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The discrimination of geoforensic trace material from close proximity locations by organic profiling using HPLC and plant wax marker analysis by GC

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ABSTRACT

There is a need to develop a wider empirical research base to expand the scope for utilising the organic fraction of soil in forensic geoscience, and to demonstrate the capability of the analytical techniques used in forensic geoscience to discriminate samples at close proximity locations. The determination of wax markers from soil samples by GC analysis has been used extensively in court and is known to be effective in discriminating samples from different land use types. A new HPLC method for the analysis of the organic fraction of forensic sediment samples has also been shown recently to add value in conjunction with existing inorganic techniques for the discrimination of samples derived from close proximity locations.

This study compares the ability of these two organic techniques to discriminate samples derived from close proximity locations and finds the GC technique to provide good discrimination at this scale, providing quantification of known compounds, whilst the HPLC technique offered a shorter and simpler sample preparation method and provided very good discrimination between groups of samples of different provenance in most cases. The use of both data sets together gave further improved accuracy rates in some cases, suggesting that a combined organic approach can provide added benefits in certain case scenarios and crime reconstruction contexts.

1. Introduction

Earth materials such as soils and sediments can be useful in crime reconstruction since their composition is a reflection of the underlying geology of their source location, the history of climatic and physical geographical processes occurring at that location, and the cumulative action of organisms living on and in the soil or sediment [1–5]. These factors result in a wide array of soil types which vary across different geographical scales, and which can be highly specific to a particular location, such as a crime scene [6–8]. Since earth materials can be readily transferred to items of forensic interest such as clothing, footwear, tools and vehicles, analysis of the components of samples taken from such items and samples taken from a crime scene can allow investigators to compare and exclude geographical areas, or compare and potentially exclude questioned items of suspect evidence from an investigation. For instance, analysis of soil adhering to an item of clothing or tool belonging to a suspect may be used for intelligence purposes to help narrow down the search area for a missing person or item, or be used in an evidentiary context to exclude an alibi location as the source of the material [9–12] or to compare samples derived from two items of interest.

The majority of the reported physical and chemical analyses performed on geoforensic evidence target the elemental composition and/or the minerals in the soil or the size and morphology of the mineral grains [13,7,4,14] in addition to determining the bulk characteristics such as colour or pH [15,16] and, with the exception of palynology [14] and the use of organic wax markers in the UK [12,17,5], there are few well established forensic techniques to study the organic fraction of soils [15,18,19]. If the organic component is not considered, there is a risk that variations in the soil composition between known and questioned samples, resulting from vegetation or micro-organisms, may not be

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detected, leading to false-positive or false-negative interpretations of the evidence [17]. Inorganic analysis generally identifies differences between locations of forensic interest, often due to different underlying geology. Since bedrock composition tends to be consistent over relatively large geographical areas (kilometre scale variations), the ability of physical and inorganic techniques can be spatially constrained [20]. There is therefore a need to consider techniques which are able to compare and exclude samples on the basis of the composition of the organic fraction of the soil and to develop an empirical evidence base to ascertain the limits of applicability of these techniques, for instance in the degree of variation in vegetation, or the spatial separation required to observe distinct, accurate differences between locations [5,17,20–23].

There are many analytical techniques used in soil science to characterise the organic composition of soils for agricultural or environmental protection purposes, or in earth sciences research. For forensic purposes however it is problematic that many of these analyses require large quantities of sample or require complex sample preparation, to the extent that they cannot provide the appropriate levels of accuracy and precision required for forensic work, nor can they be considered practical for implementation in a large scale forensic context [4,17]. There are, however, two chromatographic approaches which have been demonstrated to offer valuable data from the analysis of the composition of targeted component groups within the organic fraction of soil; the determination of wax markers by Gas Chromatography (GC) [5,12,17,21,24,25] and (currently) untargeted profiling of soil components by High Performance Liquid Chromatography (HPLC) [20,23,26–29]. Both approaches produce profiles which are known to vary across small andforensically relevant spatial scales [20,23] and for sites with different management and land uses [5,12,17,21,24–29].

The profiles of wax markers in soil have been found to reflect the composition of the compounds found in the leaves, stems and roots of the plants grown in them, and these profiles are known to remain stable over time, providing a historical record of the vegetation present at a site [17]. A database of wax marker profiles has been developed for a range of forensically relevant land use and vegetation types in the UK, and the wax marker profiles of numerous plant species are now well understood, and as such, this type of analysis is an excellent intelligence and evidential tool [21]. In addition, since the wax marker profiles can include unusual compounds, it has been demonstrated that it is possible to profile soil from a specific location at a scale relevant for evidentiary purposes [24].

HPLC has only rarely been reported in the literature as a geoforensic analysis tool, however it has long been known that soil gives rise to highly complex chromatograms that can be highly individual to specific locations [26–29]. More recent work has developed a method to improve the practicality and cost of the HPLC analysis of geoforensic samples and has shown this technique to add value when used in conjunction with more established techniques, giving highly accurate results in a forensic scenario where Quartz Grain Surface Texture Analysis, a technique that has been used successfully in many cases, was unable to provide full discrimination between locations [20], and further studies have identified that this analytical technique can offer a choice of markers for comparing known and questioned samples [23].

The importance of using a combination of independent forms of analysis for geoforensic analysis of samples has been outlined in the published literature [12], and it is therefore of significant value to incorporate the complementary analysis of the organic with the analyses of the inorganic fractions. This study aims to demonstrate the variability of GC and HPLC profiles over a forensically relevant, close-proximity spatial scale in order to evaluate the relative and complementary benefits of both HPLC and GC profiling techniques for assisting crime reconstruction.

2. Methodology

2.1. Site description

Three sites in the UK were selected for this study as outlined by McCulloch et al. [23]. All three sites were parklands in urban areas and comprised different areas where a person could legitimately come into contact with earth materials, but also contained spaces and thoroughfares that lacked natural surveillance, which could provide opportunities for crimes to be committed. These sites were Brockwell Park in London, UK, Lochend Park in Edinburgh, UK, Craigiebuckler Estate in Aberdeen, UK. In addition one additional site was chosen in the USA; Central Park in New York City in a similar manner to previous studies [20,23] to ensure comparability of the results. All the chosen sites were well-established municipal parkland, and maintained for public recreational use. At each site, four forensically relevant locations were chosen for sampling that represented potential alibi sites and potential crime scene sites (McCulloch et al. [23]). The positioning of these locations at close-proximity to one another was chosen in order to demonstrate the forensic relevance of this study compared to many previously published works, where the locations of interest had been situated several miles apart, at areas of different underlying geology and of markedly different land uses [26–28]. Large distances and significant differences in land use between sample locations may not be applicable to many crime scenarios, for instance in urban environments with similar land use, or environments where the underlying geology is less variable [22,17,20,23].

Although there were broad qualitative similarities in the land-use for each location chosen within a site, there were no additional pre-selection criteria, such as controlling the variation and species of surrounding vegetation for each type of location. This approach was, again, considered more forensically relevant since offenders are more likely to consider situational factors such as visibility, accessibility and frequency of public usage, rather than the specific vegetation planting, when selecting a location to undertake criminal activities [30–32].

The mock crime-scene at each site was characterised by having exposed soil adjacent to a fresh water pond, with resident waterfowl and miscellaneous wild vegetation, with bamboo growing immediately adjacent to the sample points in London and Aberdeen. This was chosen to represent a potentially viable site for the concealment of a murder weapon. These locations (Fig. 1) had limited pedestrian access and would therefore be unlikely to be entered as part of normal leisure activities and therefore lacked natural surveillance.

Soil from a natural path through woodland (Fig. 2) was sampled at each site, to represent a secluded route to and from the mock crime-scene. The sample location was an area of bare earth with dense tree cover and leaf litter, immediately adjacent to a residential area and used by local residents as a thoroughfare to and from the park.

The mock alibi location was a flat area of managed grassland (Fig. 3), that was well-maintained by the land owner. It was chosen to represent an alibi site where soil has been transferred as a result of sports and recreation activities. In Edinburgh and London, these areas were in use as football pitches at the time of sampling, while the primary users of these locations in New York and Aberdeen were used by dog-walkers.

The final location at each site was a sloping area of unmanaged grassland (Fig. 4), with wild vegetation, mixed grasses and wild flowers. These sites were chosen to represent an additional alibi

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Fig. 1. Locations Adjacent to Fresh Water. Mock crime scenes next to fresh water ponds at sites in London, Aberdeen, Edinburgh and New York (clockwise from top-left) Compiled from McCulloch et al. [23].

Fig. 2. Woodland locations: secluded woodland entrance/exit routes at sites in New York, London, Aberdeen, and Edinburgh (clockwise from top-left) Compiled from McCulloch et al. [23].

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Fig. 3. Managed grassland: managed, recreational alibi sites at Edinburgh, Aberdeen, London and New York (clockwise from top-left) Compiled from McCulloch et al. [23].

Fig. 4. Unmanaged land: unmanaged recreational areas in London, New York, Aberdeen, and Edinburgh (clockwise from top left) as described in McCulloch et al. [23].

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site and were well used by walkers, runners and also, for the London site, by cyclists.

2.2. Sample collection

All samples were collected in January 2014, when the soils were at or near field capacity. Five samples were collected from each location in order to assess intra-location variability, using the grid suggested for sampling footprints and tyre tracks by Pye [33] as outlined by McCulloch et al. [20,23]. In accordance with Simmons [34], surface soil samples were collected using a stainless steel spatula, removing any turf or gravel, where present. Approximately five grams of topsoil (0–1 cm depth) was collected at the corners and central point of a 1 m square grid. All samples were stored in breathable containers and allowed to air dry prior to use.

2.3. Sample preparation

HPLC sample preparation was performed using the method of McCulloch et al. [20,23]. 250 mg of dry soil was weighed using a semi-micro balance and added to 0.5 ml acetonitrile, using a calibrated pipette, in a microcentrifuge tube, sonicated for 20 min, then centrifuged for 15 min at 13,000 rpm. The supernatant was then passed through a 0.22 μm PTFE syringe filter into an HPLC vial.

The instrument parameters followed those outlined by McCulloch et al. [23]. An aliquot of 50 μl from each sample was injected onto an Agilent 1100 HPLC system with DAD detector, using a Waters Xbridge C18, 3.5 μm, 150 × 4.6 mm column at 30 °C, and UHQ water and acetonitrile for mobile phases A and B, respectively, at a flow of 1 ml/min in accordance with Table 1. The chromatograms were recorded at 254 nm with a 4 nm bandwidth and peak width of >0.1 min.

2.4. Plant wax marker determination by GC

Plant wax marker analysis was performed according to the method detailed in Morrison et al. [21], developed from Dove and Mayes [35], excluding the derivitisation steps as there was no mass spectrometry required. All solvents were redistilled prior to use and all aliquots were transferred with glass tipped, calibrated, auto-pipettes. All glassware was ashed and rinsed in *n*-heptane prior to use. All samples were crushed with a mortar and pestle, weighed, then dried in an oven at 50 °C for two hours and their moisture content calculated.

To each dry sample, internal standards of docosane (C_{22}) and tetratriacontane (C_{34}) were added at a concentration of 0.0506 mg/g in decane, then *n*-heptacosanol (1-C_{27}-ol) was added at a concentration of 0.2179 mg/g in 50:50 (v/v) *n*-heptane:ethanol. Samples were saponified in 1 M ethanolic KOH at 90 °C for 16 h then the organic layer was extracted in 3:1: *n*-heptane:water. This extract was evaporated to dryness and redissolved in heptane.

The extract was loaded onto a silica gel column that had been prepared with *n*-heptane, then the hydrocarbons were eluted with *n*-heptane, ketones were removed from the column with 97:3 *n*-heptane:ethyl acetate, and the crude alcohol fraction was collected by washing with 80:20 *n*-heptane:ethyl acetate. The hydrocarbon and crude alcohol extracts were then evaporated to dryness.

Table 1
Mobile phase gradient.

Time (min)	% mobile phase A	% mobile phase B
0.0	53	47
3.0	45	55
24.0	26	74
29.0	2	98
31.0	2	98
32.0	53	47
35.0	53	47

2.4.1. N-*Alkane sample preparation*

The hydrocarbon fraction was redissolved in *n*-heptane, transferred to a GC vial, evaporated to dryness, then dissolved in 20 μl dodecane prior to injection onto the GC.

2.4.2. *Fatty alcohol sample preparation*

Each of the crude alcohol fractions were redissolved in 100 μl *n*-heptane and 30 μl was loaded onto a capped, 1 ml glass solid phase extraction (SPE) column which was packed with 60 mg Chromasorb HP (80–100 mesh) packing material in *n*-heptane, and to which 60 μl saturated urea in ethanol had been added. The columns were placed in an oven at 70 °C for 20 min and allowed to dry overnight, then the sterol/stanol fraction was recovered by elution with *n*-heptane, the urea removed by washing with water, and the fatty alcohols subsequently eluted with *n*-heptane. Both fractions were then evaporated to dryness prior to derivatisation.

Acetate derivatives of the fatty alcohols were prepared by heating overnight with pyridine:acetic anhydride (5:1) at 50 °C. The acetate derivative was then repeatedly re-dissolved in *n*-heptane then evaporated to dryness until no acetic acid smell was observed, then dissolved in 25 μl dodecane prior to injection onto the GC. The GC instrument parameters are presented in Table 2.

3. Data analysis

3.1. HPLC data analysis

The marker sets first presented by McCulloch et al. [23] are useful for exclusionary comparison of soils at this spatial scale, which give very high accuracy when grouping samples using multivariate statistical methods. The HPLC profiles were first integrated using Agilent Chemstation software, eliminating all peaks that were below the limit of quantification, then the data for each of the peaks (Table 3) were adjusted for variations in sample quantity and analysed in SPSS.

3.2. GC data analysis

The GC data were analysed using chromquest software, then the absolute concentration of each *n*-alkane and fatty alcohol was calculated relative to internal and external standards. Normalised concentrations were calculated relative to the total concentration of *n*-alkanes or fatty alcohols in the sample, then the mean values and standard errors for each *n*-alkane and fatty alcohol were calculated and plotted using Microsoft Excel. Previous work has shown that the odd chain *n*-alkanes and even chain fatty alcohols are typically more informative and discriminatory, therefore the profiles of these markers were included for comparison [25,20,6,21].

3.3. Canonical Discriminant Function Analysis (CDFA)

Discriminant analyses were then performed on the data to determine the accuracy and precision with which the HPLC and GC wax markers allow samples to be grouped according to their source location. For this type of analysis, the software uses each wax marker or HPLC peak as a predictor variable, and each sample location as a grouping variable. The data for each sample were analysed by the software to generate functions, which were linear combinations of the variables that maximised the difference

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Table 2
GC instrument parameters.

Sample type	*n*-Alkane	Fatty alcohol
Column	SGE BP1 0.5 μm, 30 m × 0.52 mm id.	ZB 5HT Inferno 0.25 μm, 30 m × 0.25 mm id.
Temperature programme	170 °C for 4 min, 30 °C/min to 215C, 1 min hold	170 °C for 5 min 30 °C/min to 210 °C 1 min hold, 5.3 °C/min to 320 °C, 7 min hold
Injector	0.7 μl, direct injection (280 °C)	0.8 μl, direct injection (275 °C)
Detector	Flame ionisation (340 °C)	Flame ionisation(300 °C)
Carrier gas	Helium, 4 ml/min	Helium, 3.5 ml/min

Table 3
Retention times of HPLC markers (McCulloch et al. [23]).

Marker set	Peak retention times (min)
A	4.4, 9.0, 9.4, 10.0, 10.8, 11.6, 12.2, 12.6, 13.6, 14.2, 15.0, 15.5, 15.8, 18.8, 19.6, 20.3, 23.6, 24.3, 37.3, 30.4, 30.8
B	1.9, 4.4, 6.7, 12.2, 13.2, 13.7, 15.0, 19.1, 24.5, 26.9, 28.5

between each location tested. The functions were then used to assign each sample in the dataset to a particular location, based on their scores for each function, and the accuracy of classification was defined by comparing the predicted sample location to the true sample location.

The scores for each function were then used as co-ordinates for each individual sample to create a scatter plot, where samples of similar composition clustered closely together, allowing groups of samples, and the relative degree of difference between groups, to be visualised.

4. Results

4.1. Lochend Park, Edinburgh, UK

Utilising the profiles of HPLC peak set A, it was possible to discriminate all four locations within Lochend Park, Edinburgh, as presented by McCulloch et al. [23]. The presence of the peak at 10.8 min and the absence of the peak at 15.8 min appeared to be valuable markers for soils adjacent to fresh water and unmanaged land, respectively. The profiles of woodland and managed

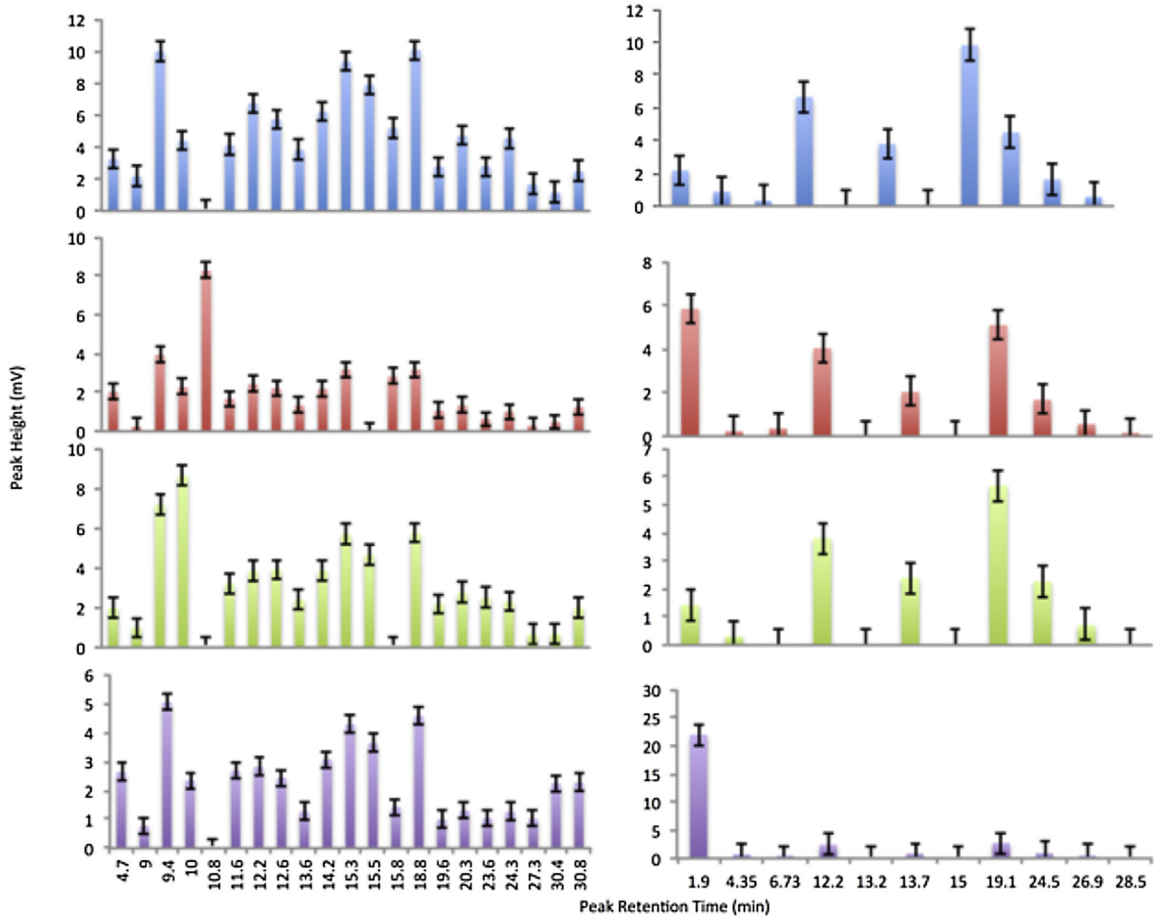

Fig. 5. Edinburgh HPLC profiles using marker set A (left) and B (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations at Lochend Park, Edinburgh. Compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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grassland were more similar to one another but could be separated by differences in the relative size of certain pairs of peaks such as the ratio of peak height at 9 min and 4.5 min as outlined fully in McCulloch et al. [23] (and see Fig. 5).

The profiles for HPLC of peak set B were not as easily distinguishable at each of the four locations in Lochend Park, Edinburgh. However, under further scrutiny, each profile could be discriminated from the others (see McCulloch et al. [23]). The woodland samples were distinctive with large peaks at 1.9 min, while soils adjacent to fresh water were distinct in having their two largest peaks at 1.9 and 19.1 min, which were similar in size to one another. It was more difficult to visually discriminate the profiles of managed grassland and unmanaged land, however, as discussed in McCulloch et al. [23] the small peaks present in the managed grassland profiles at 6.7 and 28.5 min were absent in the samples from unmanaged land (Fig. 5).

The wax marker profiles allowed clear visual discrimination of the four sample locations at Lochend Park, Edinburgh (Fig. 6). The location adjacent to fresh water and unmanaged land were distinct; C29 was the most abundant and discriminatory marker, and 1-C24-ol was present for the soils adjacent to fresh water, but was absent in the unmanaged land soils. The increase in concentration for the series C25, C27, C29 and C31 was characteristic of managed grassland and woodland soils, however the increase appeared more linear for woodland soils and exponential for the managed grassland. These two locations could also be distinguished from one another through the ratio of 1-C24-ol to 1-C26-ol, which was much higher for woodland soils.

At Lochend Park, all three sets of markers gave 100% accuracy in grouping samples to the correct location when used in a CDFA (Table 4). The functions identified for these sets of variables correctly predicted the location that each sample belonged to. The first function accounted for the greatest amount of variation between the groups for all three sets of markers, at 88.9%, 62.4% and 70.8% of the observed variance for HPLC set A, HPLC set B and the Wax Markers, respectively. With all three functions identified and included in the analysis for HPLC sets A and B and the wax markers, the sample groups were statistically significantly discriminated at the 99% confidence interval (Fig. 7).

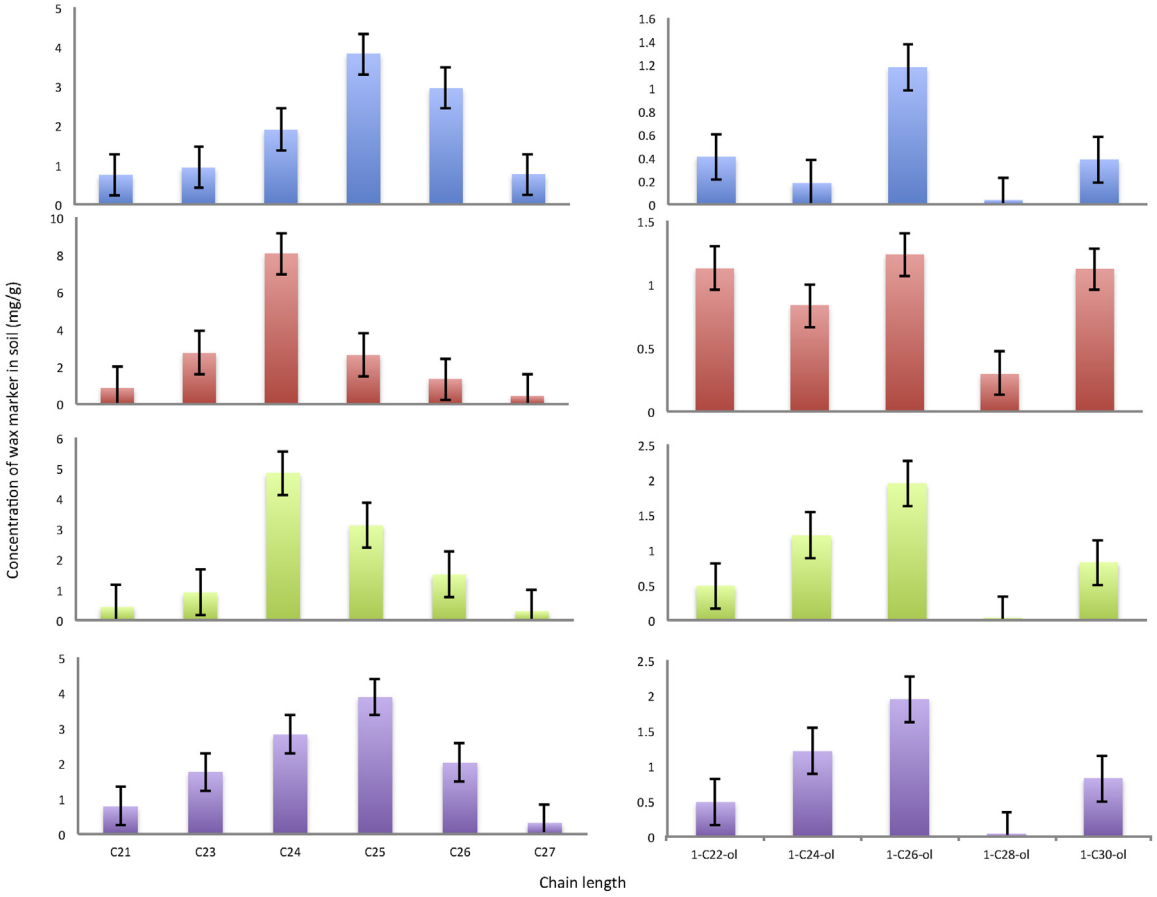

Fig. 6. Edinburgh GC profiles of *n*-alkanes (left) and fatty alcohols (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations at Lochend Park, Edinburgh. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
CDFA results for Lochend Park, Edinburgh.

Marker type	Classification accuracy %	Wilks lambda significance test of functions			% variance function 1	% variance function 2	% variance function 3
Edinburgh		1–3	2–3	3			
HPLC set A	100.0	0.000	0.000	0.022	88.9	8.3	2.7
HPLC set B	100.0	0.000	0.018	0.397	62.4	32.5	5.0
Wax markers (GC)	100.0	0.000	0.000	0.025	70.8	21.3	7.9

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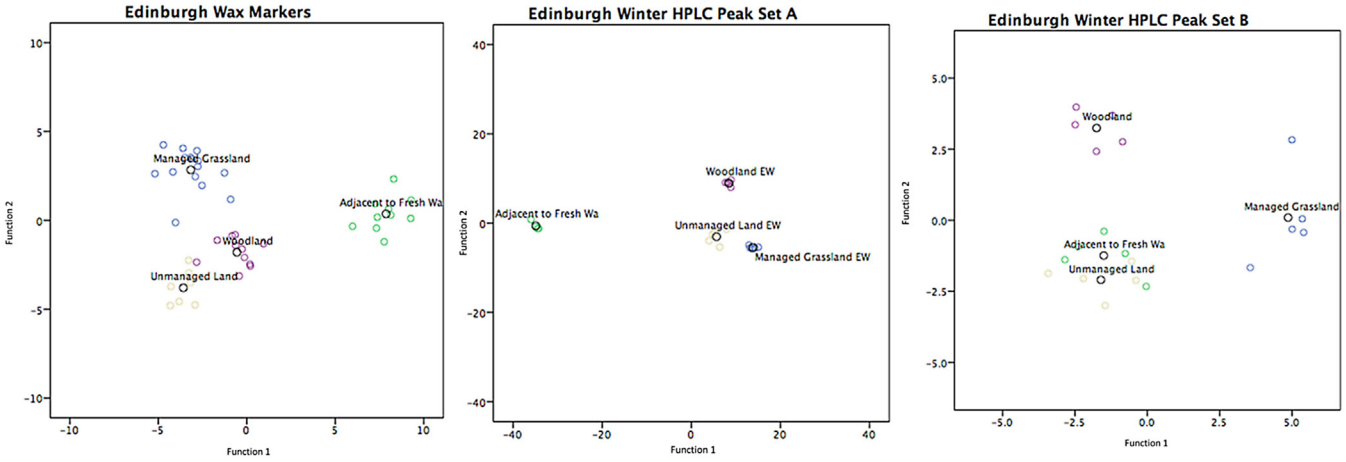

Fig. 7. CDFA Scatter Plots from Lochend Park, Edinburghfor wax marker profiles, HPLC peak set A, and HPLC peak set B at soils from locations adjacent to fresh water (Green), unmanaged land (Yellow), managed grassland (Blue), and woodland (Purple) with the position group centroids shown in black. HPLC plots compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The scatter plots for function 1 and 2 show sample groups clustering according to their sample location, with clear separation between the group centroids evident for all three sets of markers. All samples were grouped correctly.

4.2. Brockwell Park, London, UK

All four locations sampled in Brockwell Park, London could be distinguished by the HPLC profiles of peak set A. Comparison of the

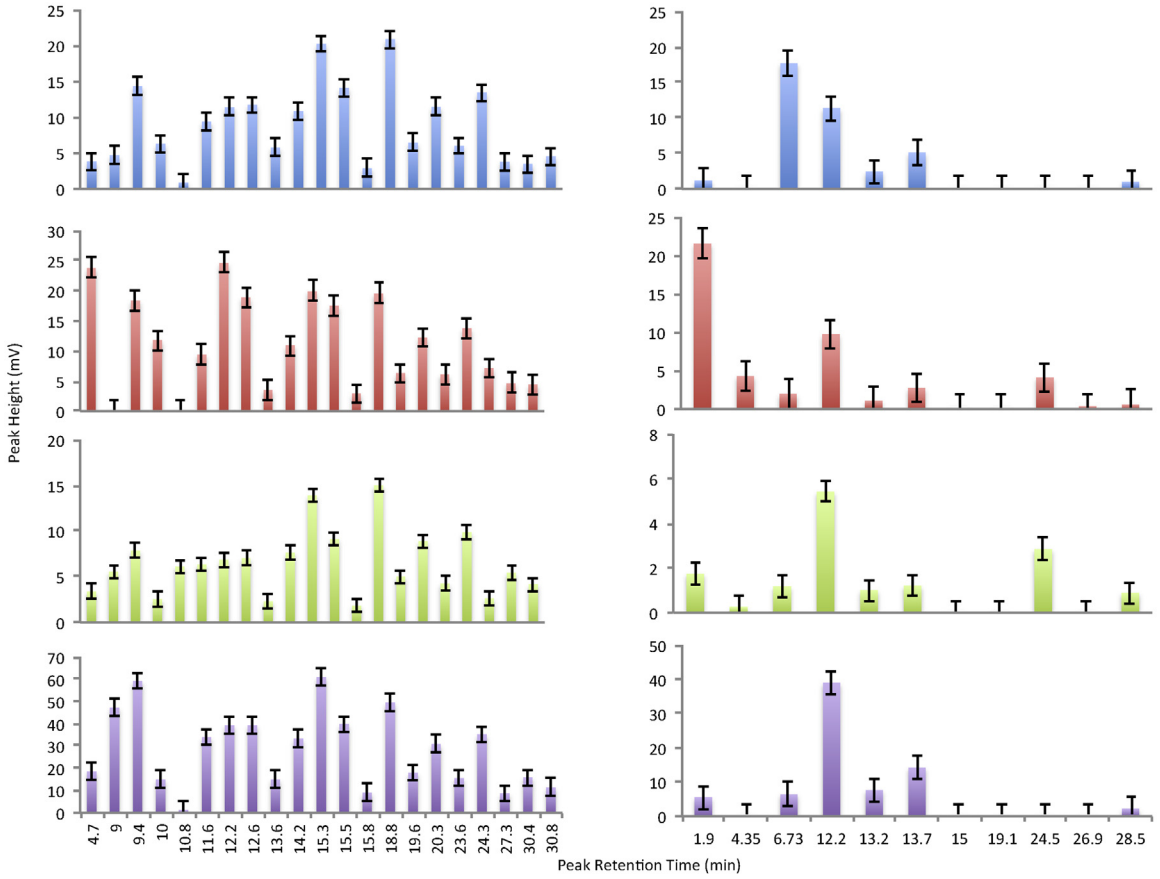

Fig. 8. London HPLC profiles using marker set A (left) and B (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations at Brockwell Park, London. Compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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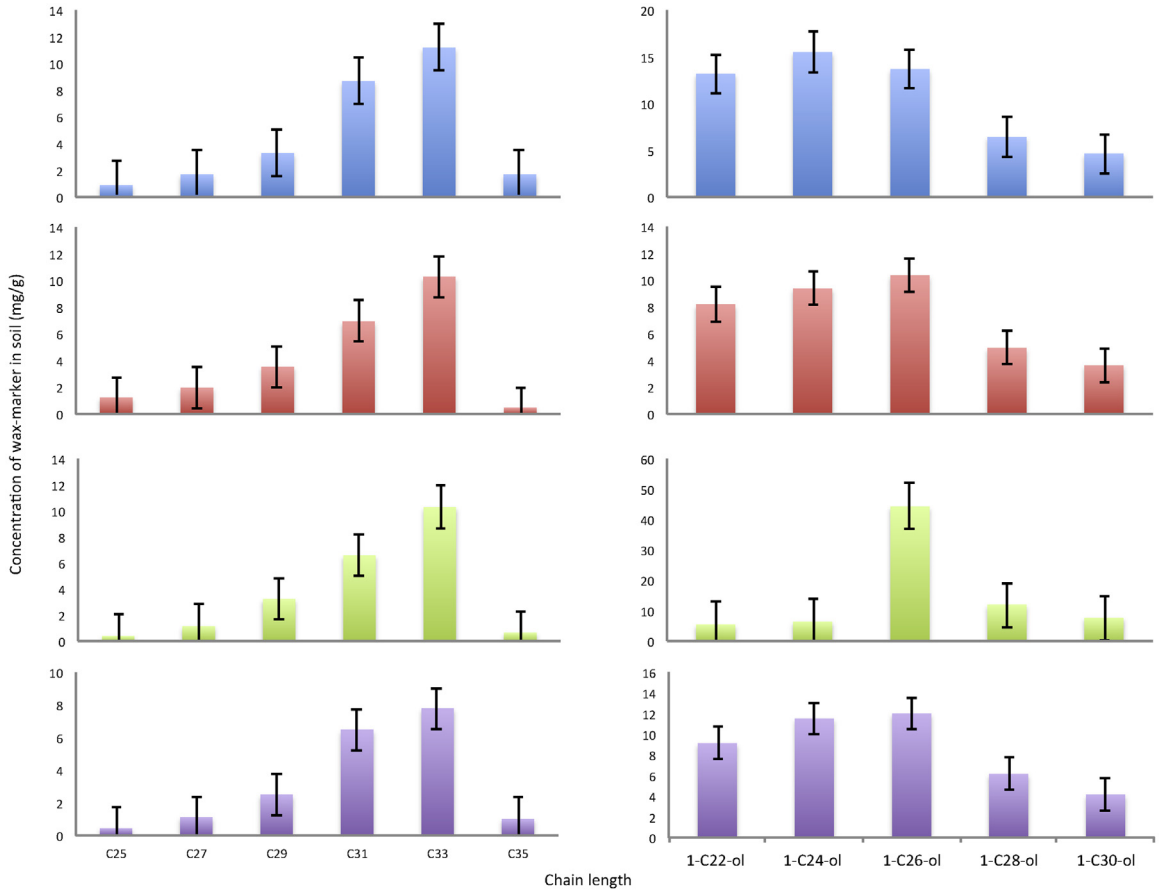

Fig. 9. London GC profiles of *n*-alkanes (left) and fatty alcohols (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations at Brockwell Park, London. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

two tallest peaks for each location was useful in discriminating the samples in this dataset (Fig. 8). For soils adjacent to fresh water the two tallest peaks were 4.7 and 12.2 min, whereas for woodland soils the peaks at 9.4 and 15.3 min were largest. In contrast the peaks at 15.3 and 18.8 min were the largest peak in the profiles of both managed grassland and unmanaged land, suggesting the possibility that these peaks are indicative of grassland soils. Peak height ratios were also used to distinguish the sites, and full discussion of the visual similarities and differences is outlined in McCulloch et al. [23]

The HPLC profiles for peak set B discriminated all four samples locations within Brockwell Park, London (Fig. 9). The large size of the peak at 6.7 min relative to the peak at 12.2 min distinguishes managed grassland from all other sample locations.The height of peaks at 1.9 min compared to all other peaks is distinctive of the profiles in soils adjacent to fresh water. The profiles of soils from unmanaged land and woodland were more similar, however they can be discriminated on the basis of the peak at 24.5 min for unmanaged land which is not present in the woodland samples examined.

The different locations in Brockwell Park were not so easily visually discriminated using their wax marker profiles. Only the profile from the unmanaged land was visually distinct from the others with an exceptionally high concentration of 1-C26-ol at 44 mg/g compared to approximately 10 mg/g for the other locations. More subtle differences in the relative concentrations of 1-C24-ol and 1-C26-ol allowed tentative discrimination of managed grassland from woodland and soils adjacent to fresh water, as the 1-C24-ol peak was 13% larger than the 1-C26-ol for the former, while it was 4% and 9% smaller than 1-C26-ol for woodland and soils adjacent to fresh water, respectively. Likewise, small differences were observed in the size difference between C31 and C33 at these two locations with an increase of 20% and 49% from C31 to C33 for woodland and soil adjacent to fresh water. It should be noted that these moresubtle differences were comparatively small in magnitude compared to the variability in the results at each location, as indicated by the size of the error bars (Fig. 9).

Table 5provides the results of the CDFA analysis of samples collected from Brockwell Park. Only HPLC set A gave 100% grouping accuracy when these variables were used in CDFA (Fig.10), while 90%

Table 5
CDFA results for Brockwell Park, London.

Marker type	Classification accuracy %	Wilks lambda significance test of functions			% variance function 1	% variance function 2	% variance function 3
London		1–3	2–3	3			
HPLC set A	100.0	0.000	0.002	0.034	89.7	7.0	3.3
HPLC set B	90.0	0.000	0.041	0.684	84.8	13.9	1.3
Wax markers (GC)	80.4	0.000	0.011	0.119	81.7	11.6	6.6

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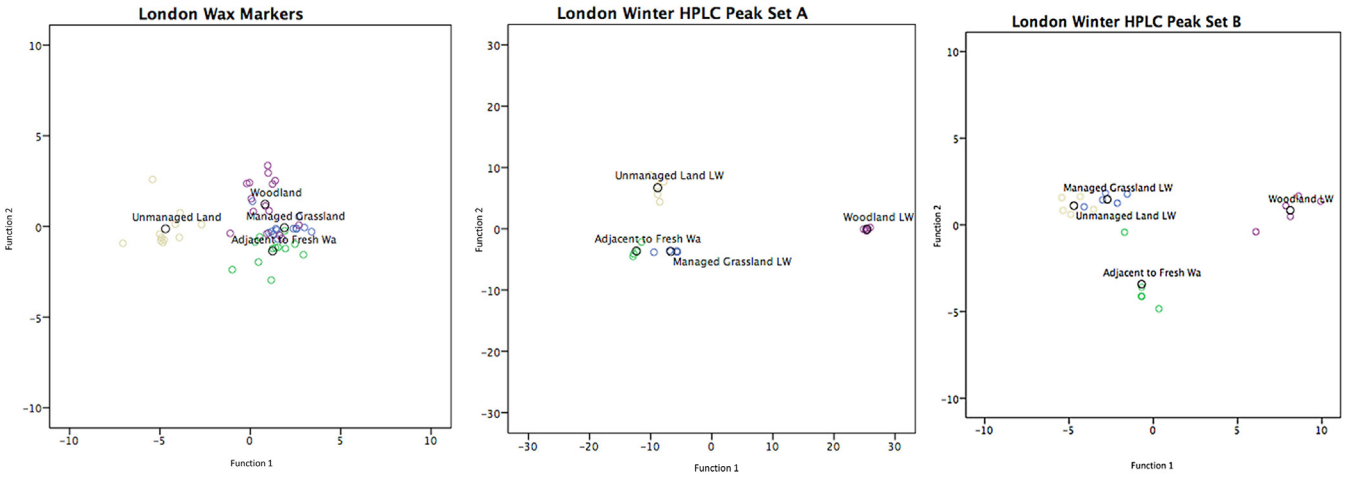

Fig. 10. CDFA Scatter Plots from Brockwell Park, London: CDFA Scatter Plots from Brockwell Park, London for wax marker profiles, HPLC peak set A, and HPLC peak set B at soils from locations adjacent to fresh water (Green), unmanaged land (Yellow), managed grassland (Blue), and woodland (Purple) with the position group centroids shown in black. HPLC plots compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 80.4% of samples were assigned to the correct group for HPLC set B and the wax markers, respectively. The first function accounted for 89.7%, 84.8% and 81.7% of the observed variance for HPLC set A, HPLC set B and the wax markers, respectively. When all three functions were used, the group differences for all three approaches were statistically significant at the >99% confidence interval.

The misclassification in the wax marker groupings at Brockwell Park were highest for the managed grassland, with four samples misclassified as adjacent to fresh water and one sample attributed to the woodland location. Woodland samples were also misclassified, one was assigned to managed grassland while a further four were grouped with soil adjacent to fresh water. Two samples were misclassified for HPLC set B, one sample from managed grassland was predicted to belong to the unmanaged land group, while one sample from the location adjacent to fresh water was incorrectly assigned to the managed grassland soil group.

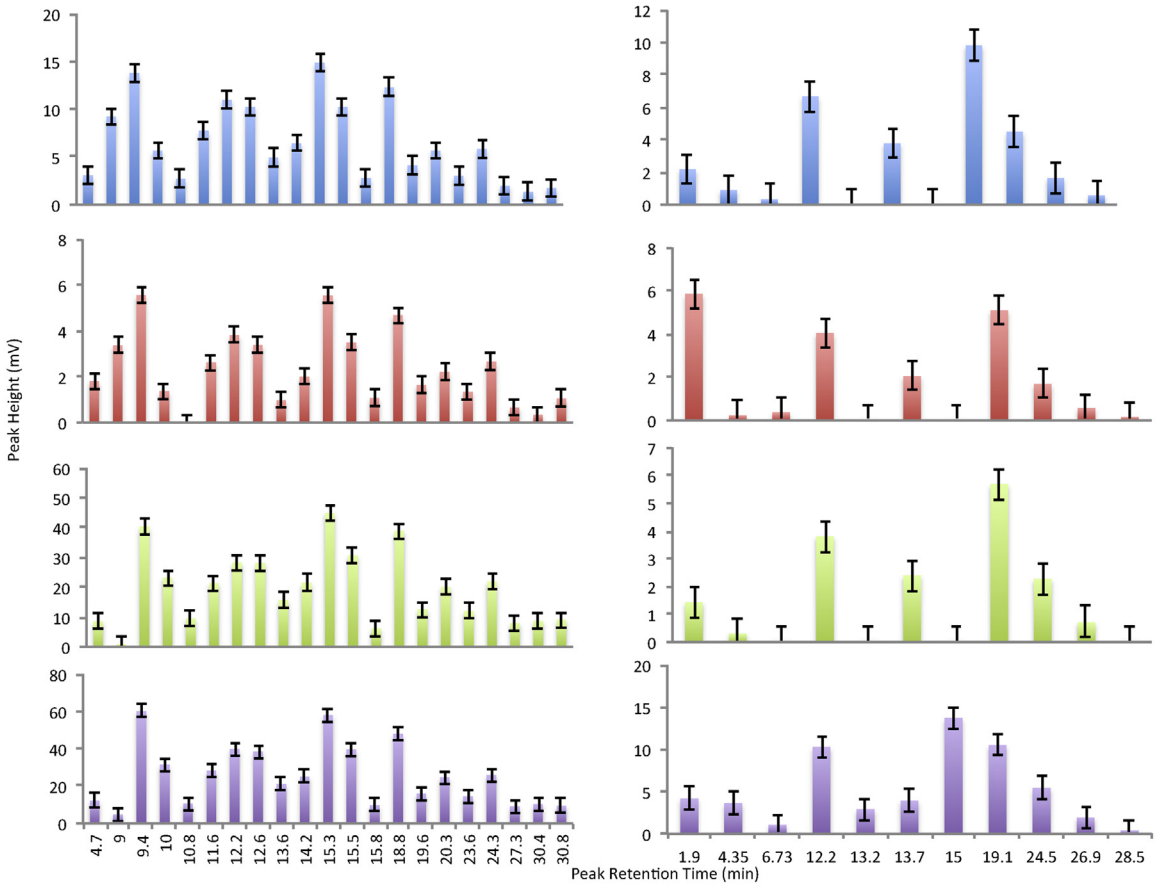

Fig. 11. New York HPLC profiles using marker set A (left) and B (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations at Central Park, New York City. Compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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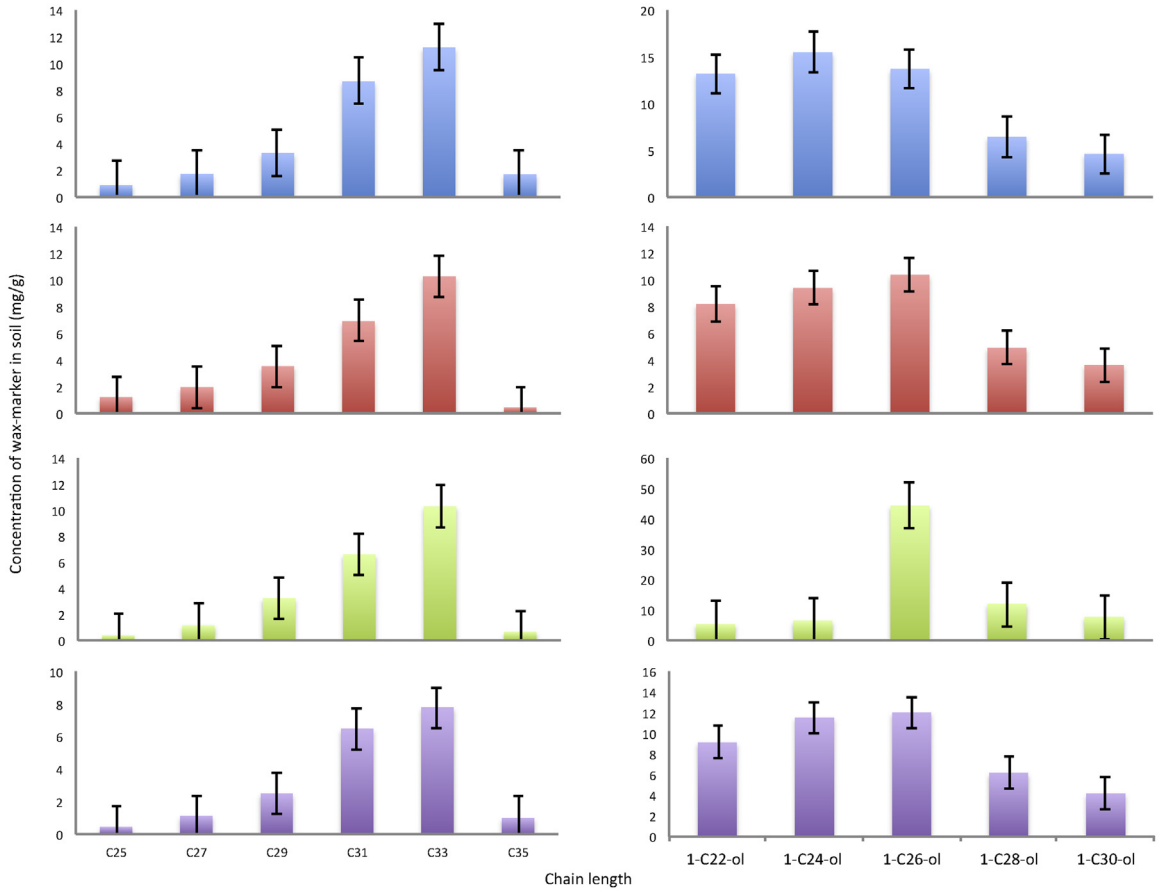

Fig. 12. New York GC profiles of *n*-alkanes (left) and fatty alcohols (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations in Central Park, New York City. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.3. Central Park, New York City, USA

All locations sampled at Central Park, New York City could be discriminated on the basis of their HPLC profiles for peak set A [21] (Fig. 11). The absence of a peak at 10.8 min distinguished samples taken from the location adjacent to fresh water, while the absence of the peak at 9 min was a distinctive feature of the samples taken from the unmanaged land site. Whilst the profiles of the managed grassland and woodland locations were visually similar, there was a difference between the two locations in the size of the peak at 9 min compared to 4.7 min.

The profiles for peak set B (Fig. 11) also enabled discrimination between the four locations. Soil profiles for unmanaged land could be separated from the other three locations by the absence of the peak at 1.9 min, while the profiles from soil adjacent to fresh water were most noticably different from the other locations in the ratio of the peak at 1.9 min compared to the peak at 12.2 min. The peaks in the managed grassland samples were generally three times as large as those for the location adjacent to fresh water, while peaks identified in the woodland samples were approximately twice the size obtained for managed grasslands. It has been shown that the use of relative peak size can provide an additional method to discriminate the groups visually [23].

The wax markers profiles allow woodland soils to be easily discriminated; in these locations C31 was present at a higher concentration than C29, whereas C29 was more abundant than C31 for the other locations. Managed grassland contained elevated levels of C35 compared to C33, whereas for the other locations, C35 was less abundant than C33. The most prominent feature distinguishing the unmanaged land from soils adjacent to fresh water was the ratio of 10-C29-ol to 1-C26-ol, which was much higher for the former, at 3.9:1, compared to 2:1 for the latter. While it was possible to visually discriminate the four profiles, many of the distinguishing features were small in magnitude compared to the variability of the data within each location (Fig. 12).

Both HPLC sets A and B gave 100% grouping accuracy at the New York site, while the wax markers correctly assigned 89.9% of the samples to their groups (Table 6). The differences between groups were statistically significant for all marker sets at the >99% confidence level with functions 1–3. Function 1 accounted for

Table 6
CDFA results for Central Park, New York City.

Marker type	Classification accuracy %	Wilks lambda significance test of functions			% variance function 1	% variance function 2	% variance function 3
New York		1–3	2–3	3			
HPLC set A	100.0	0.000	0.000	0.005	92.3	5.8	1.9
HPLC set B	100.0	0.000	0.000	0.071	73.3	24.1	2.5
Wax markers (GC)	89.9	0.000	0.000	0.003	66.1	25.3	8.6

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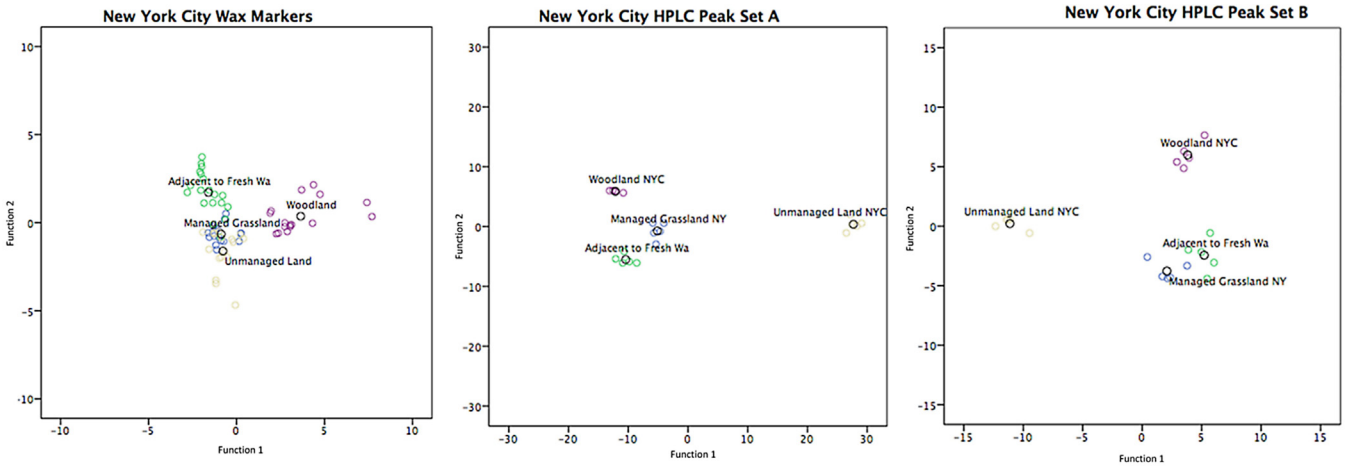

Fig. 13. CDFA Scatter Plots from Central Park, New York City: CDFA Scatter Plots for wax marker profiles, HPLC peak set A, and HPLC peak set B at soils from locations adjacent to fresh water (Green), unmanaged land (Yellow), managed grassland (Blue), and woodland (Purple) with the position group centroids shown in black. HPLC plots compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

92.3%, 73.3% and 66.1% of the variation between groups for HPLC sets A and B, and the wax markers, respectively.

The accuracy of the groupings at the Central Park site are reflected in the CDFA scatter plots (Fig. 13), with good spacing between groups evident for HPLC peak sets A and B, and some overlap of sample groups for the wax markers. CDFA on the wax marker data misclassified one sample from managed grassland as unmanaged land, two samples from the location adjacent to fresh water were assigned to managed grassland and one inaccurately placed in the unmanaged land group, while three samples from

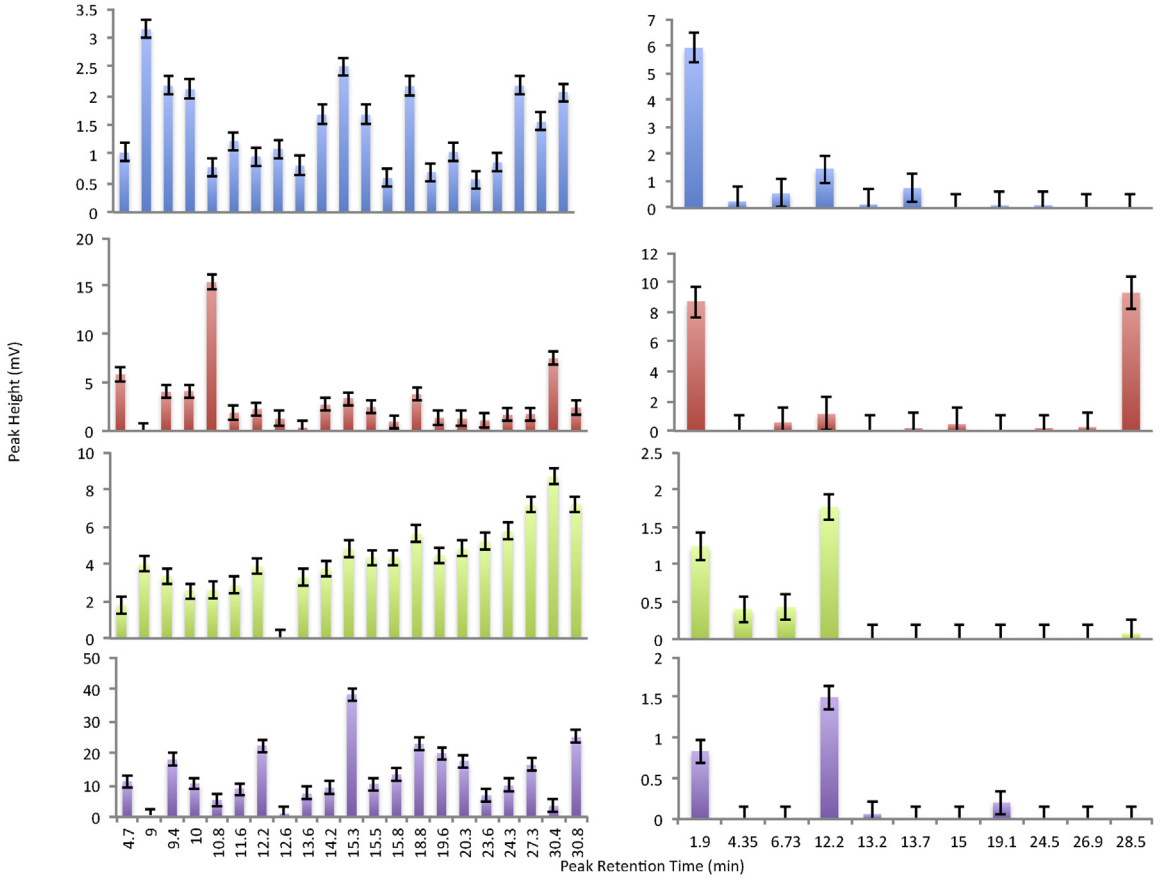

Fig. 14. Aberdeen HPLC profiles using marker set A (left) and B (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations in Craigiebuckler. Compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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unmanaged land were attributed to unmanaged land, however all the woodland samples were grouped accurately.

4.4. Craigiebuckler Estate, Aberdeen, UK

The profiles for HPLC peak set A (Fig. 14) distinguished the four locations in Craigiebuckler Estate, Aberdeen as outlined fully by McCulloch et al. [23]. The managed grassland samples were distinguished with the largest peak in the profile at 9.4 min and the profiles of soils adjacent to fresh water were distinctive with the largest peak at 10.8 min. The large relative height of the peak at 30.8 min was distinctive of woodland soil profiles, and the profile of the samples from unmanaged land was could be discriminated from samples from the other locations with the highest peak at 30.4 min.

Peak set B (Fig. 14) for the samples from the four locations also clearly distinguished the different locations within this site (see McCulloch et al. [23]). The comparison of the retention time and ratio between the two largest peaks at each location was useful in grouping the samples from managed grassland and those from soils adjacent to fresh water, while the ratio between the 1.9 min and 12.2 min peak pairs separated woodland soils from unmanaged land.

Woodland soils at the Craigiebuckler site could be easily identified using their wax marker profile, since C27 was the most abundant *n*-alkane, whereas C31 had the highest concentration of the *n*-alkanes for the other locations. Unmanaged land was distinct from the other locations since 10-C29-ol was the most concentrated of the alcohols. The remaining locations, adjacent to fresh water and managed grassland, could be differentiated through the C33:C35 ratios, which were 1.5:1 and 6.4:1, respectively (Fig. 15).

At the Craigiebuckler site, 94.7% of samples were grouped correctly using HPLC set A, while HPLC set B provided 100% accuracy and the wax markers predicted the correct group in 86.2% of cases. The differences between groups were statistically significant at the >99% confidence interval when functions 1–3 were used for all marker sets (Table 7).

Using HPLC Peak set A, all but one sample was correctly classified across all four sites. One sample from the unmanaged location in Craigiebuckler Estate, Aberdeen was misclassified as having originated from the woodland location. All samples were correctly assigned for HPLC set B. Three samples taken from managed grassland were attributed to unmanaged land using the wax marker data, in addition one sample from the location adjacent to fresh water was misclassified as unmanaged land, while one sample from unmanaged land was grouped with woodland soils, and four woodland samples were incorrectly classified as unmanaged land (Fig. 16).

4.5. Increasing group attribution accuracy

When neither of the HPLC marker or wax marker profiles were able to give 100% accuracy in all groupings, further CDFA were performed using the results of both the GC and HPLC results in conjunction with one another. Using only wax markers (alkanes and alcohols), the accuracy rates were 86.2%, 89.9% and 80.4% for the Aberdeen, New York City, and London sites respectively, using only HPLC set A the accuracy rate was 94.7% in Aberdeen, and for HPLC set B in London, the accuracy was 90%. Using both the HPLC and wax markers in the same CDFA, the grouping accuracy increased to 100% as shown in Table 8.

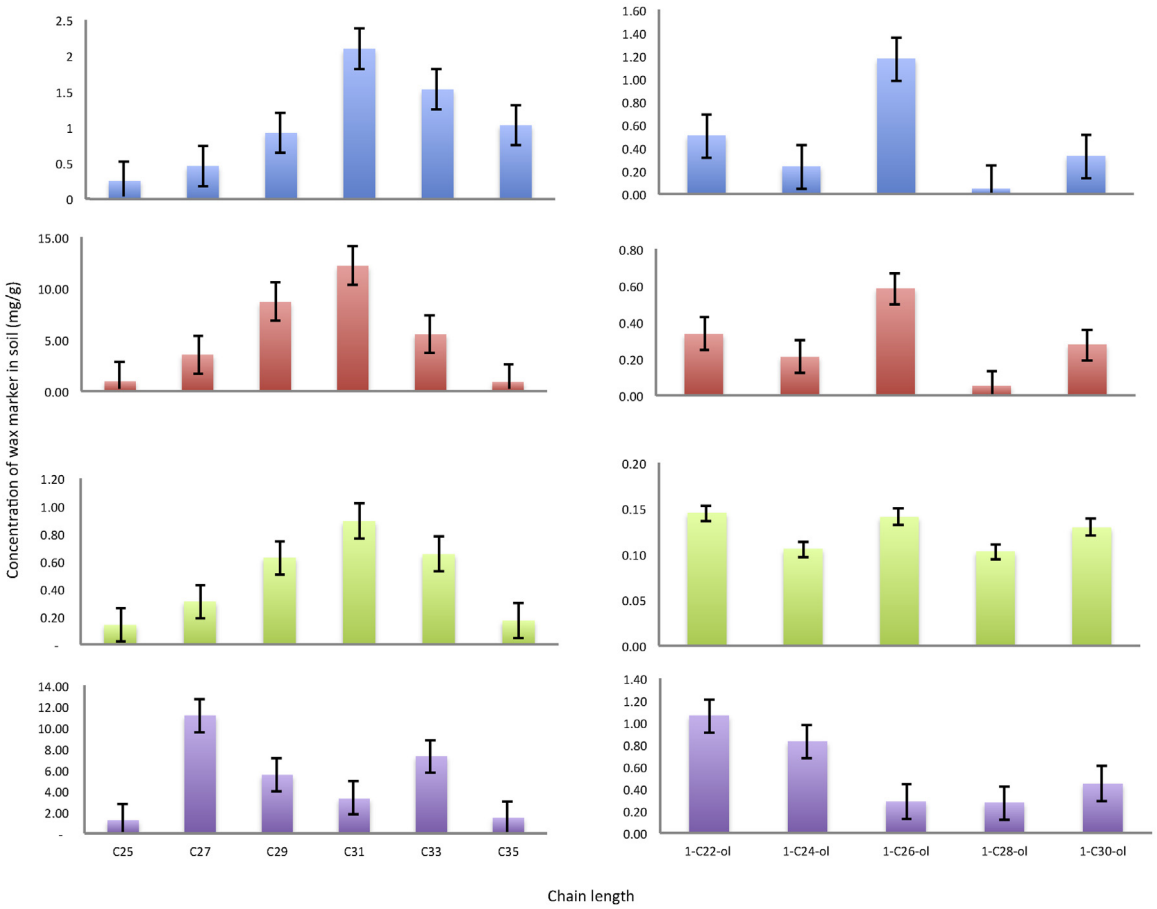

Fig. 15. Aberdeen GC Profiles of n-alkanes (left) and fatty alcohols (right) for managed grassland (blue), adjacent to fresh water (red), unmanaged land (green) and woodland (purple) locations in Craigiebuckler, Aberdeen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 7
CDFA results for Craigiebuckler, Aberdeen.

Marker type	Classification accuracy %	Wilks lambda significance test of functions			% variance function 1	% variance function 3	% variance function 2
Aberdeen		1–3	2–3	3			
HPLC set A	94.7	0.001	0.147	0.531	90.6	7.8	1.5
HPLC set B	100.0	0.000	0.000	0.014	97.4	2.4	0.2
Wax markers (GC)	86.2	0.000	0.000	0.397	68.0	26.6	5.4

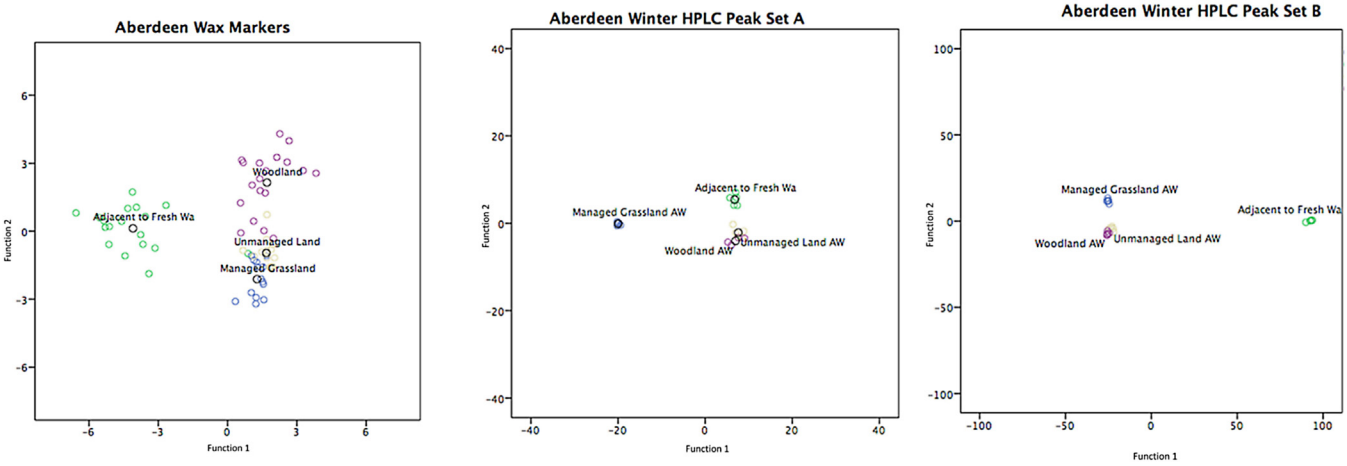

Fig. 16. CDFA Scatter Plots from Cragiebuckler, Aberdeen for wax marker profiles, HPLC peak set A, and HPLC peak set B at soils from locations adjacent to fresh water (Green), unmanaged land (Yellow), managed grassland (Blue), and woodland (Purple) with the position group centroids shown in black. HPLC plots compiled from McCulloch et al. [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 8
CDFA results for the combined use of HPLC and wax markers.

Marker type	Classification accuracy %	Wilks lambda significance test of functions			% variance function 1	% variance function 2	% variance function 3
London results for wax markers (GC) and HPLC markers		1–3	2–3	3			
Set A	100.0	0.000	0.002	0.034	89.7	7.0	3.3
Set B	100.0	0.000	0.019	0.246	82.7	14.2	3.1
Aberdeen results for wax markers (GC) and HPLC markers							
Set A	100.0	0.000	0.000	0.000	93.0	4.8	2.2
Set B	100.0	0.000	0.000	0.046	98.6	1.3	0.2
New York City results for wax markers (GC) and HPLC markers							
Set A	100.0	0.000	0.005	0.084	93.9	4.6	1.5
Set B	100.0	0.000	0.027	0.581	95.1	4.6	0.3

In all of the cases where less than 100% accuracy was achieved using only one set of markers, 100% accuracy was achieved using the combination of the two types of organic marker sets. Furthermore, the separation between groupings was statistically significant at the 99% confidence level using the combination of organic methods. At the London site, functions 1–3 explained 89.7%, 7.0% and 3.3%, respectively, of the variation between the groups using the wax markers and HPLC set A, and 82.7%, 14.2% and 3.1% of the variance, using HPLC set B. For the Aberdeen site, the first three canonical functions explained 93.0%, 4.8% and 2.2% of the variance using HPLC set A to complement the wax markers, and 98.6%, 1.3% and 0.2% using HPLC set B (Fig. 17).

5. Discussion

Both the HPLC and GC organic profiling techniques provided strong discriminatory results at a realistic crime scene scale, with high accuracy rates when discriminated using CDFA. The statistical analysis shows HPLC can offer high accuracy rates in grouping samples according to provenance location as outlined in McCulloch et al. [23]. Accuracy rates were slightly lower for the GC markers (using only alkanes and alcohols) than either of the HPLC marker sets, with the exception of the Lochend Park site where all sets of markers gave 100% accuracy. The two HPLC marker sets were able to improve the accuracy achievable when using only the wax markers, demonstrating that HPLC can add value to geoforensic investigations when used alongside established techniques such as organic wax marker analysis. Interestingly, in the two cases where neither set of HPLC markers gave 100% accuracy when used on their own, (for example HPLC set B for London and HPLC set A for Aberdeen), with the addition of the wax marker profile data, it improved the accuracy of discrimination to 100%.

Visual comparison of the profiles using both analytical methods also allowed for discrimination between the four locations at each

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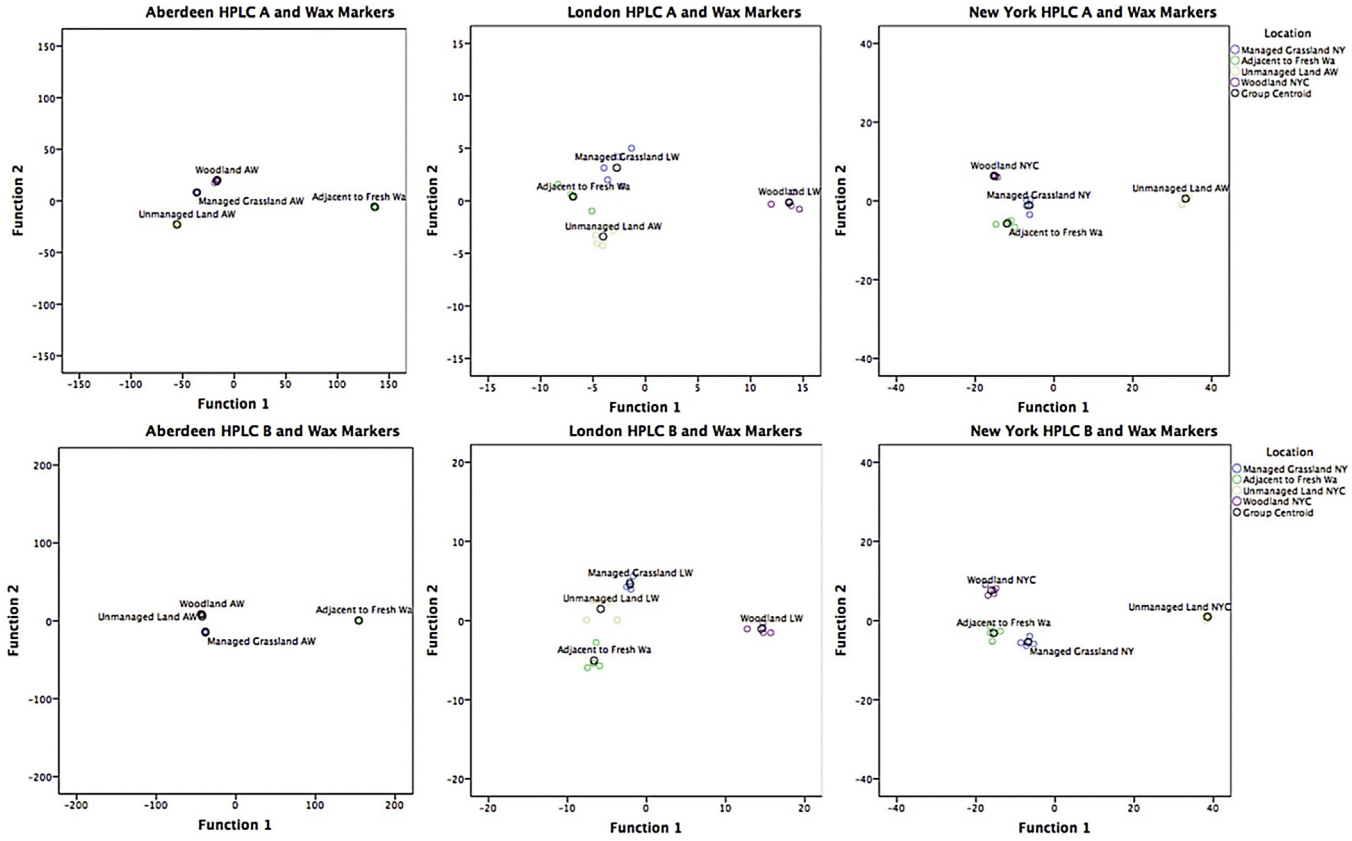

Fig. 17. CDFA Scatter Plots for the combined use of wax marker profiles and HPLC peak sets at sites where either marker set could not provide 100% accuracy, for soils from locations adjacent to fresh water (Green), unmanaged land (Yellow), managed grassland (Blue), and woodland (Purple) with the position group centroids shown in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

site. The visual differences were generally more distinct for the HPLC data, making this potentially quicker to process these data sets with appropriate available chemometric tools. Furthermore, with more points potentially available for comparison, the degree of certainty with which a visual assessment could be made was higher for the HPLC. For all sites, the GC profiles allowed for at least one of the locations to be unambiguously identified visually. It is important to acknowledge that the visual assessment of the profiles is naturally a subjective process (as is the case with many other forms of trace evidence). For wax marker data, the advantage of identifying and quantifying known compounds is invaluable. This allows interpretation of what plant material is most likely to have contributed to the profile, thus also assisting in intelligence operations with the available databases. The variability in the data within each location was such that the subtle differences in peak ratios that facilitated complete discrimination of all four locations with HPLC profiling, and were particularly useful for the wax markers, should be treated with caution when interpreting evidence. These small differences may not be reliably detected across all sample replicates. This high internal variability presents an additional challenge when sample quantities are limited such that the number of sample replicates is restricted. In this regard, wax marker profiling offers a significant advantage over HPLC, since the analysis can be performed on sample quantities as low as 13 mg [17]. This allows individual aggregates from a multiple source sample to be recovered and analysed separately from the background matrix to improve the comparability with a potential source location, in particular avoiding potential issues such as those encountered with a mixed provenance sample [6].

The high variability observed in the data is likely to be a reflection of the heterogeneous nature of soil. However, there are a number of potential sources of variability in the methodology which should be considered, for both the HPLC and GC techniques. The sample preparation technique for the GC analysis was more complex to learn at the outset as there were many more steps in the GC method in comparison to the HPLC method. The GC method comprised multiple concentration and reconstitution steps, using very small volumes of 20–60 μl sample. It could be more difficult to achieve analytical precision and accuracy when working with smaller volumes of sample since small systematic errors have a larger relative effect on the results. Furthermore, the large number of steps in the analytical method presented potential opportunities for small human errors, such as inadvertent, inaccurate weighing or imprecise pipetting to create variability in the resulting data. However, the concentrations of the compounds present in the samples is determined relative to known standards using the GC approach, providing a means of calibrating the chromatographic system and monitoring reproducibility.

The HPLC sample preparation technique was in comparison simpler to learn and to perform than the GC technique. Only one weighing and dilution step is required, allowing a batch of samples to be prepared in less than two hours compared to one day cumulatively for the GC method. Aside from the practical and financial advantages of a simpler sample preparation method, the reduced opportunity for analyst variability suggests that the error bars evident in the HPLC data arise from true differences in the samples. The variability observed possibly reflects the sensitivity of the HPLC method in detecting differences at a smaller scale resolution, or from the limitations of the instrument parameters chosen during method development. The HPLC methodology did not include a grinding step, as the technique was developed with the aim of retaining as much of the innate variation between

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samples as possible, so that rare and informative peaks were not diluted out through homogenisation. The initial method development experiments [20] showed that milling had no impact on the ability to detect and separate peaks of interest. Since milling may, however, improve extraction efficiency and the uniformity of content of the individual sample points, it is recommended that the influence of milling on the reproducibility and sensitivity of the technique is investigated in future studies, in addition to assessment of temporal variability and the effects of the moisture content of the samples on the resultant profiles.

There were no clear consistencies observed between the profiles for locations of apparently similar planting, such as woodland locations, across the four sites. This may be a result of the locations being chosen on the basis of environmental factors (such as ease of access, public usage and visibility) rather than the degree of consistency in planting between locations of the same type, or as a result of the variation in plant species at locations of the same type at the four different sites. It may be possible to reduce intra-location type variability relative to inter-location type variability through careful control of the planting at samples locations, however the ecological validity of any technique developed in this manner would be limited to situations where the vegetation at the crime scenes and alibi sites was similarly constrained. Furthermore, the degree of improvement in precision obtained using a more controlled experimental design is considered to be minimal, since in this study there were equally high levels of variability obtained for locations of homogenous vegetation. For example, the variability of the profiles generated for the managed grassland locations, where the surface vegetation was consistent at each site, was comparable to the results from woodland locations where there was a variety of plants within each location and predominantly different tree species at each of the sites. This could be a result of the organic profiles reflecting past as well as current plant and animal inputs.

6. Conclusions

This study aimed to assess the variability of organic profiles over a forensically relevant, close-proximity spatial scale. Two organic methods were used to evaluate the relative and complementary benefits of both HPLC and wax marker by GC profiling techniques for assisting crime reconstruction. It has been established that HPLC has the potential to offer an accurate and practical technique for the analysis of geoforensic evidence, provided sample sizes were not constrained, and that it is applicable for discrimination of close proximity sites across a range of geographical locations [21]. The HPLC profiles were useful not only when combined with CDFA, but also when compared visually. In addition to offering visually distinct profiles and improved accuracy in the statistical analysis compared to the GC technique (alkane and alcohols only), the HPLC analysis is quicker, cheaper and simpler to perform, which reduces potential error rates and improves the confidence with which conclusions can be made when comparing samples. Whilst it is acknowledged that the HPLC method is not currently able to quantify specific compounds as the wax marker approach can, the findings from this study indicate significant promise in developing the HPLC approach further to provide an additional technique for the comparison of samples based on the organic component of a forensic sediment sample.

The GC technique has been validated to industry standards in accordance with ISO17025 guidelines and is an accepted trace evidence technique within geoforensic casework in the UK. The wax marker profile data performed well across all four sites. It is noted that the accuracy rates achieved with CDFA were not as high as for the HPLC method, and in visual comparison the wax marker profiles were less easily discernible than for the HPLC markers, although the compounds were all individually identifiable and quantifiable. Wax marker analysis has been demonstrated as suitable for use on very small sample quantities (c. 10 mg compared to 250 mg for HPLC). It also allows the generation of intelligence given the ability to identify compounds that are dominating a profile, The GC technique can therefore, offer indications of the type of vegetation and habitat that has contributed to the soil to provide intelligence in 'seek and find' cases. In addition, while not included in this study, there are other compound groups which can also be included in the GC dataset such as sterols, stanols, aldehydes and ketones, which have the potential to broaden the number of measures within an evidence base, potentially improving the capacity for discrimination and the evidential value of an analysed soil sample.

This study has illustrated that the HPLC method presented here performs well when compared to the GC method in terms of distinguishing between different close proximity locations. The HPLC method alsooffers benefits in terms of a simpler preparation process for samples prior to analysis. There is significant potential for further testing of the HPLC method and in considering the combined use of both techniques to achieve very high accuracy rates for discriminating close proximity sample locations in particularly serious crimes.

The 100% accuracy achieved in this study using this combined approach suggests that it would be beneficial to measure both HPLC and GC markers where additional exclusionary discrimination is required, providing there is sufficient sample quantity of questioned sample and available resources. Whilst further work will be required to fully validate the HPLC profiling technique, this study demonstrates that the HPLC profiling method has significant potential to provide investigators with an accurate and simple approach, where sufficient sample quantities are available, and in cases where this level of spatial resolution is required to discriminate between samples of close proximity. To this end, HPLC could be considered as an initial screening step for exclusionary purposes. A combined approach using a range of organic characteristics has the potential to further enhance the value and/or significance of the analysis of close proximity samples generated within casework.

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