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Journal of Chromatography A, 794 (1998) 359–366

JOURNAL OF CHROMATOGRAPHY A

Cationization in electrospray microcolumn liquid chromatography–mass spectrometry

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Abstract

Post-column modification of the mobile phase in microcolumn liquid chromatography–mass spectrometry has been studied. The modification involved addition of sodium, potassium, rubidium and cesium ions to form charged adduct ions. The target molecules studied included modified cyclodextrins and oligosaccarides, bafilomycins and 18-crown-6. Relative complex constants were determined and the concentration and type of cation was evaluated in order to obtain a good sensitivity. An optimum in sensitivity was found with respect to the cation concentration. The optimum cation concentration for singly charged cations was found to be close to 5×10^{-5} *M*. This concentration was independent of the alkali metal ion used. In most cases the relative sensitivity increases slightly with the size of the cation. The technique was applied to complex samples for structure elucidation of unknown compounds. © 1998 Elsevier Science B.V.

Keywords: Mass spectrometry; Cationization; Mobile phase composition; Electrospray ionization; Cyclodextrins; Oligosaccharides; Bafilomycins; Alkali metals

1. Introduction

The combination of microcolumn liquid chromatography and mass spectrometry was initiated several years ago. This ‘pre-electrospray era’ is characterized by the many different types of interfaces that were used [1,2]. At present, the electrospray ion-source has become more or less a standard. This is a remarkable interface that accepts liquid flow-rates ranging from 1 ml/min down to the nl/min range [3] with only minor modifications to the spray needle. During the same time period that the electrospray interface was developed, microcolumn liquid chromatography became a powerful separation technique [4,5]. The packed fused-silica columns usually have column inner diameters in the range 50–320 μm. Theoretical plate numbers of more than 100 000 can fairly easy be obtained when using long columns (>1 m). Even in this range of column diameters there is a trend towards the use of the smaller I.D. mainly because of the higher efficiency obtained [6–8] and a smaller sample consumption [9,10]. Another advantage is that fairly expensive mobile phases like, for example, high-purity deuterium oxide can be used on a long time scale [11].

The low mobile phase flow-rate that is used makes it possible to do post-column modifications of the mobile phase. This technique was probably first used to introduce a suitable matrix solution for fast atom bombardment mass spectrometry [12]. Such a modification is done in electrospray ionization for several reasons. First of all to give the possibility of modifying the solvent seen by the interface. This will include, for example, changing the pH for sensitivity reasons. Many acidic compounds are better separated using a low pH in the mobile phase. These compounds, for example, carboxylic acids and phenols, are sometimes better detected as negative ions.

0021-9673/98/$19.00
PII S0021-9673(97)00921-7

Addition of, for example, ammonia to raise the pH is therefore useful. To obtain the best performance of the interface, it has to be optimized for the mobile phase in question. Both a high percentage of water or organic solvent can cause problems. This is of course especially evident in gradient elution. The sheath liquid in this case will act as a buffer against changing composition. Columns with I.Ds. in the range 50–250 μm will have flow-rates from 0.1 up to 1 μl/min. Depending on the mobile phase flow-rate and composition as well as the needle design, the sheath liquid flow for best performance is usually from zero up to 5 μl/min.

Although the electrospray interface works well with many types of compounds there are still compound classes that are more troublesome. These are the compounds that not easily can bind a proton to form a charged adduct ion, for example, non-nitrogen containing polar compounds. Of the more important compounds belonging to this class are different types of carbohydrates. Cyclodextrins were detected as sodium adducts [13] probably without adding any sodium ion to the sample. A more recent application deals with synthetic polymers. The permanently charged cation was added using the sheath liquid to form a complex with the analytes of interest [14]. Several other examples that concern cationization in electrospray ionization can be found [15–18].

The present study concerns the post-column addition of several alkali metal ions to form charged adduct ions. The cation concentration and type for obtaining good sensitivity in microcolumn liquid chromatography–mass spectrometry has been investigated.

2. Experimental

2.1. Chemicals.

LC-grade acetonitrile was from (Rathburn Chemicals, Walkerburn, UK) and used as received. Water was purified using a Millipore® system. Deuterium oxide (99.8% isotopic purity) was from Dr. Glaser AG (Basel, Switzerland). Bafilomycin A_1, Heptakis(2,6-di-O-methyl)-β-cyclodextrin, 18-crown-6 and cesium acetate were from Sigma Chemical Company (St. Louis, MO). Potassium, lithium and sodium acetate were from Merck (Darmstadt). Other compounds studied were obtained ‘in house’.

2.2. Post-column modification of mobile phase

The sheath liquid was delivered at various flow-rates by a low-pressure syringe pump (Harvard Apparatus, South Natic, MA) and mixed with the mobile phase in the spray needle. The standard electrospray needle (Finnigan) was modified by the insertion of a 5 mm long stainless-steel capillary with an I.D. of about 100 μm. The welded new spraytip ended 1 mm outside the standard needle (Fig. 1).

2.3. Micro-column liquid chromatography

The mobile phase was delivered using an ISCO model 100DM syringe pump (ISCO Instruments, Lincoln, NE, USA) operated in the constant pressure mode. The injector was a Valco CI4W (200 nl, Valco Instruments, Houston, TX, USA). A second and a third injector (Rheodyne 7010), equipped with a 500 μl loop, was placed between the pump and the sample injector. These injectors served as the mobile phase reservoirs in experiments using deuterium oxide as well as to generate various types of mobile phase gradients (Fig. 1). The columns 50–250 μm

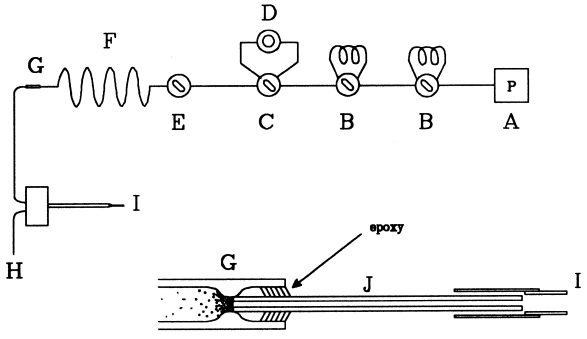

Fig. 1. Set-up for LC–MS system: A, constant pressure pump; B, injection valve, 500 μl loop; C, injection valve; D, magnetically stirred mixing chamber, 50 μl for gradient generation; E, micro column injector; F, microcolumn; G, connector; H, sheath liquid introduction; I, modified spray needle; J, fused-silica tube, 25 μm I.D. 500 mm long.

I.D. were prepared from empty fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) as described earlier [6]. The packing material was Kromasil C-8, 5 μm (EKA Nobel AB, Surte, Sweden) and Nucleosil® 100-5 C-18 (Macherey-Nagel, Düren, Germany), held in place by a 0.2 mm long section of glass wool ('frit') similar to what was described previously [19]. The columns were connected to the electrospray needle using a 300 mm long (50 μm I.D., 150 μm O.D.) empty fused-silica tube.

2.4. Electrospray mass spectrometry.

All experiments were done on a TSQ 700 triple quadrupole mass spectrometer equipped with an electrospray interface (Finnigan MAT, San José, CA, USA). All spectra were obtained using unit mass resolution.

In flow-injection measurements, the column was exchanged for an empty 50 μm I.D. fused-silica tube and the low-pressure syringe pump was used.

Single ion measurements on appropriate adduct ions were done in the studies of signal/noise. No corrections were done to compensate for the isotopic distribution.

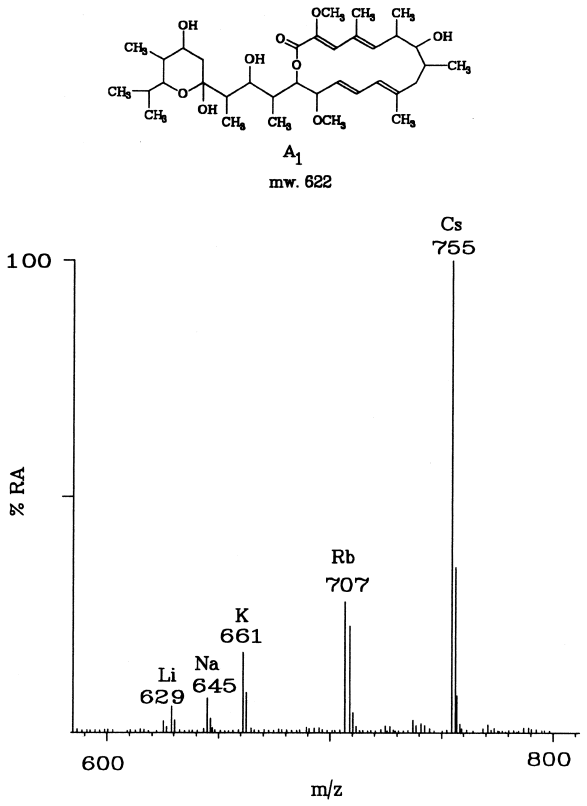

Fig. 2. Electrospray mass spectrum of bafilomycin A_1 alkali metal ion concentration 5×10^{-5} *M* in 50% acetonitrile–water.

3. Results and discussion

3.1. Relative complex constants

This study was initiated by findings showed in Fig. 2. Bafilomycin A_1 was infused to the electrospray ion source using an equimolar concentration of all the cations shown. Several other derived structures from this bafilomycin having a common large lactone ring showed a similar selectivity towards the different cations. In order to evaluate the competing nature of the cations for the target molecule (A) the concept of relative formation rate was adopted.

It was assumed that the complex formation between the cation (e.g. Na^+) and the target (A) occurs at the interface in the small droplet liquid phase and that the increase in concentration of the adduct in the gas phase $[(ANa)^+]_g$ is directly proportional to the interface concentration of the adduct ion $[(ANa)^+]_i$. Furthermore, the interface concentration of sodium is supposed to be in equilibrium with the bulk solution of the sodium ion:

$$d[(ANa)^+]_g/dt = k_1.[(ANa)^+]_i = k_1.k_{Na}\cdot[A]\cdot[Na^+] \quad (1)$$

where k_{Na} is defined by the complex equilibrium:

$$k_{Na} = [(ANa)^+]_i \cdot [A]^{-1} \cdot [Na^+]^{-1} \quad (2)$$

k_1 denotes a rate constant for the transfer of an already formed adduct ion at the droplet surface to the gas phase. This transfer rate is assumed to be equal for all the ions studied. Introducing a competing ion (K^+) and changing from concentration in the gas phase to gas phase ion intensity (*I*), the following equation for the relative formation rate will be obtained:

$$I_{(AK)^+}/I_{(ANa)^+} = k_K.k_{Na}^{-1}.[K^+]/[Na^+] \quad (3)$$

Eq. (3) shows that a plot of the intensity ratios versus the concentration ratio of the two cations in the liquid phase should give a straight line. The slope therefore should give the relative complex formation constant. Such plots are shown in Fig. 3 for some singly charged cations using dimethyl-β-cyclodextrin as target molecule. If the complex formation constant for the sodium ion is set to unity it is possible to relate all other complex constants to that of sodium. These constants are given in the figure. Using this model compound it was found that the values are all between 2 and 3. No general trend with the size of the cation was observed in this case. When the target molecule also had a methyl group in the 4-position, more selective complexation was seen. In the case of rubidium the relative complex constant increased to 4.7.

Even more selective complexation was seen in case of 18-crown-6. This compound is known to selectively bind potassium ions in solution. To measure the relative complex constants using sodium ion as a reference was not possible since the ionic strength was kept constant throughout the experiment. Fig. 4 shows the results obtained for rubidium and cesium using the potassium ion as a reference. The results show a decrease in relative formation constant with increasing size of the cation for cations larger than potassium. However, most compounds investigated so far seem to give complexes where the relative complex constant increases with the size of the cation. Similar results were also obtained in an earlier study [17].

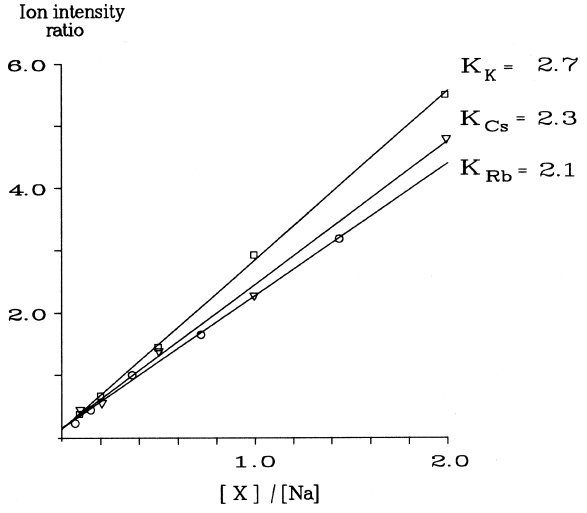

Fig. 3. Determination of relative complexation constants. X= alkali metal ion as shown.

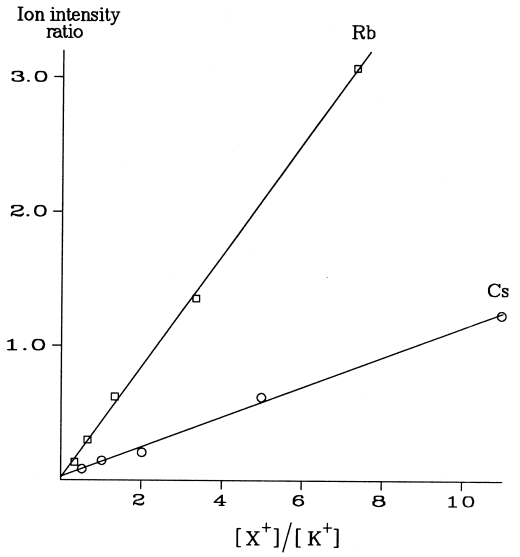

Fig. 4. Determination of relative complexation constants vs. potassium. Rb; slope 0.41 Eq. (3) O Cs; slope 0.11.

A change to a cation with a larger relative complex constant does not necessarily mean that the sensitivity will be much different. The sensitivity in a quantitative determination of 18-crown-6 using potassium and cesium, respectively, was only about 2.3 times larger using potassium. In this case the complex constant for potassium is about 10 larger than that of cesium (Fig. 4).

A qualitative explanation for this could be that there is a limited time available for a droplet to give away ions before it turns into a solid particle or is pumped away by the vacuum system after passing the heated capillary region. A high surface concentration of the complex promotes a high rate of desorption.

The complexes discussed here are probably not found in appreciable concentration in a normal solution. But the interface region in a small droplet found in the spray most likely have other properties than the bulk solution [20].

3.2. Selection of cation concentration

It is of interest to find out what concentration should be used for a sheath liquid introduced cation in order to obtain the best sensitivity. Clearly as low concentration as possible should be used to avoid a high background spray current. The lowest concentration that can be used is logically determined by the concentration of the target molecule, a slight excess will probably be necessary. Fig. 5 shows how the signal/noise varies with the concentration of the rubidium ion at three different temperatures of the heated capillary. In this case the injected concentration of the target molecule was 1×10^{-7} *M*. After mixing with the sheath liquid in the spray needle the molar excess of rubidium was about 12 at the lowest concentration of the rubidium ion. As can be seen, the signal/noise remains constant until a concentration of about 1×10^{-5} *M* is reached. The signal/noise seems to stabilize at a concentration of about 5×10^{-5} *M*. No significant difference can be observed in the temperature range studied. Very similar observations regarding signal/noise behavior were found for several other target molecules as well as for different singly charged cations. The excess of the cation to target molecule does not have to be very high. Calibration curves prepared using concentration of the target up to 1×10^{-5} *M* were perfectly linear even at a cation concentration as low as 5×10^{-5} *M* (K^+). The conclusion is therefore that the distinct raise in signal/noise has nothing to do with the compounds studied or the excess of cation concentration but rather reflects the spray process itself or, for example, spray needle dimensions. In the spray there will be a distribution of droplets having different sizes. Initially these droplets will have the same concentration of the cation, a concentration that gradually increases during evaporation of the solvent. This increase in concentration will be larger in smaller droplets due to the higher vapour pressure leading to a higher solvent evaporation rate (the Kelvin effect). Therefore, conditions leading to ion emission occurs earlier in smaller droplets. If one assumes that the ionization efficiency is higher in smaller droplets then the results showed in Fig. 5 could reflect the droplet distribution in the spray. Larger droplets might be pumped away by the vacuum system before giving away ions or they do not enter the heated capillary as easy as smaller droplets.

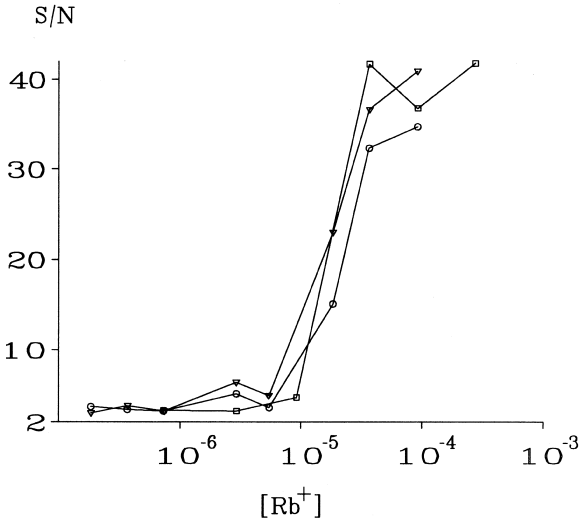

Fig. 5. Signal-to-noise vs. Rb^+ concentration in spray liquid. Sample: 200 nl, 1×10^{-7} *M* heptakis (2,6-di-O-methyl)-β-cyclodextrin dissolved in solvent stream; flow injection: 3 μl/min; 50% acetonitrile–water; capillary temperature: 200°C, O 210°C, 230°C.

Doubly charged ions like calcium behave slightly different. A maximum in signal/noise occurred already at a concentration as low at 7×10^{-6} *M* (Fig. 6). The reason for this shift in concentration might be that the surface charge necessary for ion emission will occur earlier with a doubly charged ion. The cyclodextrin concentration was in this case three times lower in order to have a comparable excess of the cation. The sensitivity using rubidium and calcium was almost the same, MDQ was found to be 10 fmol at a signal three times the noise.

3.3. Application to microcolumn liquid chromatography

It has been shown earlier [13] that cyclodextrins can be detected as sodiated molecular ions. Fig. 7 shows a separation of a commercially available cyclodextrin sample. It is supposed to have two methoxy groups, in the 2- and 6-positions, respectively, in every glucose unit. In order to identify the impurities in the sample a separation was first done using an acetonitrile–water-based mobile phase, then

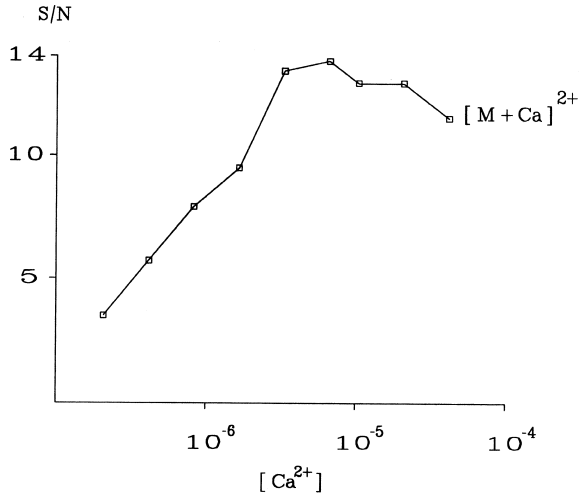

Fig. 6. Signal-to-noise ratio vs. Ca^{2+} concentration; capillary temperature: 230°C; other conditions as in Fig. 5; flow injection: 3 μl/min; 50% acetonitrile–water with Ca^{2+}; capillary temperature: 230°C.

water in mobile phase and sheath liquid is exchanged for deuterium oxide [11]. The separation (Fig. 7) was done on a 50 μm I.D. column using a continuous gradient from 20 to 75% ACN/deuterium oxide. Potassium ions were added to the sheath liquid and used to ionize the sample molecules. The mass

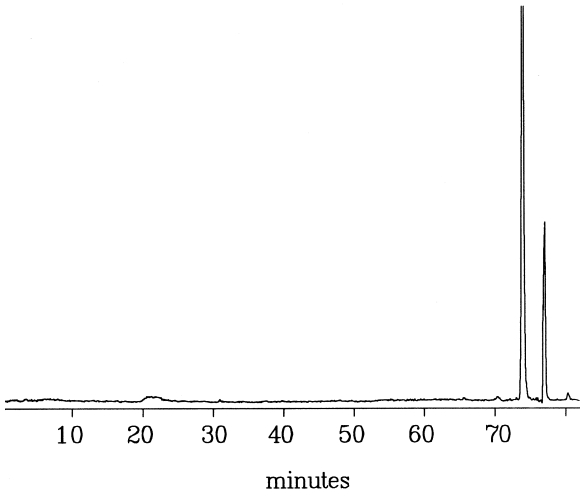

Fig. 7. Separation of cyclodextrins. Column: 1000 mm×50 μm I.D. packed with 5 μm Kromasil C-8; gradient: 20–75% acetonitrile–deuterium oxide; sheath liquid: 3 μl/min 50% acetonitrile–deuterium oxide, 0.5 m*M* potassium acetate; sample: commercially available heptakis (2,6-di-O-methyl)-β-cyclodextrin.

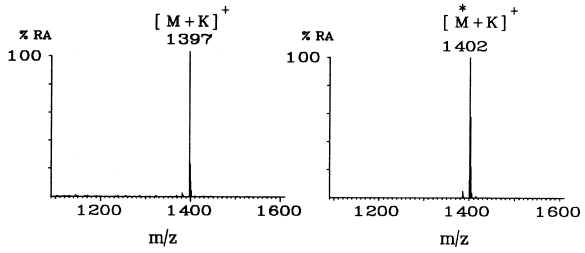

Fig. 8. Mass spectra obtained for impurity peak. Left, watersystem; right, deuterium oxide system.

spectra for the major impurity (77 min) obtained from water and deuterium oxide, respectively, are shown in Fig. 8. The peak-shift in the mass spectrum was found to be 5, showing that only 5 unreacted hydroxygroups still remain. The mass difference to the correct molecular mass was found to be 28 in the water-based system. The impurity is therefore most likely formed by addition of two more methylgroups in the 3-positions in two of the glucosresidues. The small peak eluting after 80 min corresponds to a compound that have a total of 4 extra methoxygroups.

The separation was done on a 1 m long 50 μm I.D. column that ended about 0.3 mm outside a standard spray needle. It was necessary to spray directly from the very end of this small I.D. column to avoid excessive bandbroadening. Even though such a column normally should produce more than 100 000 theoretical plates [6] one still has to assume that the impurity peak contains several unresolved isomers.

Another interesting area to microcolumn chromatography involves the separation of compounds formed during chemical degradation of ethylcellulose (acidic hydrolysis). The resulting mixture of compounds are quite complex as can be seen from Fig. 9. The sample mainly comprises compounds having from three up to four glucose units with varying degree of substitution. Ionization in this case was done by using rubidium ions. The rubidium ion has a molecular weight of 85 and an isotope peak at 87 with a relative abundance of 30%. The isotopic pattern given by the adduct ions will be very similar to what is seen with organic chloride compounds. This is particularly attractive since it makes it easy to distinguish between, for example, protonated molec-

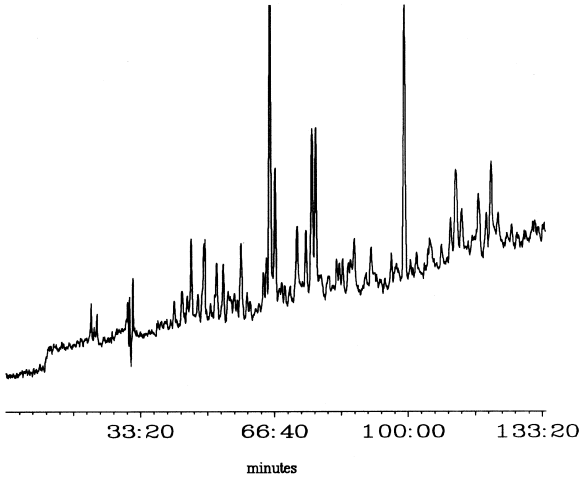

Fig. 9. Separation of degraded ethylcellulose sample. Column: 1000 mm×250 μm I.D., Kromasil C-8, 5 μm; gradient: 80% acetonitrile–water to 100% acetonitrile; sheath liquid: 3 μl/min 50% acetonitrile–water, 0.5 m*M* rubidium acetate.

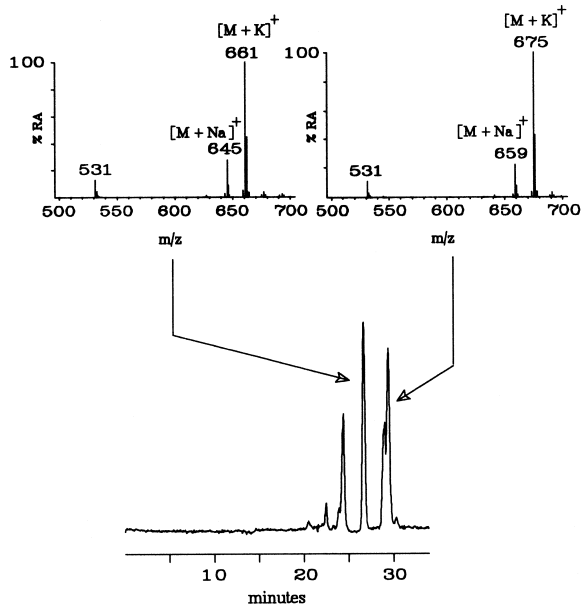

Fig. 10. Separation of bafilomycin A_1 related substances. Column: 1000 mm×250 μm I.D., Kromasil C-8, 5 μm step; gradient: 80% acetonitrile–water to 100% acetonitrile; sheath liquid: 3 μl/min 50% acetonitrile–water, 0.1 m*M* sodium and potassium ions.

ular ions, if present, and rubidium adducts. Attempts to ionize unmodified oligosaccarides using alkali metal ions seems to be more difficult. A requirement for a successful ionization seems to be that the molecule is hydrophobized using, for example, methylation.

Fig. 10 shows the separation of some compounds resulting from a fermentation process. In this case two cations were added to ionize the compounds, sodium and potassium. The reason again was to find out what type of adduct ions would be formed. A very large difference in the intensity ratio of the two cation adducts would probably indicate a major difference in the molecular structure. Such judgments are more reliable if one uses cations like, for example, cesium and rubidium which are not so commonly found naturally in samples of biological origin. Addition of known concentrations of several cations could perhaps form the basis for fingerprinting (masses and relative intensities of adduct ion) of molecules. The fingerprint will then depend on the concentration and complex formation constants for the cations in question.

The major peak in the chromatogram (Fig. 10) is Bafilomycin A_1 (mw. 622) but some other closely related compounds could also be found. The fragment ion at m/z 531 is from the large ring structure and is common for both compounds. A change to deuterium oxide in the mobile phase gave a mass shift of four for the molecular ions. The information obtained indicates that the molecules differ in a $-CH_2-$ group somewhere in the six-membered ring of the molecule.

4. Conclusions

It has been shown that post-column addition of permanently charged cations can be used successfully to form charged complex ions that can be detected with good sensitivity using the electrospray interface. The optimum concentration of a singly cation in the solution sprayed was about 5×10^{-5} *M* and independent of the cation (alkali metals). It was proposed that the optimum concentration is dependent of the droplet size, decreasing if smaller droplets can be generated. Relative complexation constants was often shown to increase with the size of the cation with the expected exception of 18-crown-6.

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