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JMS Letters

Dear Sir,

Use of Matrix-assisted Laser Desorption/Ionization Mass Spectrometry for the Rapid Detection of Low-mass Components in the *Alkanna tinctoria* Pigments Fraction

Matrix-assisted laser desorption/ionization (MALDI)[1] mass spectrometry (MS) has emerged as a powerful method in the analysis of large biomolecules such as proteins and nucleotides with molecular masses (M_r) up to 500 kDa[2] and 60 kDa,[3] respectively. The use of an aromatic compound as a MALDI matrix, which usually produces ions that predominate in the low-mass region, accounts for the limited utility of MALDI in the analysis of involatile, low-mass compounds. There are only a few reports of the utilization of MALDI for M_r determination of small molecules, such as taxanes[4] and indoles[5] with $M_r \sim 800$ and 1000 Da, respectively. These MALDI spectra, obtained under continuous extraction conditions, mainly exhibit signals corresponding to sodiated and radical molecular ions and a significant amount of fragmentation down to m/z 400. Nevertheless, the most abundant signals in these spectra are the matrix ions in the m/z 200–400 range.

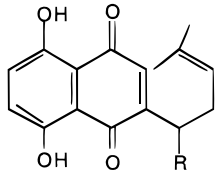
In this letter, we report the application of delayed extraction[6] linear MALDI/MS to the rapid screening of the naphthaquinone pigment components present in the root hexane extract of *Alkanna tinctoria*. This mainly consists of a mixture of non-volatile alkannin esters and small amounts of polymeric pigments, which makes it difficult to analyze by the traditional methods of electron impact (EI) and chemical ionization (CI) MS. Repeated column chromatography has been employed previously to isolate the acetate, isobutyl and isovalerate esters of alkannin from the hexane extract of *Alkanna tinctoria*.[7,8] Full structural characterization of these fractions has been carried out by CIMS[7] and NMR spectroscopic methods.[7–9] Nevertheless, there has been no information on any medium- and high-mass pigment components of these chromatographic fractions. Therefore, we decided to evaluate whether MALDI/MS can be used as a rapid and simple means to provide insights into the complexity of these mixtures.

The isoexenylnaphthazarins, commonly known as alkannins, are lipophilic red pigments.[10–12] They are found in the outer surface of the roots of at least 150 species that belong to the genuses *Lithospermum*, *Echium*, *Onosma*, *Anchusa*, *Arnebia*, *Macrotomia* and *Cynoglossum* of the family Boraginaceae. In addition to being used as pigments in food and cosmetics, they have been reported to possess antibacterial,[13–15] anti-inflammatory,[16–18] antitumor[19–21] and wound-healing properties.[17,22] Even though the wound-healing properties of these roots were described by Dioscorides,[23] 'The root is of a binding nature, being good for burnings and old ulcers,' it was not until 1977 that this medicinal property was confirmed and the active components of the root were determined.[24,25] Only recently the ointment Helixderm received approval by the Hellenic National Drug Organization for the treatment of chronic skin ulcers. As far as we know, this is the first preparation of its kind and is considered to fill a considerable gap in the therapeutic arsenal, because it provides effective treatment for indolent ulcers (ulcus cruris) and exhibits antibiotic action.[13]

Roots of *Alkanna tinctoria* Tausch were collected in Anatolia, Turkey, and were purchased from P. N. Gerolymatos Pharmaceutical (Athens, Greece). A 200 g amount of powdered dried roots of *Alkanna tinctoria* was subjected to the extraction procedure described previously.[25] MALDI/MS analysis of the pigments fraction of *Alkanna tinctoria* was carried out on a PerSeptive Voyager RP-DE reflectron time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with a nitrogen laser (wavelength 337 nm). The mass measurement of the pigments fraction of *Alkanna tinctoria* was performed in the linear mode using sinapinic acid as the matrix. Samples were generally dissolved in 0.1% aqueous trifluoroacetic acid (TFA) at a concentration of 0.1–0.5 mg ml^{-1}, while the sinapinic acid matrix was dissolved in 0.1% aqueous TFA–acetonitrile (2:1, v/v) at a concentration of 5–10 g l^{-1}. To 1 μl of the sample solution were added 10 μl of the matrix solution, and 0.5–1 μl thereof was deposited on the sample plate and air-dried prior to mass spectrometric analysis. Analysis of the pigments fraction of *Alkanna tinctoria* by fast atom bombardment (FAB) MS was carried out on a MAT 95 double-focusing mass spectrometer operating at 5 kV accelerating voltage. Xenon fast atoms at 8 kV (2 μA) were used for ionization. *Alkanna* samples were dissolved in 1–2 μl of dimethyl sulfoxide or methanol and deposited on a stainless-steel probe tip, followed by addition of ~1 μl of thioglycerol or glycerol–thioglycerol matrix.

MALDI/MS analysis of the hexane extract of *Alkanna tinctoria* provided a preliminary insight on the structure of the naphthaquinone pigments that are characteristic of the *Alkanna tinctoria* Tausch. Even though the goal of this study was to detect specifically any medium- and high-mass pigment components, most of the MALDI signals were in the m/z 200–800 range. Several low-M_r compounds that have been characterized previously, such as deoxyalkannin and the isobutyl/isovalerate esters of alkannin,[7,8] were detected by MALDI/MS (Table 1). In addition, the MALDI mass spectrum exhibited signals that probably correspond to higher M_r analogs that have not been detected previously by the more traditional methods of EIMS and CIMS. The most abundant ion in the MALDI mass spectrum shown in Fig. 1 is that at m/z 272, which corresponds to the radical molecular ion of the deoxyalkannin (**1**, R = H in Table 1). Deoxyalkannin has been

Table 1. Observed M_r values[a] and proposed structures of the R group in alkannin structure 1



1

M_r	R	Ref.[b]
272	—H	8, 9
358	—$OCOCH(CH_3)_2$	8, 26
370	—$OCOCH{=}C(CH_3)_2$	7
372	—$OCOCH_2CH(CH_3)_2$	7, 8
386	—$OCOC_5H_{11}$	—
402	—$OCOC_5H_{11}O$	—
416	—$OCOC_6H_{13}O$	—
430	—$OCOC_7H_{15}O$	—
448	—$OCOC_6H_{13}O_3$	—

[a] Molecular mass (M_r) values deduced from the MALDI mass spectrum in Fig. 1.
[b] Previous reports on structure characterization of certain alkannin pigments.

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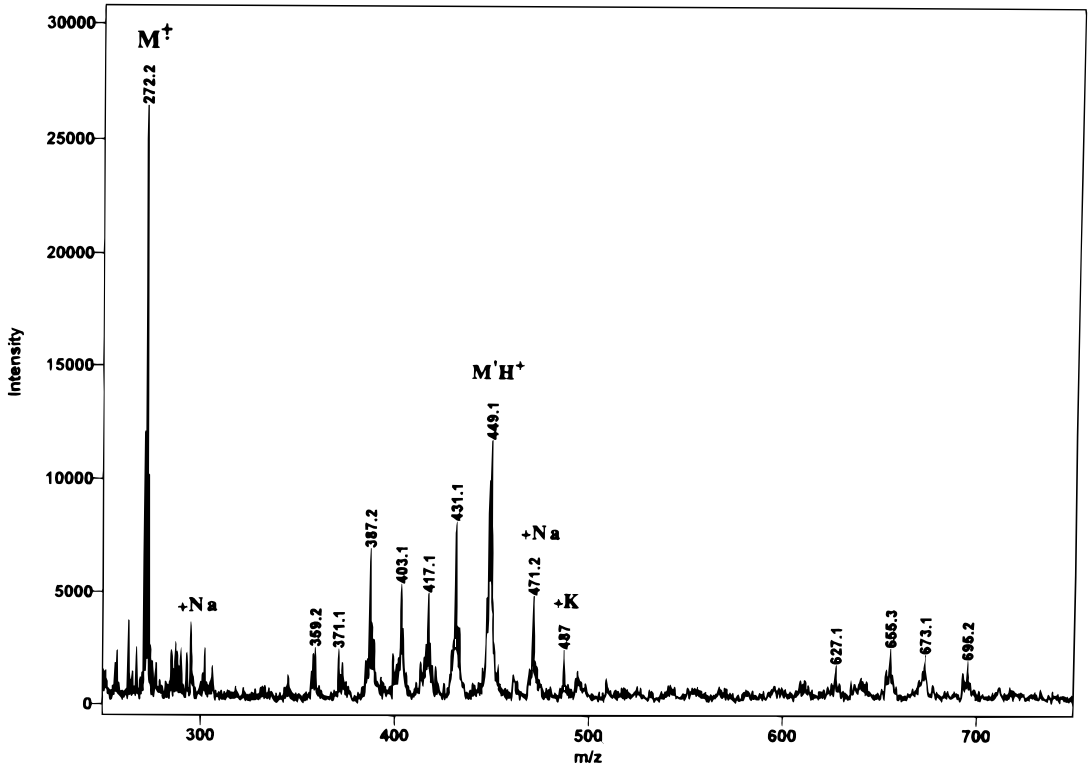

Figure 1. Delayed extraction MALDI mass spectrum of *Alkanna tinctoria* mixture using sinapinic acid matrix. The MALDI signals in the *m/z* 600–700 range correspond to adduct ions of basic alkannin structures and the sinapinic acid matrix.

isolated previously by column chromatography and analyzed by isobutane CIMS, yielding radical and protonated molecular ions,[7] and fully characterized by proton and carbon-13 NMR spectroscopy.[8] The formation of abundant radical molecular ions has been observed previously in the MALDI mass spectra of chromophore-containing compounds,[27] which also show protonated molecules of lower abundance (e.g. *m/z* 273 for the deoxyalkannin). The ion of *m/z* 271 was formed from the molecular ion via loss of hydrogen, or from fragmentation of higher mass alkannin components. Higher M_r species formed predominantly protonated MH^+ molecules and $[M + Na]^+$ adducts, presumably owing to the higher proton affinity as the size of the R group increases. Proposed structures for some of these ions are listed in Table 1, with the R group of the common naphthaquinone structure **1** ranging from $C_4H_7O_2$ to $C_8H_{15}O_3$.

The ions at *m/z* 359 and 373 correspond to the protonated molecules of the isobutyl (**1**, R = —$OCOCH(CH_3)_2$) and isovaleryl (**1**, R = —$OCOCH_2CH(CH_3)_2$) alkannin moieties, respectively. An additional unsaturation site at the R chain of the alkannin isovalerate gives rise to the *m/z* 371 ion (angelate). All isobutyl, isovalerate and angelate esters of alkannin have been observed previously in the analysis of chromatographic fractions of the *Alkanna tinctoria* hexane extract,[7,8] but not in the analysis of the unseparated hexane extract. The MALDI signal at *m/z* 387 arises from the dimethylbutyl alkannin, while its hydroxylated derivative yields the MH^+ ion at *m/z* 403. Further aliphatic chain extensions in the R group of the latter structure are also present in the mixture, as evidenced by the MALDI signals at *m/z* 417 and 431. The ion at *m/z* 449 probably arises from the addition of two hydroxyl groups to the aliphatic chain (**1**, R = —$OCOC_6H_{13}O_3$), with the sodium and potassium adducts giving rise to satellite signals at *m/z* 471 and 487, respectively (Fig. 1). The fact that all these higher M_r components have not been observed previously by other MS ionization methods makes MALDI/MS an ideal method for screening pigment fractions of *Alkanna tinctoria* for new components.

In order to confirm the superior performance of MALDI for the direct analysis of natural product extracts, we analyzed the *Alkanna tinctoria* hexane extract by FABMS.[28] The FAB mass spectrum provided much less information on the presence of all the aforementioned components, because it was dominated by the abundant deoxyalkannin molecular ion of *m/z* 272. It is worth noting the formation of the deoxyalkannin–thioglycerol adduct and the dimeric deoxyalkannin moiety followed by hydrogen elimination, as evidenced by the strong FAB signals at *m/z* 378 and 541, respectively (Fig. 2). Accurate mass measurement of the corresponding ions corroborated these assignments (378.1047 and 541.1848 u, respectively). The addition of one thioglycerol molecule was observed for both the deoxyalkannin and its dimeric component, giving rise to the *m/z* 378 and the 649 ions, respectively (Fig. 2). Both the dimer and the matrix adducts were only observed in a thioglycerol-containing FAB matrix, and not with other common FAB matrices such as glycerol or *m*-nitrobenzyl alcohol. Therefore, it seems that the presence of a strong nucleophile, such as the thioglycerol anion, promotes the oxidative dimerization of deoxyalkannin, which probably occurs through a Michaels-type addition followed by loss of hydrogen. The addition of an acidic medium, such as TFA, to the thioglycerol matrix results in a decrease in the dimer's abundance, thus showing that its formation is favored by basic conditions. The formation of dimeric and even trimeric components has been observed previously in the electrospray MS analysis of the acetate and isovalerate isolated constituents.[29]

These results show the importance of delayed extraction MALDI over other desorption MS techniques, as an excellent screening method for confirming known, in addition to determining new, pigment components directly from the analysis of

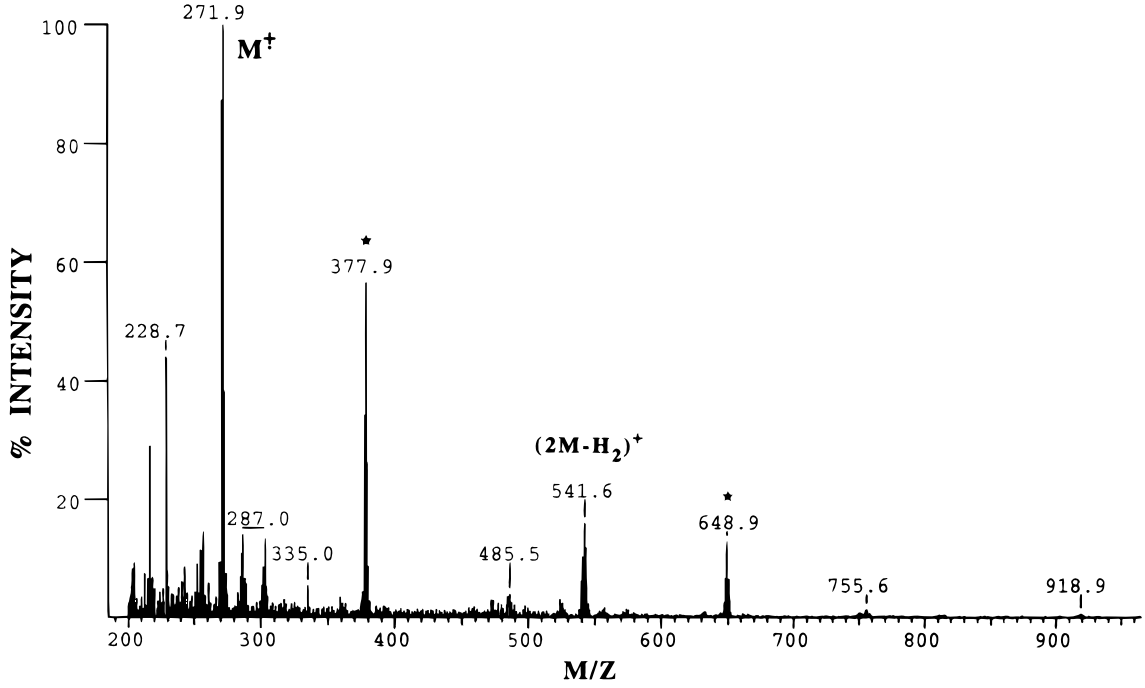

Figure 2. FAB mass spectrum of *Alkanna tinctoria* mixture using glycerol–thioglycerol (1 : 1) matrix. The peaks marked with asterisks correspond to thioglycerol adduct ions of deoxyalkannin and its dimeric component.

the extraction mixture. Some of these components, such as those with M_r 402, 416, 430 and 448, have been observed for the first time, and we are currently trying to isolate them for further characterization. This work demonstrates that MALDI/MS is an ideal method for analyzing natural product extracts, and it can be an invaluable addition to a large number of natural product chemists who currently limit their MS approaches to FAB or EI ionization methods.

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