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Particle Beam Liquid Chromatography/Mass Spectrometry with Negative Ion Chemical Ionization for the Confirmation of Ivermectin Residue in Bovine Milk and Liver

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Particle beam liquid chromatography/mass spectrometry (LC/MS) using negative ion chemical ionization was applied to the analysis of ivermectin residue in bovine milk and liver. Samples were prepared by liquid/liquid extraction followed by alumina B solid-phase extraction clean-up. On-line LC/MS of extracts was carried out on a C-18 bonded silica column. Signals were observed from on-column injections of 4 ng dihydro-avermectin B_{1a} (H_2B_{1a}) in extracts equivalent to 2 ml milk or 0.3 g liver. The specificity required for a regulatory confirmation procedure was achieved by monitoring the H_2B_{1a} molecular ion and four fragment ions. Ion chromatogram peak areas were at least three times greater than control samples integrated over the same time window. Coeluting matrix compounds enhanced the response and altered the abundance pattern of H_2B_{1a}. To compensate for this matrix effect, control milk extracts were spiked with H_2B_{1a} standard and used for the abundance matching requirement of regulatory confirmation.

INTRODUCTION

Ivermectin is the common name for a veterinary medication consisting of a mixture of two structural homologs, dihydro-avermectin B_{1a} and B_{1b} (H_2B_{1a}, H_2B_{1b}) in at least an 80:20 ratio. Ivermectin is approved for treatment of parasitic infections in beef cattle, but only if given many weeks before slaughter to allow drug residues to deplete below regulatory tolerances. Ivermectin is currently not approved for lactating dairy cattle.

The US Food and Drug Administration (FDA) has set a tolerance for ivermectin residue levels in beef liver, and the FDA can bring legal action if this tolerance is exceeded. A validated analytical method is used to determine if a violative residue level occurs. Legal action is further supported by a confirmation step that definitively shows the residue compound is present. The high specificity of mass spectrometry makes it the preferred approach to regulatory confirmation.

The H_2B_{1a} homolog (Fig. 1) is the 'marker' for ivermectin residues, and its concentration must fall below 15 ng g^{-1} (p.p.b.) in beef liver.[1] The marker is a single compound whose concentration correlates to the total residue level. Drug depletion studies have shown that dosed H_2B_{1a} is secreted through the mammary gland over many weeks,[2] underscoring the concern that residues might also occur in retail milk. Extrapolation from tissue levels indicates that a regulatory analytical method should be capable of detecting 2 ng ml^{-1} (p.p.b.) H_2B_{1a} in milk.[3] Several methods have been developed for measuring H_2B_{1a} levels in milk and liver. These assays employ reverse-phase liquid chromatography (LC) with fluorescence[4–7] or ultraviolet (UV) detection.[8,9]

Regulatory confirmation is a qualitative step which is applied after a marker residue has been measured at a violative level. In the past, the presence of ivermectin residues has been confirmed by a lengthy method based on partial hydrolysis, derivatization, LC and fluorescence detection.[10] To date there have been no literature reports of a *mass spectrometric* method to confirm ivermectin residues at the levels required for liver and milk monitoring. This lack led the FDA to pursue new approaches to the confirmation of ivermectin residue by mass spectrometry.

Previous approaches to mass spectral analysis of H_2B_{1a} have shown limited sensitivity. The negative ion, filament-on thermospray mass spectra showed molecular ions and fragment ions due to loss of one or two sugar moieties.[11] However, this analysis used 500 ng H_2B_{1a}. Thermospray mass spectrometry was previously used by the FDA, but detection limits were above the current requirements.[12] Direct injection thermospray mass spectrometry in negative ion mode was used to support an LC UV method for ivermectin in milk, but detection limits were not reported.[7] Direct exposure chemical ionization tandem mass spectrometry (MS/MS) was used to confirm H_2B_{1a} added to liver at 38 p.p.b.,[13] but direct probe introduction of extracts is

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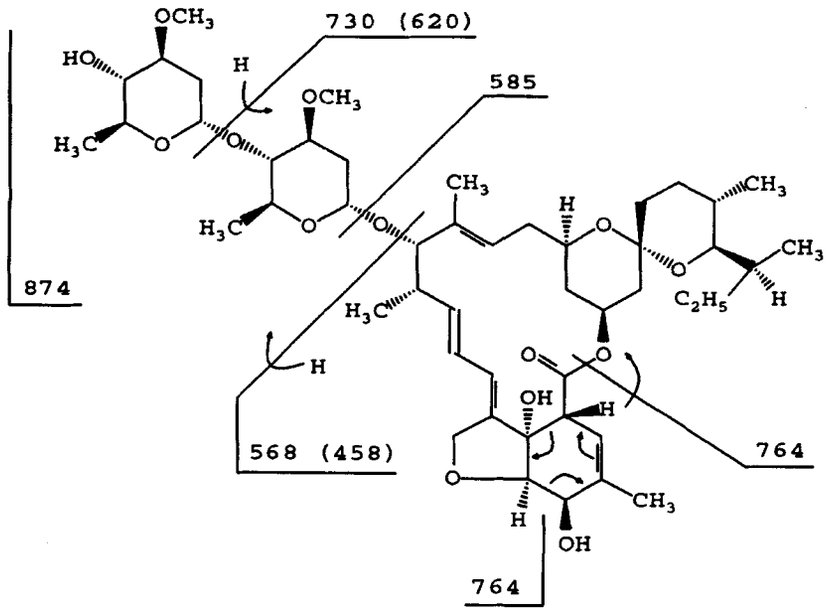

Figure 1. Structure of H_2B_{1a}, showing fragment ions observed in NICI.

complicated by matrix interferences. A tandem mass spectrometer is required to overcome this problem.

Particle beam liquid chromatograhy/mass spectrometry (PB LC/MS) with negative ion chemical ionization (NICI) has now been shown to detect H_2B_{1a} at lower levels than was possible with previous mass spectral approaches. This detection scheme was combined with existing extraction and clean-up procedures for H_2B_{1a} in milk and liver. (The development of these procedures has been reported in part.)[14] On-line LC was used to separate H_2B_{1a} from most matrix interferences. The molecular ion and diagnostic fragment ions were detected from as little as 2 ng H_2B_{1a} using selected ion monitoring (SIM). Use of PB LC/MS enables detection of H_2B_{1a} in milk and liver at levels necessary for regulatory monitoring: 15 p.p.b. in liver and 2 p.p.b. in milk.

EXPERIMENTAL

Apparatus

All LC/MS data were acquired on a 5988A mass spectrometer equipped with the 59980A Particle Beam Interface and the 59970C MS Pascal Series Chemstation data system (Hewlett-Packard, Palo Alto, California). Methane gas pressure was adjusted so the source pressure gauge read 1 torr. Source temperature was 300 °C. The instrument was tuned and calibrated in negative ion mode using ions from perfluorotributylamine (PFTBA). Source voltages were adjusted to maximize the signal from the PFTBA parent ion at *m/z* 633. Full-scan mass spectra were acquired with peak widths adjusted to 0.5 u at half height. Peak widths were adjusted to 0.6 u for SIM. The exact *m/z* values for H_2B_{1a} were used for SIM: 585.34, 764.47, 838.49, 856.50, and 874.51. Dwell time per ion in SIM was set at 500 ms.

Relative abundances of monitored ions were calculated from ion chromatogram peak areas, which were integrated as follows: the start and end points of the H_2B_{1a} peak were determined by inspecting the total ion chromatogram. Baseline points were forced at these times for computerized integration. The same time points were forced in control ion chromatograms if no H_2B_{1a} peak was apparent.

For 100% organic mobile phase (acetonitrile or methanol), the particle beam desolvation temperature was 30 °C and helium nebulizer pressure was 25 p.s.i. When 20% aqueous phase was combined with acetonitrile at a flow rate of 0.4 ml min^{-1}, desolvation temperature was set at 50 °C and helium pressure at 35 p.s.i. Unless otherwise noted, chromatographic separation was carried out on a Novapak C-18 column, 2 × 150 mm, 4 μm particles (Waters, Milford, Massachusetts), with a mobile phase of acetonitrile–50 mM ammonium acetate adjusted to pH 4 (80:20) at 0.4 ml min^{-1}.

Reagents and tissues

Ivermectin standard was obtained from the manufacturer (Merck, Rahway, New Jersey) at a known weight percent of H_2B_{1a} and H_2B_{1b} in glycerol. A measured weight of this standard was diluted in methanol to an H_2B_{1a} concentration of 100 ng μl^{-1}. The stock solution was stored at 4 °C. A working solution was prepared by diluting stock solution with methanol to a concentration of 1 ng μl^{-1}. Analytical standard was prepared at about 0.08 ng μl^{-1} in methanol–water (80:20). The working solution was also used to fortify milk at 2 p.p.b.: 20 μl was added to 10 ml of milk.

Mobile-phase solvents included LC-grade acetonitrile and methanol. Distilled, deionized water was prepared with a resistivity of greater than 17 MΩ cm. A buffer solution was prepared with 50 mM ammonium acetate in water, adjusted to pH 4 with glacial acetic acid.

Aqueous solvents were filtered through 0.22 μm nylon membranes before use.

All milk samples consisted of raw, whole bovine milk which had been stored frozen before use. Control milk was collected at the FDA's Center for Veterinary Medicine, Office of Science, Beltsville, and stored at −80 °C. Milk containing biologically incurred residues was obtained by dosing a cow with ivermectin and sampling the raw milk. The concentration of incurred H_2B_{1a} was determined, and the milk was stored frozen at −40 °C or below. Various samples were diluted with control milk to obtain residue levels close to the target concentration of 2 p.p.b. Control liver was obtained from a local retail market and was found to be negative for ivermectin residues.

Extractions

(i) A determinative procedure for ivermectin in bovine milk was recently approved by the FDA.[4] The same liquid/liquid extraction was used, up to the point of derivatizing the sample.

(ii) A simpler liquid/liquid extraction of milk was developed in our laboratory: a 10 ml milk sample was combined with 10 ml hexane and 20 ml acetone in a 50 ml centrifuge tube. The tubes were shaken for 30 s and centrifuged for 10 min at 1000 × *g*. The milk proteins formed a band just below the bilayer interface. The upper layer was removed to a second 50 ml tube. Another 10 ml hexane was added to the milk, which was vortexed, sonicated, and shaken for 30 s. The centrifugation was repeated, and the upper layers were combined and evaporated under nitrogen at 60 °C to yield about 0.5 ml yellow oil, which was dissolved in 3 ml hexane, vortexed and sonicated. Then 5 ml acetonitrile was added and the tube was shaken for 30 s. The upper (hexane) layer was discarded and the lower layer was extracted with another 3 ml hexane. The lower layer was evaporated to dryness under a nitrogen stream at 60 °C.

(iii) A determinative assay for H_2B_{1a} in liver also has been approved, based on liquid/liquid extraction.[5] This procedure was used as described, up to the point of derivatizing the sample.

(iv) The recently developed technique of matrix solid-phase dispersion (MSPD) has been used in an assay for H_2B_{1a} in liver.[6] This extraction was used as described and was stopped before the extract was derivatized. In MSPD, tissue is manually blended with octadecylsilyl-bonded silica particles and packed in a reservoir. Drug residues are eluted with various solvents.

(v) The alumina B procedure developed by Schenck[6] for clean-up of MSPD eluent was combined with extractions (i), (ii) and (iii) to further remove interferences. An alumina-B Sep-Pak was fitted with a 6 ml polypropylene syringe barrel and a 100 μl Eppendorf-type disposable pipet tip to convert it to an SPE column. The column was mounted on a vacuum manifold, washed with 5 ml methylene chloride, and dried by briefly drawing air through it. Samples were loaded on the column in 1 ml acetonitrile. A 0.5 ml acetonitrile rinse of the centrifuge tube was passed through the Sep-Pak. Excess acetonitrile was drawn off under slight vacuum and discarded. The column was washed with 3 ml hexane, 5 ml methylene chloride and 5 ml acetone. Ivermectin was eluted with 5 ml methanol. The methanol was evaporated under a nitrogen stream at 60 °C.

After extraction, the dried residue was taken up in methanol. Water was added to yield a solvent composition of methanol–water (80:20). Injection volume was 100 μl. To inject 10%, 20% or 40% of the sample, final volume was adjusted to 1000, 500 or 250 μl, equivalent to injecting 1, 2 or 4 ml milk, respectively. Samples were stored in autoinjector vials at 4 °C at least 16 h before analysis. This storage period allowed turbid samples to clarify by formation of a precipitate, so that filtration was not necessary. Samples were stable for many weeks if stored in this way.

RESULTS

Mass spectrometry

Strong signals were observed when H_2B_{1a} was introduced via the particle beam interface for NICI. Flow injection analysis in 100% methanol showed that the molecular ion response was maximized at a source temperature of 300 °C and methane pressure of 1 torr. The NICI mass spectrum of H_2B_{1a} shown in Fig. 2(a) was obtained from 250 ng of standard injected on the Novapak C-18 column and chromatographed in acetonitrile–methanol–pH 4 ammonium acetate buffer (70:10:20) at 0.4 ml min^{-1}. Retention time was 8.6 min under these conditions. The H_2B_{1b} homolog differs by the substitution of $-CH_3$ for the $-C_2H_5$ group in Fig. 1, and its retention time in the same run was 6.4 min. The NICI mass spectrum of H_2B_{1b} was analogous to H_2B_{1a} (Fig. 2(b)). This spectrum corresponds to 20 ng H_2B_{1b}.

Figure 1 includes fragmentations proposed for some of the ions observed using NICI. The H_2B_{1a} molecular ion at *m/z* 874 was the base peak. Ions arising from loss of one and two water molecules were also observed at *m/z* 856 and 838, respectively. Loss of sugar moieties resulted in a diagnostic ion at *m/z* 585. The constant mass difference of 110 u (shown in parentheses) between several pairs of ions was due to a retro-Diels–Alder reaction combined with macrolide ring cleavage. This mechanism, which has been described for the electron ionization (EI) fragmentation of H_2B_{1a},[15] resulted in a fragment ion at *m/z* 764 (Fig. 1).

The relative abundance of the retro-Diels–Alder fragment at *m/z* 764 varied over a wide range during this study, from less than 1% to more than 200% of the M^- ion. The abundance was dependent on the amount of material coeluting with H_2B_{1a}. This relationship was demonstrated by several experiments.

Milk was fortified at 100 p.p.b., extracted by method (i) above, and analyzed under the same conditions as the 250 ng standard shown in Fig. 2. The background-subtracted mass spectrum of H_2B_{1a} is shown in Fig. 3, representing the extract of 1 ml milk containing 100 ng H_2B_{1a}. Molecular ion response is similar to Fig. 2 although less H_2B_{1a} was injected. The abundance of *m/z*

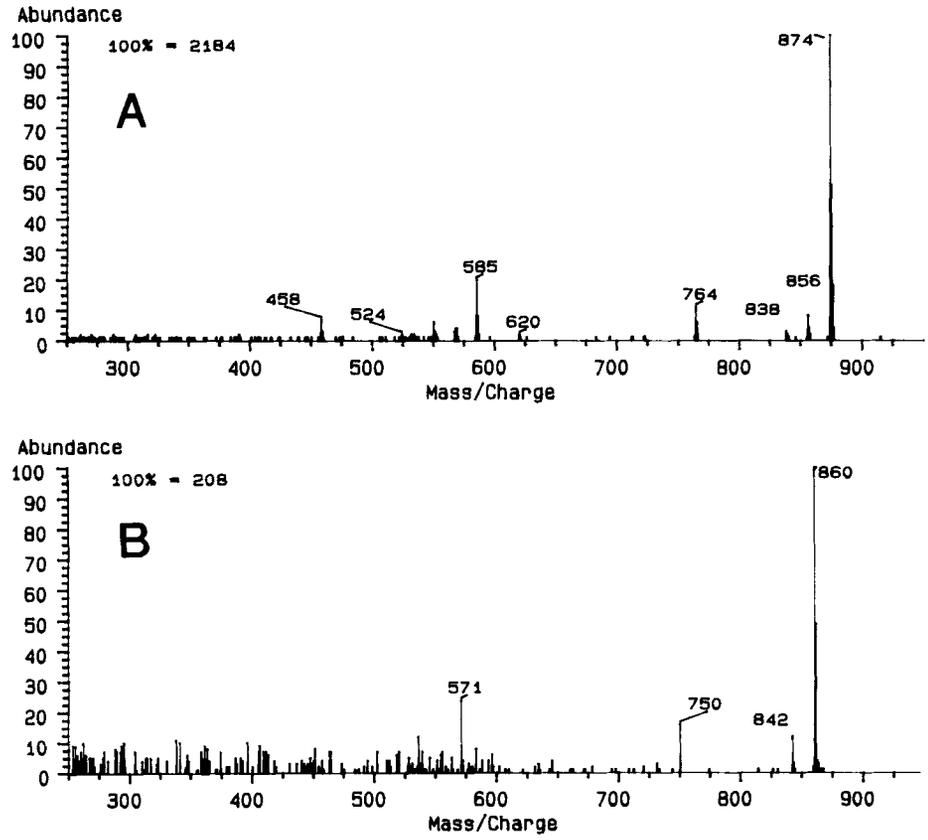

Figure 2. NICI mass spectra of ivermectin components, separated on a Novapak C-18 column and introduced via particle beam interface: (a) 250 ng H_2B_{1a} standard; (b) 20 ng H_2B_{1b} standard.

764 relative to *m/z* 874 has changed from about 12% to over 100%. Other fragment ion abundances were enhanced as well. The signals subtracted from Fig. 3 were due to acylate anions and acyl-glycerol fragment ions typical of conjugated lipids (e.g. phospholipids). These ions appeared at lower *m/z* than the major fragments from H_2B_{1a}.

As larger amounts of extract were injected, the increased matrix material caused the relative abundance of *m/z* 764 to increase. The influence of the coeluting matrix lipids was a consistent phenomenon throughout method development. Many 10 ml milk samples fortified at 2 p.p.b. were analyzed after extraction by liquid/liquid methods (i) or (ii) with alumina B clean-up (iv). Various percentages of the extracts were injected, usually 10%, 20% or 40%, equivalent to 1, 2, or 4 ml milk coinjected. In addition, each day's analyses of extracts were preceded by injection of H_2B_{1a} standard, usually 4 or 8 ng. These sample sets allow relative abundance to be studied as a function of equivalent milliliters of milk coinjected (with standards considered as zero milliliters of milk). The relative abundances were

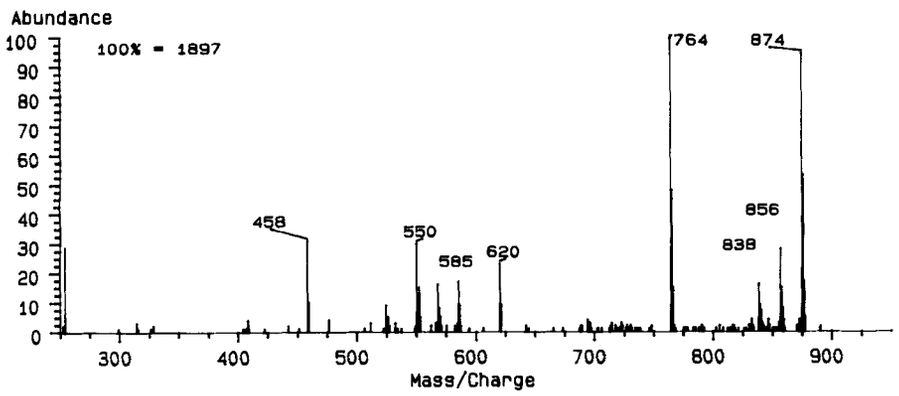

Figure 3. Background-subtracted NICI mass spectrum of H_2B_{1a} extracted from 10 ml milk fortified at 100 p.p.b., analyzed using the same conditions as Fig. 2.

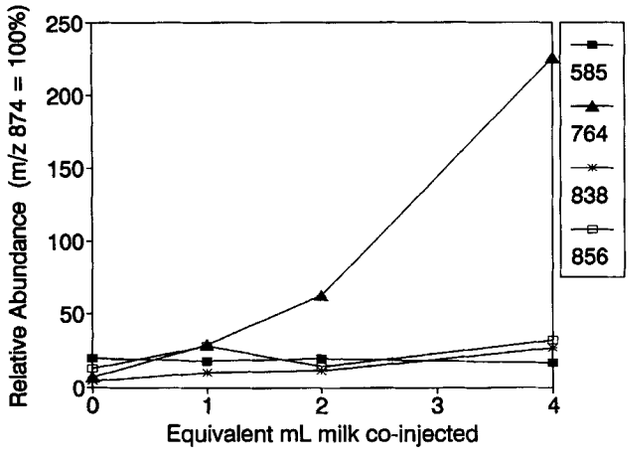

Figure 4. Averaged relative abundances of major H_2B_{1a} fragment ions as a function of equivalent milliliters of milk extract co-injected. Milk was fortified to 2 p.p.b., so levels of 1, 2 and 4 ml correspond to 2, 4 and 8 ng H_2B_{1a}. Zero level is pure standard, either 4 or 8 ng H_2B_{1a}.

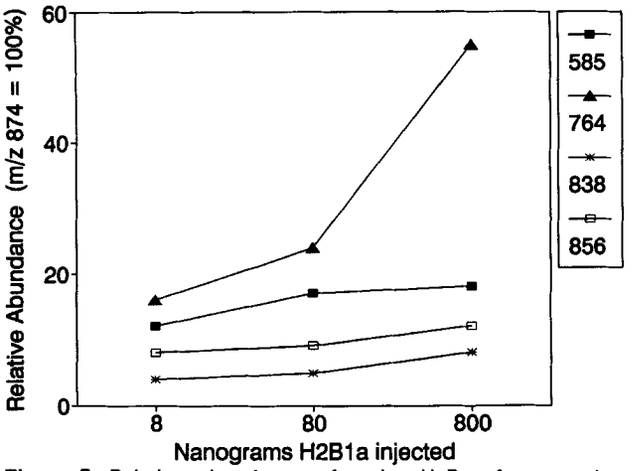

Figure 5. Relative abundance of major H_2B_{1a} fragment ions observed at three levels of standard injected.

averaged within each set, and the results are graphed in Fig. 4 (m/z 874 = 100%). The relative abundance of m/z 764 was only 7% in standards (n = 17), but it increased to 29% with 1 ml milk (n = 3), 62% with 2 ml milk (n = 17), and over 200% with 4 ml milk (n = 7). The abundance of m/z 585 was insensitive to the coeluting material, and the ions at m/z 838 and 856 were slightly enhanced.

The matrix enhancement effect was not limited to milk matrix lipids. Various amounts of H_2B_{1a} standard were analyzed by PB LC/MS with SIM of major ions. As the amount injected increased from 8 to 800 ng, the relative abundance of m/z 764 increased markedly, while relative abundance of other fragments showed slight increases (Fig. 5).

Extractions and chromatography

Method (i), used for the determination of ivermectin in milk, is an established technique designed to work with 10 ml milk. Liquid/liquid extraction (ii) was developed as an alternative since it was somewhat easier to perform. Extracts using these methods were found to contain many UV and mass spectral interferences. We used the alumina B solid-phase extraction developed by Schenck (v) to clean the extracts further. However, we found that complete chromatographic separation of H_2B_{1a} from matrix compounds was not necessary for PB LC/MS confirmation because of the specificity of SIM.

For example, Fig. 6 shows aspects of the analysis of milk fortified at 2 p.p.b. and extracted by method (i) with alumina B clean-up (v). Reconstructed ion chromatograms (RIC) for the m/z ranges 400–500, 500–600, 700–800 and 800–900 u are shown in Fig. 6(a). Selected ion chromatograms in Fig. 6(b) from the acyl anions myristate (m/z 227), palmitate (m/z 255) and oleate (m/z

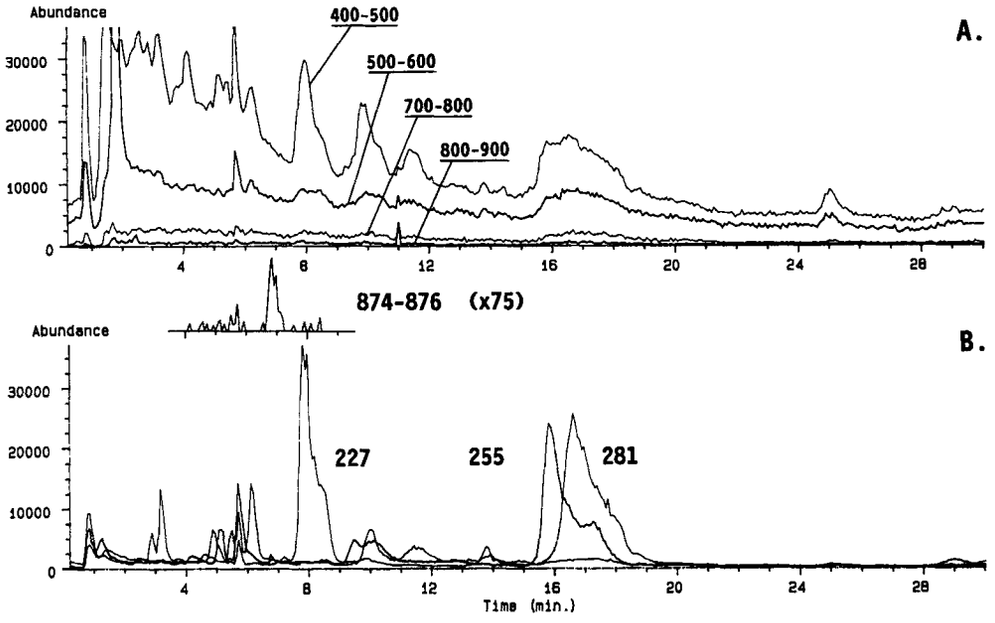

Figure 6. Ion chromatograms of milk fortified at 2 p.p.b. and extracted by liquid/liquid extraction (i) and alumina B clean-up (v), full-scan acquisition: (a) RIC from selected mass ranges; (b) acyl anion chromatograms: m/z 227 (myristate), 255 (palmitate), 281 (oleate). Inset: RIC of ivermectin molecular ion cluster.

281) show that many matrix peaks arise from conjugated lipids. The inset between (a) and (b) is the reconstructed ion chromatogram for the H_2B_{1a} molecular ion cluster, *m/z* 874–876, plotted on the same time scale and an expanded vertical axis. The existence of material coeluting with H_2B_{1a} is apparent in the lower-mass ion traces. However, above *m/z* 500 these background signals amount to chemical noise, and they are very minor above *m/z* 700.

By monitoring five high-mass ions from H_2B_{1a} it was possible to detect low-nanogram amounts in milk extracted by method (ii) followed by alumina B clean-up (v). In Fig. 7 ion chromatograms from control milk and milk fortified at 2 p.p.b. are superimposed. The fortified sample corresponds to 4 ng H_2B_{1a} injected. Figure 8 shows the ion chromatograms of milk containing incurred residues extracted by the same method. The incurred sample contained 2.6 p.p.b. ivermectin. Milk

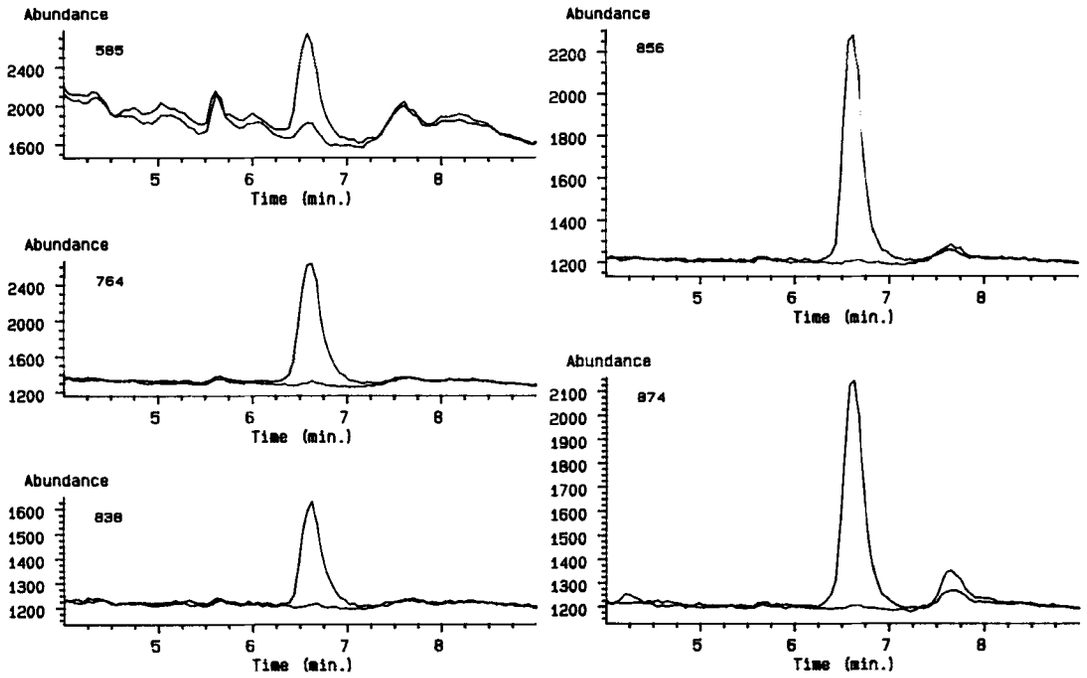

Figure 7. Overlay of selected ion chromatograms of control milk and milk fortified at 2 p.p.b., extracted by method (ii) with alumina B clean-up (v) (equivalent to 2 ml milk injected).

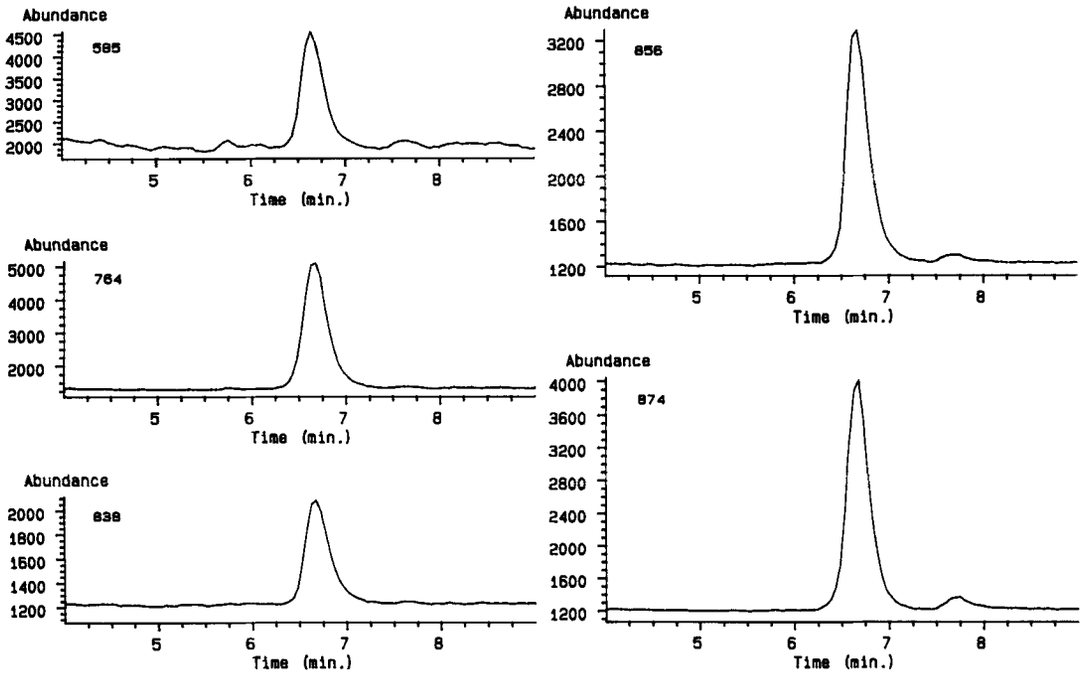

Figure 8. Selected ion chromatograms of milk containing 2.6 p.p.b. incurred H_2B_{1a}, extracted by method (ii) with alumina B clean-up (v) (equivalent to 2 ml milk injected).

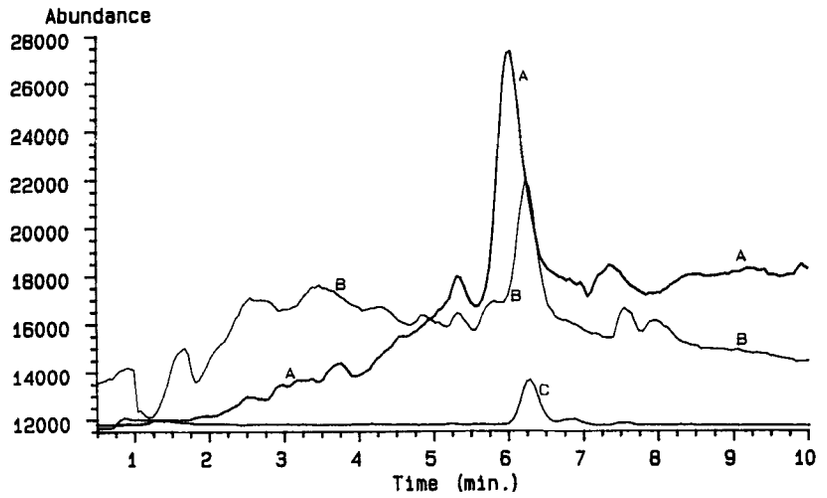

Figure 9. Total ion chromatograms from SIM analyses of H_2B_{1a} in liver extracts: (A) extraction (iii); (B) MSPD procedure (iv); (C) extraction (iii) with alumina B clean-up (v).

taken 16 h after a cow was dosed had been previously determined to contain 13.2 p.p.b. H_2B_{1a}. This milk was diluted 1:4 with control milk before extraction.

We found that the Waters alumina B Sep-Pak Vac, which contains only one-third the packing of a conventional Sep-Pak, lacked the capacity for 10 ml milk. We also tried loading ivermectin standard on Supelco alumina B in acetonitrile, but LC/MS analysis of the eluent showed three additional peaks appearing in the chromatogram after H_2B_{1a}. The first two peaks exhibited molecular ions at *m/z* 874, and could be due to isomerization of the macrolide ring. The third peak corresponded to a compound with molecular weight 856, resulting from dehydration on the SPE column. To avoid this problem, the extracts shown in Figs 7 and 8 were prepared by loading extracts on the alumina B Sep-Paks in methylene chloride.

The PB LC/MS system was also applied to bovine liver. Samples of fortified liver were extracted by three different techniques, and the total ion chromatograms from SIM analyses are shown in Fig. 9. Control tissue was fortified at 15 p.p.b. for liquid/liquid extraction (iii) and at 20 p.p.b. for the MSPD extraction (iv). Both resulted in an excessive level of background (traces 9(a) and 9(b), respectively). However, if tissue fortified at 15 p.p.b. was extracted by method (iii) followed by alumina

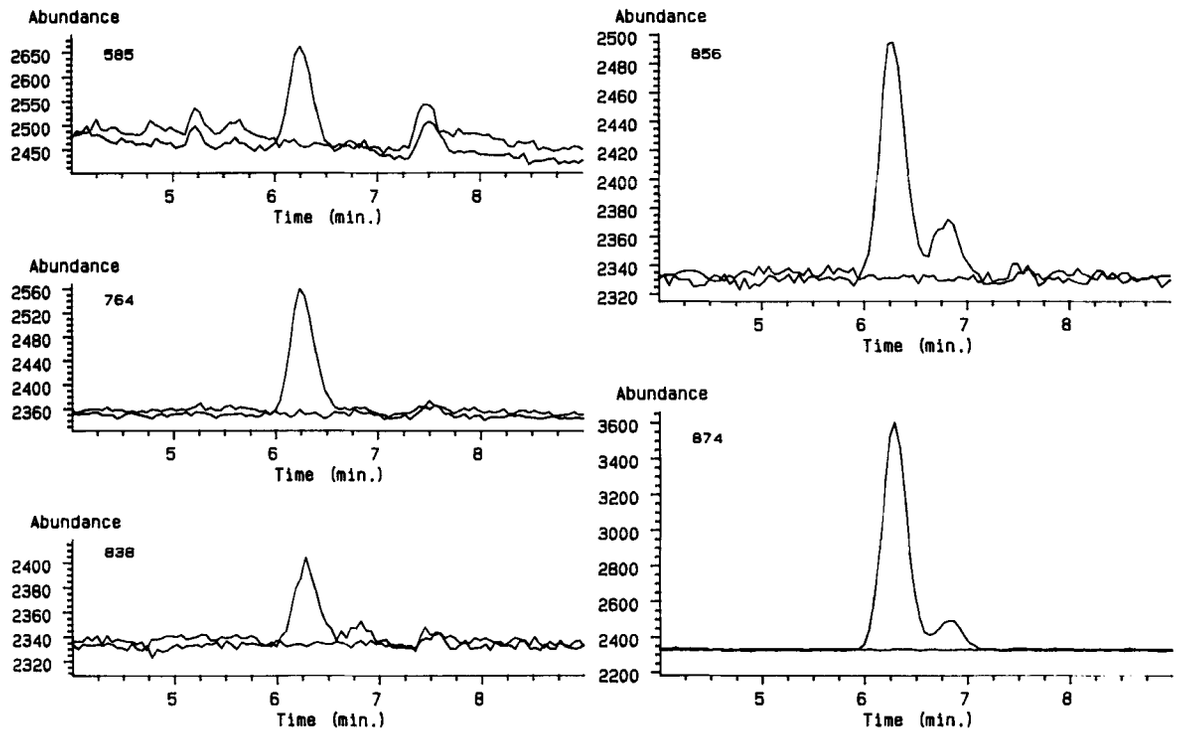

Figure 10. Overlay of selected ion chromatograms of control liver and liver fortified at 15 p.p.b., extracted by liquid/liquid extraction (iii) with alumina B clean-up (v) (equivalent to 0.3 g liver injected).

B clean-up (v), a very clean sample was obtained (trace 9(c)). The relatively larger signals for H_2B_{1a} in traces 9(a) and 9(b) are due to the increased abundance of *m/z* 764 caused by the coeluting material. The single-ion chromatograms from run 9(c) and a corresponding control sample are superimposed in Fig. 10. The amount injected was equivalent to 0.3 g liver, or about 4.5 ng H_2B_{1a}.

DISCUSSION

An LC/MS identification should be minimally based on retention time and measurement of molecular weight and characteristic fragment ion masses. The specificity required for confirmation of H_2B_{1a} is achieved by monitoring the molecular ion and four structure-specific fragment ions. It was emphasized by Sphon that the ions' relative abundances must match within a certain tolerance to those obtained from a standard analyzed under the same conditions.[16] A tolerance on relative abundances of diagnostic ions was also required by deRuig *et al.*[17]

The chromatographic conditions used for analyzing milk extracts allowed lower-mass matrix compounds to coelute with H_2B_{1a}. The monitored ions were relatively free of interferences because of their higher mass. However, the coeluting compounds caused significant changes in the relative abundance of some fragment ions compared to pure standard. This effect must be compensated for so the requirements for abundance matching can be met.

It is proposed that standards for comparison consist of H_2B_{1a} spiked into extracts of control milk. The concentration of the test sample would be known from a prior determinative step, so the spiking can be done to a similar concentration. The result would be a comparison of two samples run under similar conditions, which satisfies the abundance matching requirements. To minimize any other source of variability, test and comparison samples could be run consecutively and compared only to one another.

This approach to confirmation was tested with the samples shown in Figs 7 and 8. The control, fortified and incurred samples were analyzed in a series bracketed by two control extracts spiked *after extraction* with the equivalent of 2 p.p.b. H_2B_{1a} (20 ng total). A sample of 8 ng H_2B_{1a} standard was analyzed at the start of the day's analyses. The retention time and relative abundance data from these samples are shown in Table 1. The relative abundances of the fortified and incurred samples match closely to the average of the two spiked control extracts, but they are quite different from the pure standard. The control sample produced small peaks at similar retention time, but with relative abundances that differed greatly from the spiked controls.

Electron capture NICI is known for its sensitivity to minor variations in source conditions.[18] Even under the tightly controlled conditions in the above experiment, relative abundances were not reproducible within a

Table 1. Spiked control milk extracts compared to standard and extracts of control, fortified and residue-incurred milk

Sample	Retention time (min)	585	764	*m/z* 838	856	874
				Peak area		
8 ng standard[a]	6.669	136772	19628	8810	48232	85492
Spiked control	6.629	335952	493186	130786	323299	363926
2.6 p.p.b. incurred	6.678	465316	693088	157657	358464	479800
Control	6.629	21481	5747	1596	2441	2373
2 p.p.b. fortified	6.630	147106	216550	64219	156591	145188
Spiked control	6.581	541753	987378	234827	521772	619409
				Area ratio versus control		
2.6 p.p.b. incurred	6.678	22	121	99	147	202
2 p.p.b. fortified	6.630	6.8	38	40	64	61
				Relative abundance		
8 ng standard		100.0	14.4	6.4	35.3	62.5
Spiked control		68.1	100.0	26.5	65.6	73.8
2.6 p.p.b. incurred		67.1	100.0	22.7	51.7	69.2
Control		100.0	26.8	7.4	11.4	11.0
2 p.p.b. fortified		67.9	100.0	29.7	72.3	67.0
Spiked control		54.9	100.0	23.8	52.8	62.7
Spiked controls, ave.	6.605	61.5	100.0	25.2	59.2	68.2
				Difference from spiked controls		
8 ng standard	0.064	38.5	−85.6	−18.8	−23.9	−5.7
2.6 p.p.b. incurred	0.073	5.6	0.0	−2.5	−7.5	1.0
Control	−0.024	38.5	−73.2	−17.8	−47.8	−57.2
2 p.p.b. fortified	0.025	6.4	0.0	4.5	13.1	−1.2

[a] First analysis of the day. Several other extracts were analyzed before the remaining samples listed in this table.

margin of 10%. Two ions in the spiked controls (*m/z* 585 and 856) differed by about 13% from one run to the next. In the fortified sample, *m/z* 856 differed from the averaged spiked controls by 13.1%. This result implies that a narrow matching tolerance which is suitable for a more reproducible technique such as EI may not be appropriate for confirmation of ivermectin residue by NICI. Using a matching tolerance of 15% with the data in Table 1, the presence of ivermectin H_2B_{1a} is confirmed in the incurred and fortified samples, while it is excluded from the control sample.

The peak areas of the control sample were taken as the background level so that signal-to-noise levels could be calculated. The peak area from milk fortified at 2 p.p.b. was about seven times greater for the lightest ion, *m/z* 585, implying that the limit of confirmation using all five ions is under 2 p.p.b. in milk. The corresponding area ratios for the other four ions were well above the 3:1 level.

Signal enhancement by coeluting material

Material gradually accumulated on the skimmer cones in the particle beam interface after multiple injections. The cones became clogged if samples were injected continuously over about two days. This problem was minimized by placing a diverting valve between the LC column and the interface to admit effluent to the interface only when H_2B_{1a} eluted.

The skimmer cones exhibited an oily film after use from the deposition of residual milk lipids. However, unlike the experience of Hsu,[19] we did not find this to be detrimental to the procedure. First, the high-mass ions produced by H_2B_{1a} were relatively free of interferences from sample carry-over, especially above *m/z* 700. Second, a higher response for H_2B_{1a} was often observed after several extracts had been injected with new skimmer cones. In Table 1, the total response for the initial standard is well below that observed after multiple extract injections. Signal enhancement was attributed in part to slow bleeding of the accumulated milk fat. Presumably the infusion of material bleeding from the cones had the same effect as coeluting compounds. This possibility is supported by the observation of increased abundance of *m/z* 764 in pure standards injected *after* multiple milk extracts (data not shown). Higher abundance is an indication that more milk lipids were entering the source with H_2B_{1a}. The abundance of *m/z* 764 was quite low in standards injected *before* extracts (Fig. 4).

The signal enhancement is also apparent in the mass spectra in Figs 2 and 3, where the counts observed from the molecular ions are similar although the extract contained about 2.5 times less H_2B_{1a} than the standard. The enhancement effect of coeluting material has been reported by other particle beam users, who found it to be a mixed blessing. Kim *et al.* reported enhanced signals and more linear response from daminozide when malic acid was added to the mobile phase. They also observed changes in the daminozide mass spectrum as malic acid concentration increased.[20] Bellar *et al.* described enhanced ion response due to both ammonium acetate and coeluting compounds.[21] Mattina used a generic carrier molecule to reduce detection limits for phenylurea herbicides.[22] Brown and Draper reported a matrix enhancement effect from coelution of isotopically labeled and native compounds.[23] These authors, Doerge *et al.*[24] and Ho *et al.*[25] concluded that the ratio between labeled and unlabeled compounds was unaffected by coeluting compounds. Most of these reports attribute the enhancement effect to improved transfer through the interface. In the context of regulatory confirmation, the matrix enhancement effect is an advantage for detecting low levels of incurred residues.

On the other hand, the change in H_2B_{1a} ion *relative abundances* described here implies a matrix effect in the ion source in addition to the interface. Material condensed in the particles may cause localized changes in source conditions that influence the electron capture NICI process. Condensed material may affect H_2B_{1a} vaporization or cause a pressure burst when the particle beam strikes the source. Alternatively, this material may produce reagent ions which react with H_2B_{1a} to yield different fragment ions than are produced from the capture of thermal electrons.

CONCLUSION

Particle beam LC/MS with NICI can detect ivermectin residue in milk and liver extracts at levels not attainable by other mass spectrometric methods. The PB LC/MS technique has the capability to confirm the presence of ivermectin residue at levels needed for regulatory action. The conditions for comparing relative abundances must be tightly controlled, because coeluting matrix compounds were found to alter the abundance pattern of H_2B_{1a} fragment ions as well as to enhance total response. Control extracts spiked with standard after extraction can be used for abundance matching to compensate for the changes in relative abundances caused by coeluting material.

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