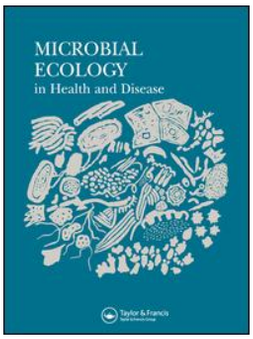

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ORIGINAL ARTICLE

Newly identified vitamin K-producing bacteria isolated from the neonatal faecal flora

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Abstract
Fat-soluble vitamin K is an essential component of the blood clotting process. Menaquinones are the naturally occurring form of vitamin K identified in bacteria. Lipid extracts were made from three bacteria originally isolated from the human neonatal gut and identified as *Enterobacter agglomerans, Serratia marcescens* and *Enterococcus faecium*. Following preparative thin layer chromatography (TLC), the lipid extracts were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. Peak analysis of the LC-MS data showed that the three bacteria produce various forms of menaquinone.

Key words: *vitamin K, menaquinone, Enterobacter agglomerans, Serratia marcescens, Enterococcus faecium, neonatal gut microflora*

Introduction

Vitamin K is a generic term for a family of fat-soluble vitamins. Two of the naturally occurring quinone forms are phylloquinone (vitamin K_1 or 2-methyl-3eicosa-2′-ene-1,4-naphthoquinone), which is obtained from plants, and menaquinone (vitamin K_2 or 2-methyl-3-multiprenyl-1,4-naphthoquinone), which is produced by certain bacteria during anaerobic respiration (1,2). Menaquinone variants are differentiated on the basis of the number of isoprene-5-carbon prenyl units on their side chains. Chain length varies from 2 to 15 isoprene units (3). The variations in the structure of the menaquinones produced by bacteria have been used in bacterial classification (4). Several bacteria that have been isolated from the human intestine have previously been identified as producing menaquinones of various chain lengths and it is thought that these bacteria contribute to the vitamin K requirements of the human body (5). This paper discusses the identification of three previously unknown menaquinone-producing bacteria isolated from faecal samples of neonates.

Materials and methods

Cultures and cultivation

Bacterial strains were isolated from faecal samples obtained from neonates aged between birth and 6 weeks old in a study carried out previously within our laboratory (6). The isolates were identified using selective media, Gram stain and API tests. Several bacterial species were identified as producing vitamin K using the techniques as outlined in this paper; however, the data presented in this paper only relate to the three bacterial species *Enterobacter agglomerans, Serratia marcescens* and *Enterococcus faecium*. Purified cultures were grown in 1 litre Duran containing tryptone soy broth powder (Lab M) dissolved in deionized water with haemin (10 mg) and cysteine (0.05% w/v) (3). The cultures were incubated anaerobically at 37°C for 72 h. Cultures were checked for purity using Gram stain as well as selective and non-selective agar. The bacterial pellets were harvested in a Sorvall RC-5B centrifuge at 16 266 g for 10 min at 4°C. The pellet was washed three times in 20 ml volumes of 0.01 M sterile phosphate-buffered saline (PBS) and centrifuged

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again under the same conditions. Menaquinone 4 standard was purchased from Sigma, UK.

Extraction, purification and analysis of menaquinones

Extraction of suspected menaquinones from the wet bacterial pellets was carried out using the modified Bligh and Dyer method (7) developed by Hammond and White (8), which involves the extraction of total lipid content. Menaquinone 4 (currently the only commercially available menaquinone) was used as a standard, as menaquinones of different side chain lengths migrate similar distances on thin layer chromatographic (TLC) plates (9). The extracted

Table I. Conditions used for monitoring of menaquinones on the Bruker Esquire-LC Ion Trap LC/MS.

HPLC system	Bruker Esquire-LC Ion Trap LC/MS
Column	Synergi Hydro Reverse Phase C18 Column (250 × 4.6 mm)
Detector	Ion trap
Mobile phase	Dichloromethane:methanol (30:70)
Monitored wavelength	270 nm
Flow rate	0.3 and 0.4 ml/min
Sample size loaded	Various
Scan begin	50.00 m/z
Scan end	2200.00 m/z
Ion polarity	Positive
Ion source type	Electro-spray ionization
Sample run time	45–60 min

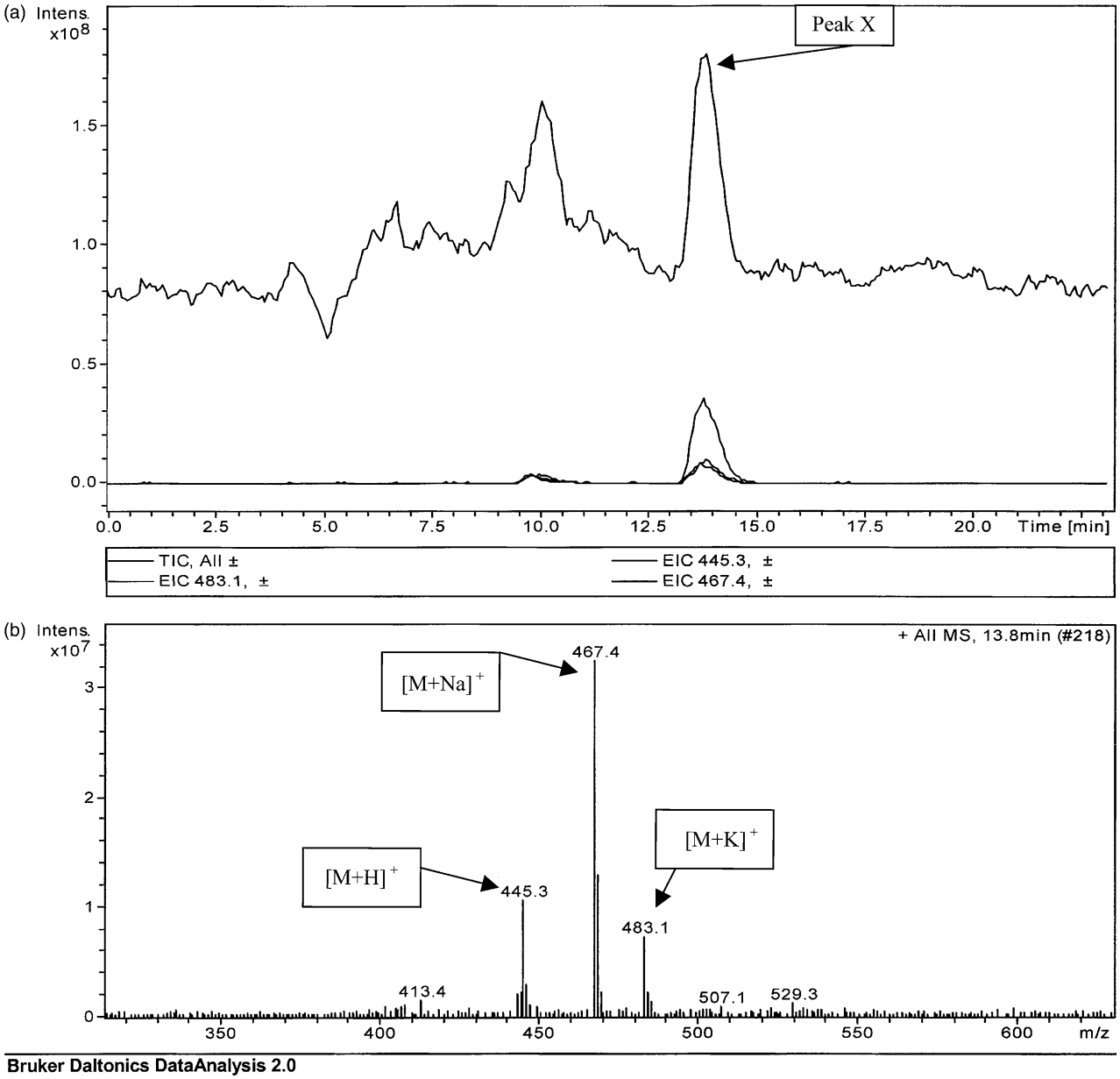

Figure 1. (a) Total ion count of a directly injected menaquinone 4 standard (6 μg/ml). (b) Mass spectrometric analysis of peak X in (a) detected at 13.8 min.

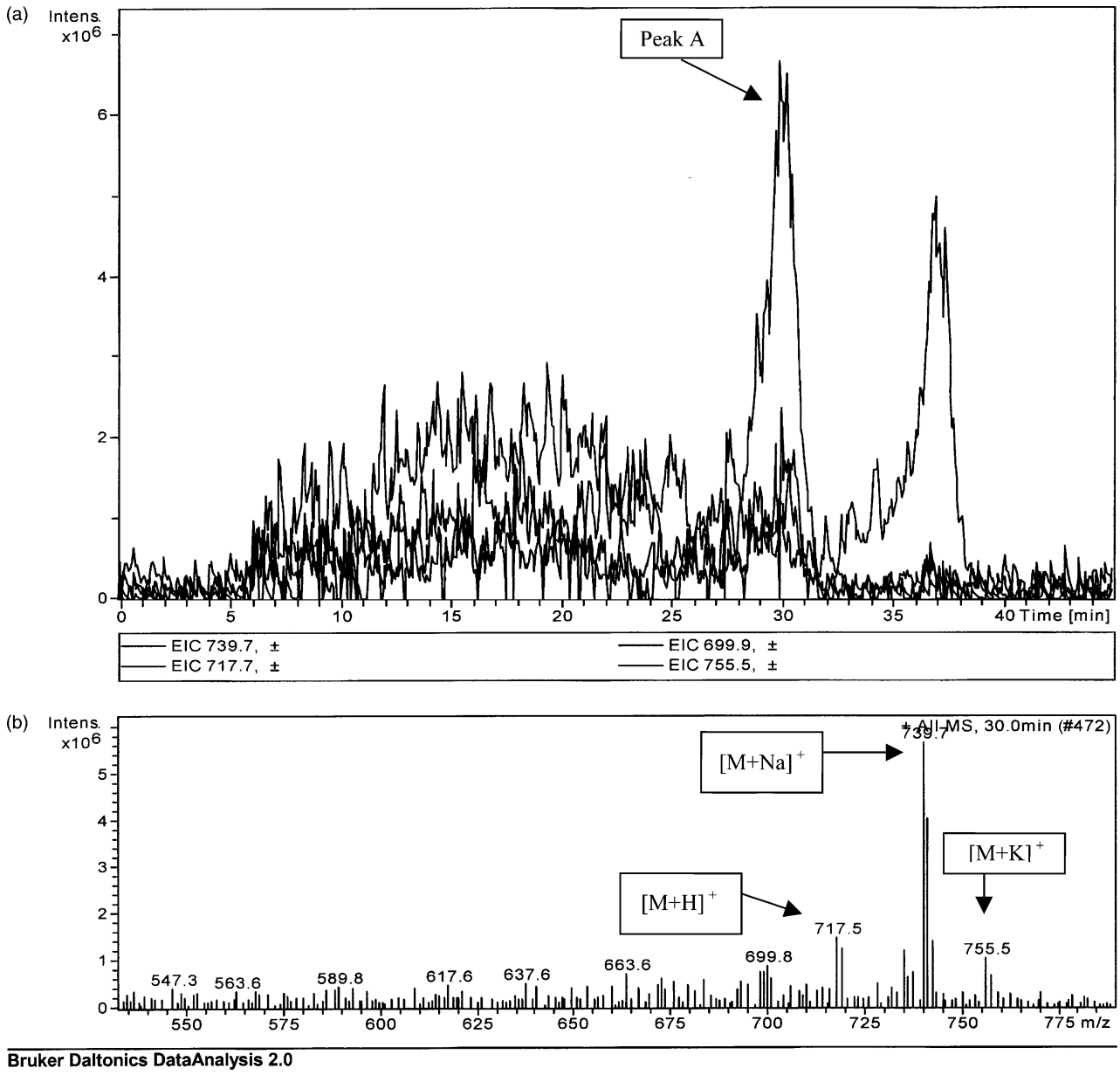

Figure 2. (a) Extrapolated ion count for a TLC extract prepared from an *Enterobacter agglomerans* extract. (b) Mass spectrometric analysis of peak A detected at a retention time of 30 min.

menaquinones were then purified using the procedure as described by Fernandez and Collins (9), on preparative silica gel GF_{254} prep TLC plates. The suspected menaquinones were viewed under a UV light box emitting light at 254 nm. Bands from the lipid extracts of the bacteria that were in line with the menaquinone 4 standard were removed and dissolved in dichloromethane:methanol (30:70), as was the menaquinone 4 standard, and all were subsequently analysed by mass spectrometry (MS). Liquid chromatography-MS (LC-MS) analysis was carried out using a Bruker Daltronics Esquire LC ion trap mass spectrometer with a Synergi Hydro C18 reverse phase (4.6 μm × 250 mm) HPLC column (00G-4375-E0) supplied by Phenomenex (Cheshire, UK) using the conditions outlined in Table I.

Results

Several Gram-positive and Gram-negative bacterial strains were identified in the study as vitamin K producers using the techniques as outlined in this paper. These included the strains *Bacteroides ovatus*, *Enterobacter agglomerans*, *Enterococcus faecalis*, *Escherichia coli*, *Prevotella buccae*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus warneri*, all of which were

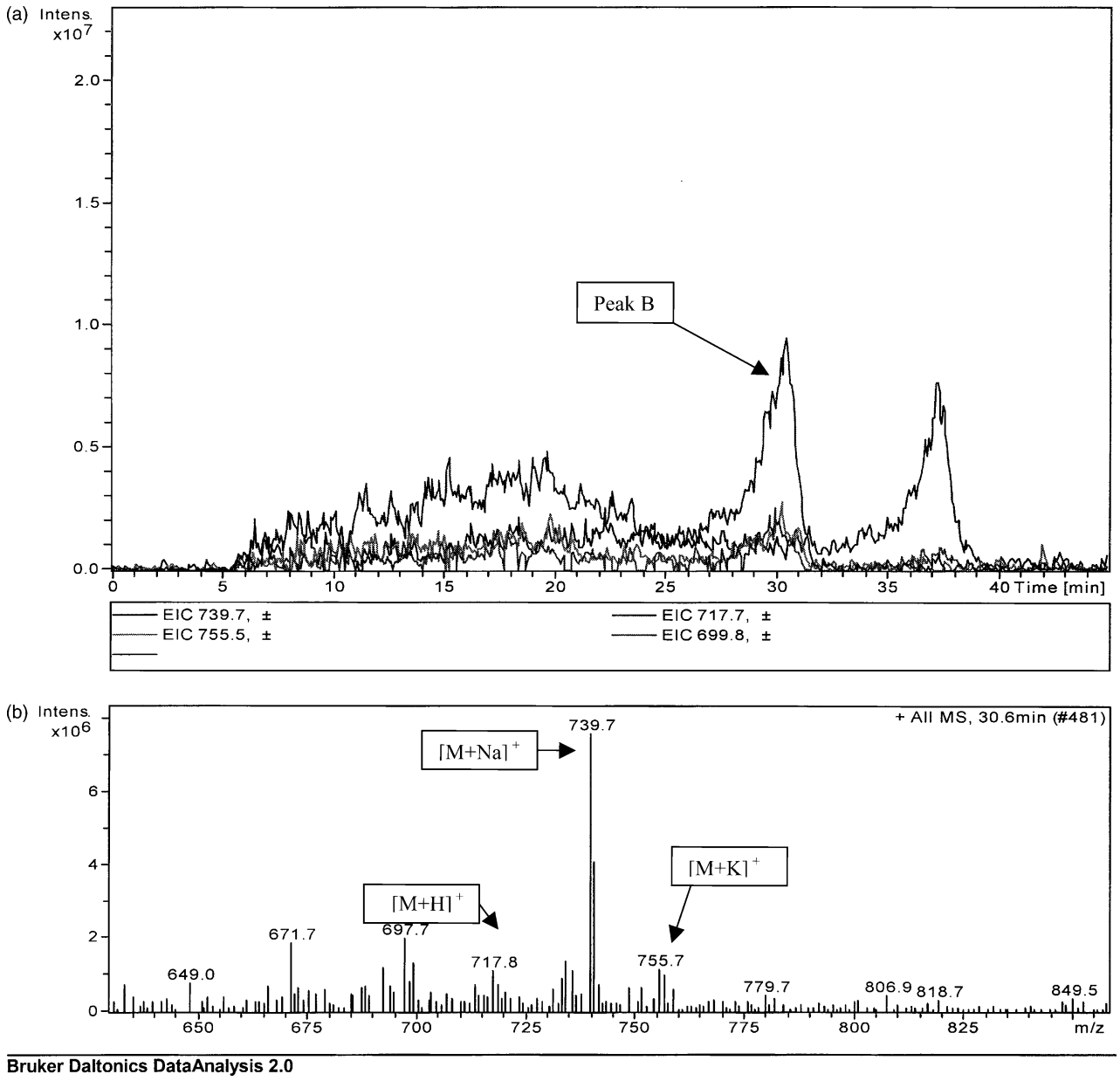

Figure 3. (a) Extrapolated ion count for a TLC extract of *Serratia marcescens* extract. (b) Mass spectrometric analysis of peak B detected at a retention time of 30.6 min.

isolated in faecal samples from breast-fed infants, while vitamin K production was also identified in the following strains isolated from infants fed exclusively on formula – *Bacteroides* sp., *Citrobacter freundii*, *Enterococcus faecium*, *Serratia marcescens*, *Staphylococcus capitis* and *Staphylococcus warneri*. However, the data presented in this paper only relate to the three bacterial species *Enterobacter agglomerans*, *Serratia marcescens* and *Enterococcus faecium*, which were selected as they have not previously been reported to produce vitamin K.

Previous studies have shown that menaquinones of differing side chain length give similar Rf values following TLC analysis (7). Therefore lipid extracts with bands which co-migrated with the menaquinone 4 standard (unpublished results) were subjected to further analysis to determine the exact menaquinone form produced by the respective bacteria being studied.

The LC-MS data obtained for the standard menaquinone 4 are presented in Figure 1. Peak X, indicated in the total ion count shown in Figure 1a, was further analysed, yielding the three significant peaks shown in Figure 1b. The molecular weights of the three peaks in Figure 1b correlated with the molecular weights of the protonated (mass = 445.3), sodiated (mass = 467.4) and potassiated (mass = 483.1) forms of menaquinone 4.

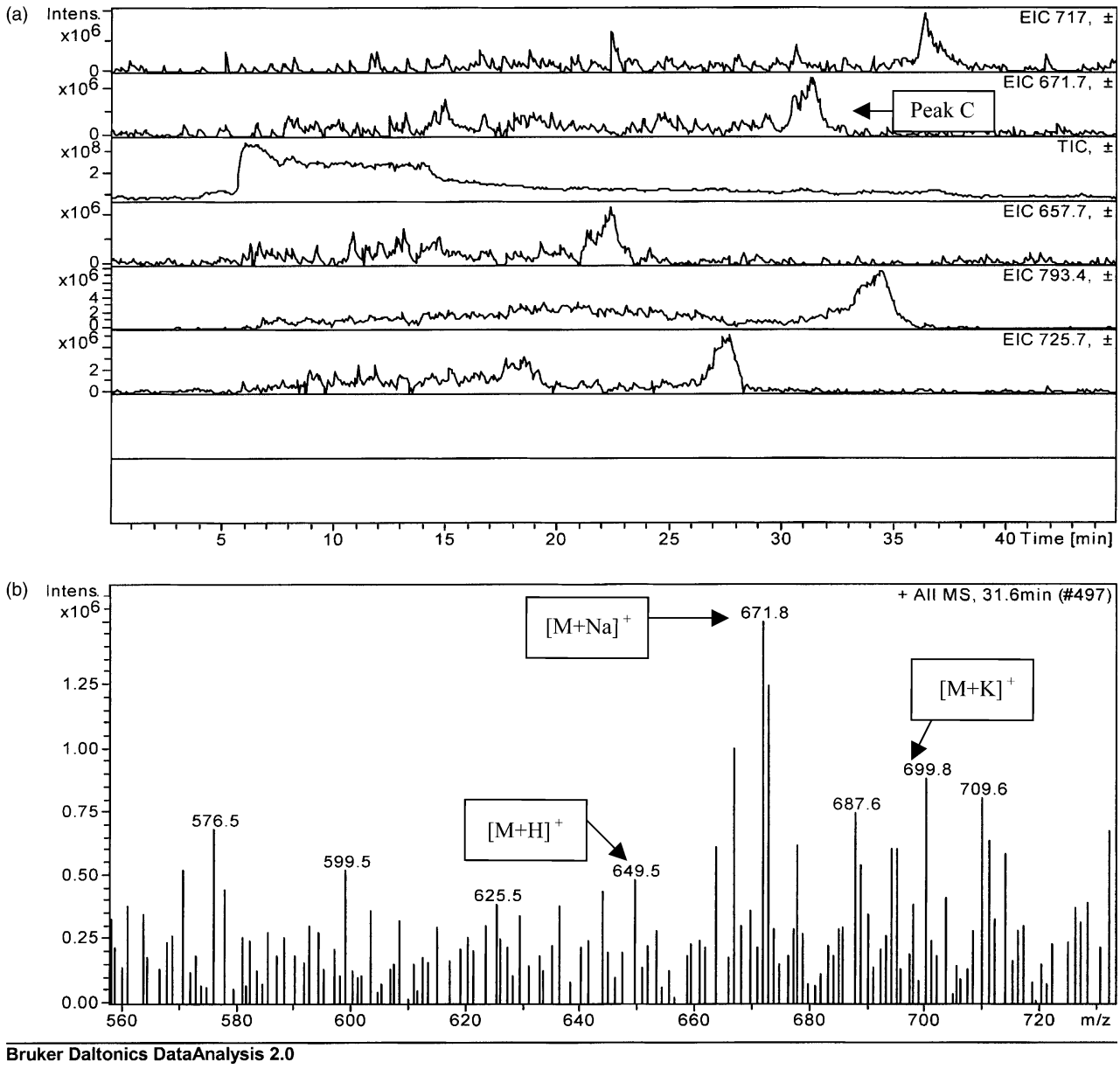

Figure 4. (a) Extrapolated ion count for individual peaks and total ion counts for a TLC extract obtained from an *Enterococcus faecium* extract. (b) Mass spectrometric analysis of the peak C detected at a retention time of 31.6 min.

LC-MS analysis of the extract from *Enterobacter agglomerans* (Figure 2a, peak A) showed molecular weight bands at 717.5 (protonated), 739.7 (sodiated) and 755.5 (potassiated) (Figure 2b) correlating to the expected molecular weight for menaquinone 8 analogues. The sodiated analogue of menaquinone 8 forms the base peak analogue, as was detected for the menaquinone 4 standard (Figure 1b).

In addition to *E. agglomerans*, a strain of *Serratia marcescens* was also found to produce menaquinone 8 (Figure 3a). As with the analysis of *E. agglomerans,* the MS analysis showed molecular weights corresponding to the protonated, sodiated and potassiated forms and, as with the other bacterial extracts, the base peak was the sodiated form of menaquinone 8 (Figure 3b).

The peak detected for *Enterococcus faecium* TLC lipid extract (Figure 4a, peak C), although of weak intensity, gave sufficient signal to allow for identification as menaquinone 7. As with the analysis for the previous strains, the protonated, sodiated and potassiated forms of menaquinone were detected, with the sodiated analogue forming the base peak.

Discussion

The LC-MS results presented in this paper for the three bacterial strains *E. agglomerans*, *S. marcescens*

and *E. faecium* show peaks correlating with sodiated, protonated and hydrogenated derivatives of the menaquinones. Molecules of sodium, potassium and hydrogen are abundant in the environment and in this experiment they could be sourced from the glass vials used to store the samples. Ion contaminants have been reported previously to form apparently stable complexes with the menaquinones during electro-spray ionization (10). The sodium, which forms the base peak analogue, is most likely forming weak covalent bonds with the oxygen on the ring structures of the menaquinones, as are the hydrogen and potassium.

This previously unreported identification of the strains *E. agglomerans, S. marcescens* and *E. faecium* as menaquinone producers will in the first instance contribute to aiding in their microbiological classification (11). In addition, the sourcing of these additional vitamin K-producing strains from the neonatal gut flora shows their potential as bacteria that could contribute in the neonate to the overall requirements of vitamin K, an essential vitamin in the human blood clotting process (6). In the study presented here, the newly identified vitamin K-producing strains together with the several other previously reported vitamin K-producing strains identified in this study namely, *Bacteroides ovatus, Citrobacter freundii, Enterococcus faecalis, Escherichia coli, Prevotella buccae, Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus haemolyticus* and *Staphylococcus warneri*, were predominantly isolated only in the 6-week-old faecal samples rather than in the 0–1- or 2–5-day-old samples analysed (unpublished results). This study looked at the ability of isolated bacterial strains to produce vitamin K; however, further quantitative studies would be required to confirm whether the bacteria listed above contribute significantly to the overall vitamin K requirement of the newborn infant in the age group studied, which could have implications in relation to the duration of hospital regimes for oral administration of vitamin K (9,10) as a prophylactic treatment for the prevention of haemorrhagic disease of the newborn (12–14). The bacterial strains reported as vitamin K producers in this paper are predominantly facultative anaerobes rather than strict anaerobes, which is reflective of the young neonatal gut only developing a more anaerobic environment at the age of the neonates involved in this study. It would be interesting to further develop the study to other age groups in a more quantitative manner over a longer period to determine the contribution of strictly anaerobic bacteria to the vitamin K concentration in the gut.

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