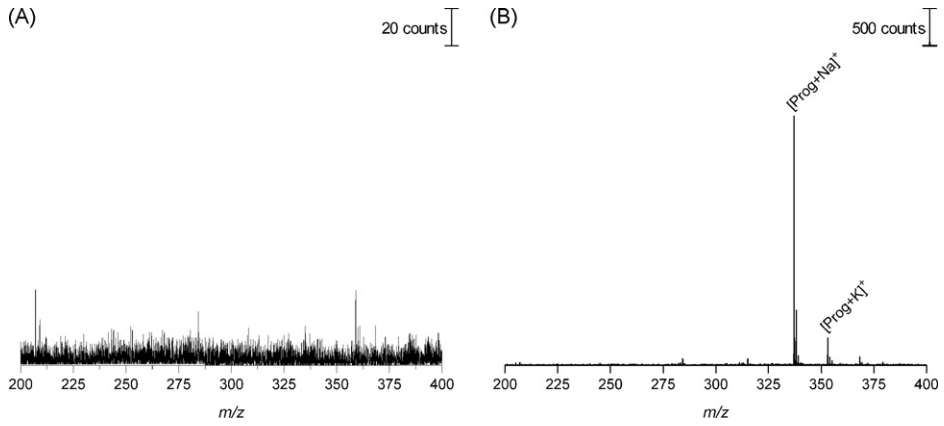

Fig. 2. SALDI mass spectra of (A) activated carbon surface and (B) 0.1 mM progesterone from activated carbon surface .

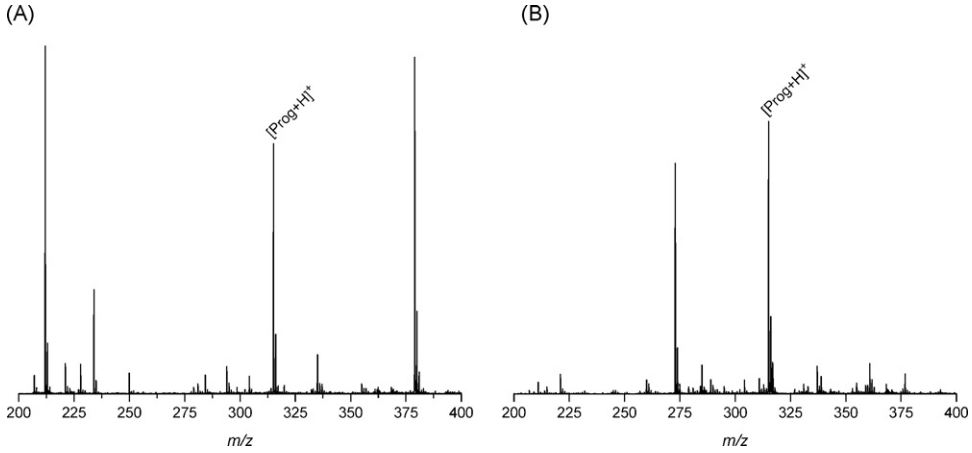

Fig. 3. MALDI mass spectrum of 0.1 mM progesterone with matrices (A) α-cyano and (B) DHB with protonated progesterone adduct labelled, $[Prog+H]^+$.

to the sodiated and protonated adducts ($[M'+H]^+$ and $[M'+Na]^+$ at $m/z = 155.03$ and 177.02 respectively) and the formation of protonated DHB with the loss of water ($[M'H–H_2O]^+$ at $m/z = 137.02$) and the dimer adduct, $[2M'+H–2H_2O]^+$ at $m/z = 273.04$.

3.2. Steroid detection

The detection of progesterone from the activated carbon surface is evident in Fig. 2B illustrating the detection of the progesterone with minimal background ion contributions to the spectra. Unlike MALDI analysis of progesterone (Fig. 3A and B), where the protonated adduct ($[Prog+H]^+$ at $m/z = 315.2$) is the dominant analyte ion, in SALDI this ion is not observed. Instead, the sodiated adduct, $[Prog+Na]^+$ at $m/z = 337.2$, dominates the spectrum with a less abundant potassiated adduct ($[Prog+K]^+$: $m/z = 353.2$) also evident. However unlike MALDI where gas phase interactions between the matrix and analyte are required for analyte ionisation, there is no possibility for such interactions with SALDI. Other SALDI techniques, such as DIOS, have reported the presence of protonated adducts when analysing small molecules. However, the porous silicon surface used in DIOS has a hydroxy functionalisation, which is proposed as the proton source for these adducts. The carbon surface used in this research has no such functionalisation on the surface which will inhibit the formation of the protonated adduct.

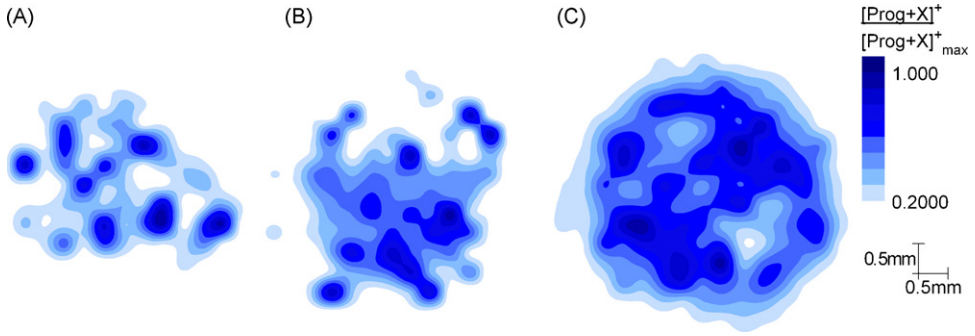

Fig. 4. Comparison of analyte ionisation distribution for progesterone analysis with MALDI and SALDI. With steroid ionisation distribution over target well of 0.1 mM progesterone with MALDI analysis with matrix of (A) α-cyano, (B) DHB and (C) SALDI analysis from activated carbon surface. Each ion distribution plot is acquired at each point of a 19 × 19 raster range, the $[Prog+H]^+$ (A and B) and $[Prog+Na]^+$ (C) abundance is plotted relative to the maximum ion abundance acquired over the well.

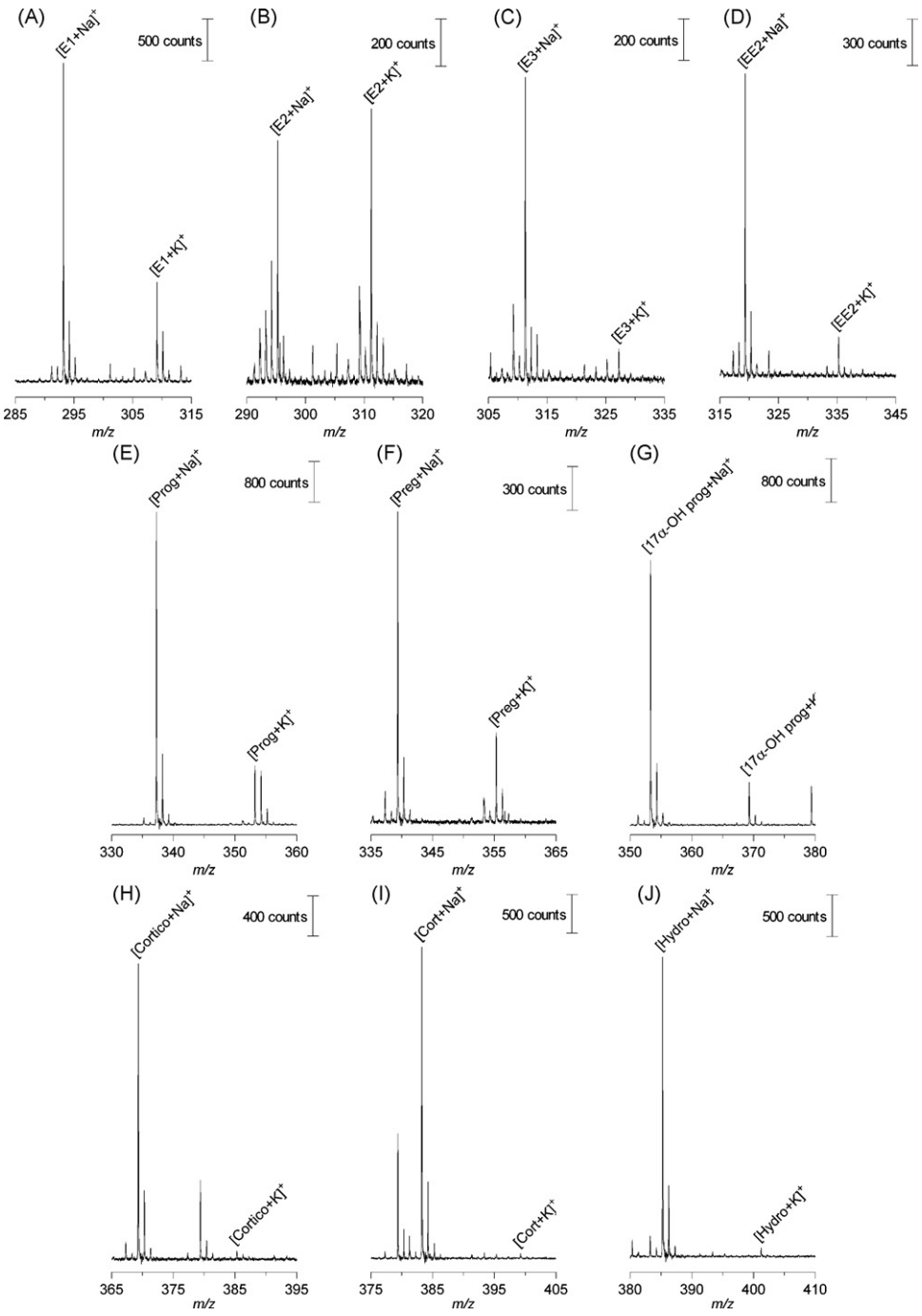

Fig. 5. SALDI mass spectra from activated carbon surface of 10 steroids of interest: (A) estrone (E1), (B) 17β-estradiol (E2), (C) estriol (E3), (D) 17α-ethynyl estradiol (EE2), (E) progesterone (Prog), (F) pregnenolone (Preg), (G) 17α-hydroxy progesterone (17α-OH prog), (H) corticosterone (Cortico), (I) cortisone (Cort) and (J) hydrocortisone (Hydro) with sodiated and potassiated adducts labelled, $[X+Na]^+$ and $[X+K]^+$ respectively.

The presence of sodiated and potassiated adducts have been reported in previous SALDI research [1,51] and are proposed to be due to the high environmental abundance of these elements. Furthermore, matrix peaks dominate the MALDI spectrum and whilst none of these peaks interfere directly with the progesterone ion, these ions can complicate the identification of the ions of interest, and can be particularly problematic when analysing unknown compounds, multiple analytes, or low concentrations.

A significant advantage of this technique is the speed of detection. The samples acquired shown in each of these mass spectra are the sum of 5 spectra each of which is the result of 10 laser shots, and acquired in approximately 20 s. The small sample volumes required ($\leq$1 μL) is another advantage to this technique, and as suggested by Kosanam et al. [23] for the analysis of steroids with MALDI, each laser ablation consumes approximately 2 nL of original sample. The potential of high throughput analysis with the use of an automated spotter and auto-samplers further emphasises the benefits of LDI for screening purposes. Furthermore, the small amount of sample consumed, together with the speed of analysis allows LDI techniques to be used synergistically with other conventional analytical techniques for further analysis, as required.

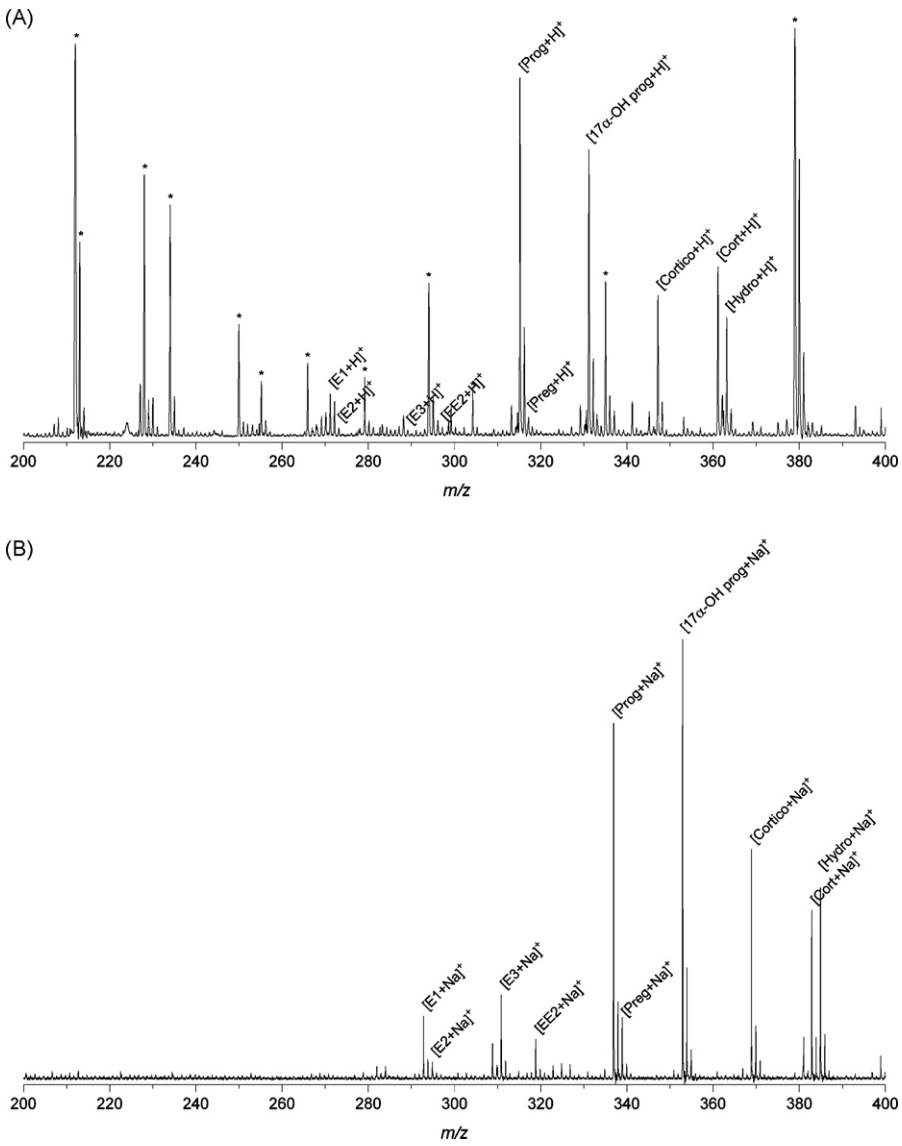

Fig. 6. Mass spectrum of equimolar concentrations (0.1 mM) of 10 steroid mix analysed with (A) MALDI with α-cyano matrix (* denote matrix related ions) and (B) SALDI with an activated carbon surface with protonated and sodiated adducts denoted $[X+H]^+$ and $[X+Na]^+$ respectively, for MALDI and SALDI analyses.

3.3. Reproducibility

Reported widely in the MALDI literature is the "sweet spot" phenomenon whereby reproducibility across the target well is highly variable [14,15,28,52]. It has been previously reported that the dried-droplet method is suitable for MALDI steroid detection [18–20,23]. As can be seen in Fig. 4A and B the use of this technique with α-cyano and DHB (MALDI) resulted in significant variation in the analyte ion intensity ($[Prog+H]^+$: $m/z = 315.2$) over the surface of the well (commonly termed "sweet spots"). Conversely, as SALDI requires no analyte/matrix co-crystallisation and the analyte distribution over the sample well is relatively uniform as shown in Fig. 4C. It is evident that the analyte distribution over the target well is much more uniform with the use of this SALDI method when compared to the conventional dried-droplet MALDI approach. This has the added advantage of improved shot-to-shot reproducibility and fewer "sweet spots" which have the potential to improve the reliability for steroid screening with this method.

3.4. SALDI detection of 10 steroids

Fig. 5 shows that this SALDI technique is amenable for the individual detection of all 10 steroids of interest, with the sodiated and potassiated adduct detected for all 10. It is also evident that the abundance of each of the steroid ions is different, with the ionisation of the estrogens and pregnenolone significantly lower than that observed for the other steroid groups. The lower ionisation of these steroids is likely due to the phenolic group, resulting in the formation of a negatively charged ion, $[M-H]^-$, or related negative adducts due to the inherent acidity of this functionality. Detection of estrogens have been demonstrated with (−)-LDI in previous reports [48], and confirm the formation of the $[M-H]^-$ ion for this group of steroids, however instrumental limitations prevented similar investigations for this research.

The detection of a steroid mixture with MALDI and SALDI with equimolar concentrations of each of the 10 steroids: estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethynyl estradiol (EE2),

progesterone (Prog), pregnenolone (Preg), 17α-hydroxy progesterone (17α-OH prog), corticosterone (Cortico), cortisone (Cort) and hydrocortisone (Hydro) is shown in Fig. 6A and B (MALDI and SALDI analyses respectively). These results indicate a number of advantages for the use of SALDI (Fig. 6B), allowing simple analyte ion identification with the analyte ions easily identified from the dominant sodiated adduct. The benefits of a carbon surface rather than chemical matrix to promote analyte desorption and ionisation is further illustrated with the MALDI analysis of the 10 steroid mix with α-cyano matrix (Fig. 6A) the low estrogen ionisation along with the presence of matrix ions complicates interpretation of the resulting MALDI mass spectrum. In Fig. 6B it is also evident that the ion abundances do vary significantly between the different steroids, despite the equimolar concentrations analysed, however the sodiated adducts of each of the steroids are detected. Further investigations into steroid ionisation with SALDI indicated the limits of detection (S/N > 5) ranging from 3.14 to 36.2 $\mu g\,mL^{-1}$ (corresponding to 1.57–18.1 ng on-plate) for progesterone and hydrocortisone respectively for the simultaneous detection of the 10 steroids. Whilst these detection limits are above those reported for conventional steroid detection techniques (i.e. GC–MS, LC–MS), the rapid analysis time and simple sample preparation indicate the potential benefits of this approach for steroid screening.

4. Conclusions

Ten underivatised steroid compounds have been analysed simultaneously for the first time on an activated carbon surface using SALDI. The rapid sample preparation and analysis shows the great potential of this method has for steroid screening. A number of advantages of using SALDI rather than the more conventional MALDI have been illustrated and indicate this method is a suitable alternative offering a much cleaner background spectra with limited analyte interference, along with the presence of two analyte adduct peaks (sodiated and potassiated), separated by $m/z = 16$ which can provide more conclusive analyte molecular weight confirmation than a single protonated peak. For the first time we have also illustrated the homogeneous analyte distribution over the target well improves spot-to-spot reproducibility when compared to analogous MALDI analyses.

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