Mass Spectrometric Study for the Analysis of Quaternary Ammonium Pesticides

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Declaration

I hereby declare that the work described in this thesis is my own, except where otherwise acknowledged, and has not been submitted previously for a degree at this or any other university.

Catherine Evans

Abstract

Legislative demands require the development and application of methods for the quantitative analysis of agrochemicals and their metabolites in food, water and the environment. Quaternary ammonium pesticides (quats) used in agriculture include paraquat, diquat, difenzoquat, mepiquat and chlormequat. Analyses of the quats present particular analytical challenges due to their high aqueous solubility, cationic character and low volatility. Although many techniques for the analysis of individual quats have been reported, those employing mass spectrometric detection are probably the most flexible and powerful. This study investigates the application of mass spectrometric approaches to the analysis of the five quats.

A liquid chromatographic–mass spectrometric (LC-MS) method capable of analysing all five quats either simultaneously or consecutively was developed and employed a non-endcapped cyano stationary phase and salt/solvent gradient elution. It was compared with a capillary electrophoretic–mass spectrometric method. The LC-MS method was shown to be more suited to regulatory analysis, due to its wider linear dynamic range and lower detection limit. For the determination of chlormequat in pear matrix, the detection limit (1.7 ng mL^{-1} ≡ 0.009 mg kg^{-1}) was well below that required for regulatory monitoring (3 mg kg^{-1}). An alternative LC-MS method using a non-endcapped C_{18} stationary phase and isocratic elution with a four component mobile phase was also investigated for the analysis of chlormequat. This isocratic method gave a significantly lower detection limit (1 pg mL^{-1}) than that achieved on the cyano column and would be appropriate for direct analysis of chlormequat in drinking water.

The behaviour of the quats during mass spectrometric detection was investigated. Their full scan mass spectra revealed the presence of cluster species; these were identified and the conditions for their formation characterised. Increased specificity in detection can be achieved through the incorporation of MS^n strategies. The MS^n fragmentation pathways of the quats were investigated and highly specific transitions

for the individual quats were identified. Simplex optimisation was used to tune the conditions employed during MS^n fragmentation to favour these specific fragmentation pathways. Inclusion of these optimised MS^n transitions within a flow injection – mass spectrometric (FI-MS^n) method allowed the rapid, quantitative determination of the quats with high specificity.

For the analysis of chlormequat in pear samples, an off-line solid phase extraction (SPE) protocol using a cyano phase sorbent and tetraethylammonium chloride as internal standard was developed and applied prior to the FI-MS^n analysis. This off-line SPE-FI-MS^n method resulted in linear dynamic ranges spanning over two orders of magnitude and achieved limits of detection for chlormequat in SIM, MS^2 and MS^3 modes (13, 36, 125 ng mL^{-1}, respectively) below that required for regulatory monitoring (600 ng mL^{-1} ≡ 3 mg kg^{-1}). The FI-MS^n method has a short analysis time per injection (0.3 min) and has potential to achieve triplicate sample analysis in less than one minute. This technique could be applied to the rapid quantitative analysis of pesticides for regulatory purposes.

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Preface

This thesis is presented in seven chapters. Chapter 1 provides an overview of pesticide usage and analytical methods employed for the determination of quaternary ammonium pesticides (quats). Chapter 2 describes the development of chromatographic and electrophoretic separations for the analysis of quats. Chapters 3 and 4 detail mass spectrometric analyses of the quats and Chapter 5 describes the development of a rapid quantitative flow injection mass spectrometric method for their determination. Chapter 6 provides an overview of the work, and the experimental and analytical procedures employed are outlined in Chapter 7.

Work already published is as follows:

Evans CS, Startin JR, Goodall DM, Keely BJ (2000) "Optimisation of ion trap parameters for the quantification of chlormequat by liquid chromatography/mass spectrometry and the application in the analysis of pear extracts" *Rapid Commun. Mass Spectrom.*, **14**; 112 - 117.

Evans CS, Startin JR, Goodall DM, Keely BJ (2000) "Improved sensitivity in detection of chlormequat by liquid chromatography-mass spectrometry" *J. Chromatogr. A*, **897**; 399 - 404.

Evans CS, Startin JR, Goodall DM, Keely BJ (2001) "Mass spectrometric analysis of quaternary ammonium pesticides" *Rapid Commun. Mass Spectrom.*, **15**; 699 - 707.

Evans CS, Startin JR, Goodall DM, Keely BJ (2001b) "Formation of gas-phase clusters monitored by ion-trap electrospray ionisation mass spectrometry: a study of quaternary ammonium pesticides" *Rapid Commun. Mass Spectrom.*, 15; 1341 – 1345.

Evans CS; Startin JR, Goodall DM, Keely BJ (2001) "Towards a generic method for the analysis of quats" in *Advances in Mass Spectrometry – Volume 15* (Gelpi E. ed.) Wiley USA.

Glossary

AA	activation amplitude
APCI	atmospheric pressure chemical ionisation
API	atmospheric pressure ionisation
AQ	activation Q
A_s	peak asymmetry factor
AT	activation time
a_z	a Mathieu trapping parameter
BDS	base deactivated silica
CE	capillary electrophoresis
CEC	capillary electrochromatography
CID	collision induced dissociation
CQ	chlormequat
DC	direct current
DF	difenzoquat
DQ	diquat
EOF	electroosmotic flow
ES	electrospray
FAB	fast atom bombardment
FI-MS	flow injection mass spectrometry
GC	gas chromatography
i.d.	internal diameter
IEC	ion exchange chromatography
IW	isolation width
k	retention factor
KE	kinetic energy
LC	liquid chromatography
LMCO	low mass cut off
LOD	limit of detection
LOL	limit of linearity

MALDI	matrix assisted laser desorption ionisation
MIR	maximum ion response
M_q^+	quaternary ammonium cation
MQ	mepiquat
MRL	maximum residue level
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multiple stage mass spectrometry
m/z	mass to charge ratio
ODS	C_{18} stationary phase
PB	particle beam
PQ	paraquat
q_z	a Mathieu trapping parameter
R^2	coefficient of determination for linear regression
RF	radio frequency
r_0	radius of ring electrode in ion trap
RSD	relative standard deviation
SCX	strong cation exchange stationary phase
SIM	selected ion monitoring
SPE	solid phase extraction
SRM	selected reaction monitoring
TEA	tetraethylammonium
t_M	dead time
t_R	retention time
TSP	thermospray
UV	ultra violet
W_h	peak width at half height
z_0	distance from centre of ion trap to end-cap electrode
β	boundary of ion trap stability diagram

It is amazing what one can achieve,
when one doesn't know what one can't do.

(Anon)

Chapter 1

Introduction

Chapter 1: Introduction

1.1 PESTICIDE USAGE

This chapter describes pesticide usage worldwide and within the UK, outlines analytical methods for the analysis of a particular group of pesticides and finally introduces the aims of this project.

After the end of the Second World War, agricultural practice was revolutionised due to the need to feed a rapidly growing world population. Agricultural production was increased through the introduction of intensive farming and the use of agrochemicals such as fertilisers and pesticides (Newbold *et al.*, 1998). Each year an estimated 2.5 million tonnes of pesticides are applied to crops worldwide (Newbold *et al.*, 1998) in order to protect them against the activity of crop destroying insects (insecticides), to control weeds (herbicides) and prevent fungal (fungicides) and bacterial (bactericides) attack. Nevertheless *ca.* one third of agricultural crop production is lost annually (Pimentel, 1990) and so to maintain an adequate global food supply continued use of pesticides is required (Newbold *et al.*, 1998).

Within the UK approximately 30,000 tonnes of pesticide are applied annually, equivalent to *ca.* 5.4 kg of active ingredient per hectare of agricultural land (Newbold *et al.*, 1998). It is reported that 80% of the total amount of pesticides used on crops in developed countries are herbicides or plant growth regulators, for example, glyphosate and chlormequat, respectively (Sherma, 1995). One particular group of pesticides possessing both herbicidal and plant growth regulating properties is that of the quaternary ammonium pesticides, which includes the plant growth regulator chlormequat, the third most widely applied pesticide in the UK (Table 1.1).

Pesticides are biologically active compounds designed principally to kill target organisms. Although their use has resulted in increased agricultural production through greater crop yields and has led to subsequent increases in food production, there has been much concern over their side effects. These effects include environmental contamination due to the presence of environmentally persistent chemicals, effects on non-target organisms, the possible emergence of resistance to

certain pesticides, and the potential for long-term harmful effects on man (Plimmer, 1996). Over time concern has evolved into regulation and legislation.

Position	Active ingredient	Tonnes applied	Position	Active ingredient	Tonnes applied
1	Sulfuric acid	12,997	6	Mecoprop	957
2	Isoproturon	2,382	7	Chlorthalonil	841
3	Chlormequat	2,335	8	MCPA	724
4	Mancozeb	1,191	9	Glyphosate	606
5	Sulfur	1,113	10	Mecoprop-P	578

Table 1.1: Top Ten UK pesticides used in 1994 by weight applied (Newbold *et al.*, 1998).

Within the UK, the main statutory instrument, which aims to ensure that pesticides are safe and effective, is the Food and Environmental Protection Act 1985 (FEPA). From this, the Control of Pesticide Regulations 1986 (COPR) and the Pesticides (Maximum Residue Levels in crops, food and feeding stuffs) Regulations as amended 1997 were derived. COPR requires that new pesticides undergo rigorous registration and approval procedures, with the manufacturer proving that the pesticide poses minimum risk to human health, safety and the environment. The Maximum Residue Levels Regulations specify the maximum residue level (MRL) of pesticide (MRL) likely to occur in or on foods (Table 1.2; Statutory Instrument, 1997) after the pesticide has been used according to the codes of good practice (Newbold *et al.*, 1998). Pesticide residues present at or below the MRL will not pose an unacceptable health risk to anyone who consumes the food. As seen from Table 1.2, the MRL specified is dependent not only on the pesticide but also on the commodity. These MRLs are considered an enforcement tool and are intended as a check to ensure that good practice guidelines are followed (Newbold *et al.*, 1998). With respect to water pollution and drinking water standards, relevant legislation is

provided by the Water Resources Act and the European Union Water Quality Directive 1980 (80/778/EEC), and as subsequently amended (Council Directive, 1998). A limit of 0.1 μg L^{-1} of individual pesticides is set for drinking water.

Pesticide	Commodity	MRL / mg kg^{-1}
Chlormequat	Carrot	0.05
	Pears	3
	Wheat	2
Paraquat	Pears	0.05
	Olives	0.05
	Hops	0.01

Table 1.2 UK maximum residue levels (MRLs) in crops for two quaternary ammonium pesticides, chlormequat, a plant growth regulator, and paraquat, a non-selective herbicide.

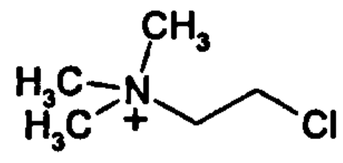
Due to the legislation in force within the UK, the use of pesticides by the agricultural industry is continuously monitored and surveyed. Surveys are performed biennially on arable crops and on a four year rolling programme for other commodities (Newbold *et al.*, 1998). Furthermore, the levels of pesticides in crops and food are monitored to ensure compliance with legislation, minimise the potential risk to the consumer and maintain consumer confidence. From an environmental perspective, the Environment Agency monitor the majority of the UK's surface waters, ground water, marine waters and effluent discharges for pesticides. The individual water companies in the UK monitor drinking water resources for pesticide contamination, thus safeguarding the quality of our drinking water and ensuring it meets EU standards (Council Directive, 1998).

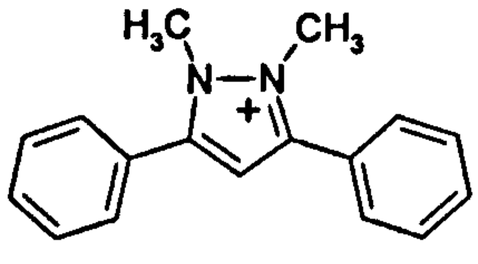
Due to the need to monitor and quantify the levels of pesticides in the environment, in food and in water, development of suitable analytical methods is required.

Furthermore, the need to carry out many analyses in order to monitor compliance with legislation means that the methods employed must be specific, rapid and cost-effective.

1.2 QUATERNARY AMMONIUM PESTICIDES

The herbicidal activity of the quaternary ammonium compounds was discovered in the 1950s, and since then these compounds have been used extensively for the control and management of terrestrial and aquatic vegetation. The structures of the quaternary ammonium pesticides (quats), paraquat (PQ; 1,1'-dimethyl-4,4'-bipyridinium ion), diquat (DQ; 1,1'-ethylene-2,2'-bipyridyldiylium), difenzoquat (DF; 1,2-dimethyl-3,5-diphenyl-1H-pyrazolium), chlormequat (CQ; 2-chlorethyltrimethylammonium, also known as chlorocholine) and mepiquat (MQ; 1,1-dimethylpiperidinium), are shown in Figure 1.1.





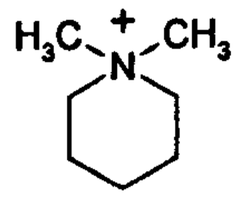


Figure 1.1: Structures of the quaternary ammonium pesticides.

All five quats are positively charged, three being monocations (CQ, MQ and DF) and two dications (PQ and DQ). All the quats are highly water soluble (Table 1.3), preferring aqueous media to organic media as demonstrated by negative values of the logarithms of their octanol-water partition coefficients (log P_{ow}). The partition coefficients are the equilibrium constants for partitioning of the quat between octanol

and water. A positive value of log *P* indicates preference for organic media, whilst a negative value indicates preference for aqueous media. In addition, all quats are stable in acid (Pico *et al.*, 2000b). Although DF, CQ and MQ are stable to hydrolytic attack (Pico *et al.*, 2000b), DQ degrades slowly at pH levels greater than 9 and PQ hydrolyses at pH levels greater than 12 (Chichila and Walters, 1991). All five quats are thermally stable and, as revealed by their very low vapour pressures at 298 K, are involatile (Table 1.3). Of the five quats, three possess UV chromophores (PQ, DQ and DF). In general, the quats possess similar characteristics and physical properties, in particular their positive charge, involatility and high aqueous solubility.

Pesticide	Relative molar mass	Melting point / °C	Vapour pressure at 298 K / mPa	log P_{ow}	Solubility in water / g kg^{-1}
Paraquat	186	300 (d)	<0.1	-4.7	700
Diquat	184	300 (d)	<0.13	-4.6	700
Difenzoquat	249	150-160	<0.01	-0.62	765
Chlormequat	122	235	<0.01	-1.59	1000
Mepiquat	114	223	<0.01	-2.82	500

Table 1.3: Characteristic physicochemical properties of quaternary ammonium pesticides; (d) indicates decomposition.

Of the quats, the bipyridinium herbicides PQ and DQ are probably the best known and are non-selective, quick-acting herbicides and desiccants (Tomlin, 1997). They are absorbed by the foliage and translocated through the plant; during photosynthesis a superoxide is produced, which damages cell membranes and cytoplasm, causing desiccation to occur. PQ and DQ are often applied together and are used for the pre-harvest desiccation of a range of crops including cotton, flax, soya beans and cereals; for the control of annual broad-leaved weeds in vines, olives, vegetables and other crops; for weed control on non-cropland, and for the control of emergent and

submerged aquatic weeds (Tomlin, 1997). PQ and DQ are highly toxic, and due to their widespread use they are often included in pesticide monitoring programmes (Pico *et al.*, 2000b).

DF is a selective herbicide used for the post-emergence control of wild oats in barley, rye, maize and flax (Tomlin, 1997). It is also used as a fungicide to control powdery mildew in cereals. It can be applied in combination with broad-leaved herbicides, such as PQ and DQ, and also with CQ (Tomlin, 1997).

The non-aromatic compounds CQ and MQ are both plant growth regulators, and are sometimes applied to crops together. CQ inhibits the biosynthesis of gibberellin, preventing cell elongation, and results in the development of sturdier plants due to their shorter, stronger stems (Tomlin, 1997). This effect also influences the developmental cycle of the plant and leads to increased flowering and harvest periods (Tomlin, 1997). As a result CQ is used to increase yields of wheat, rye, oats; to promote flower formation; improve fruit setting in pears, vines, olives and tomatoes, and to prevent premature fruit drop in pears, apricots and plums (Tomlin, 1997). MQ is used on cereals, grains, grass, seed crops and flax to reduce vegetative growth on cotton, and to prevent sprouting in leeks, onions and garlic (Tomlin, 1997). Although CQ is usually applied to the plant early in the growing season, measurable residues can remain at harvest time (Evans *et al.*, 2000a). Due to its widespread use within the UK (Table 1.1) and abroad, the UK authorities established MRLs for CQ in food commodities (some are given in Table 1.2). Although an MRL is specified for CQ in pear, the use of CQ within the UK for the production of pears intended for consumption is illegal. For these reasons CQ is often included in pesticide monitoring programmes.

1.3 ANALYTICAL METHODOLOGY USED IN ANALYSIS OF THE QUATS

1.3.1 Overview

Since the quats are often included in pesticide monitoring programmes, analytical methods for their determination need to be developed. The analysis of the quats is made difficult by their high aqueous solubility, cationic character and low volatility (Table 1.3). Despite these characteristics many analytical techniques have been employed for their determination and are discussed below.

1.3.2 Chromatographic methods

Gas chromatography (GC) with mass spectrometric (MS) detection is the most widely used technique for the analysis of pesticide residues and offers high separation efficiency, good sensitivity and selectivity combined with the capacity for multi-residue determinations (Hogendoorn and van Zoonen, 2000). As a result GC-MS has been used in the quantitative determination of pesticide residues in a variety of matrices including rainwater (Hueskes and Levsen, 1997), soil (Papadopoulou-Mourkidou *et al.*, 1997) and food (Lehotay and Eller, 1995). In keeping with this general trend, several methods have reported the use of GC for the analysis of quat pesticides: MQ (BASF, 1994); CQ (Tafuri *et al.*, 1970; Allender, 1992); DF (Tsukioka *et al.*, 1998; Steller, 1980); DQ (Lukaszewski, 1985; Hajslova *et al.*, 1989) and PQ (Kahn, 1975; Krawse *et al.*, 1984; Lukaszewski, 1985; Hajslova *et al.*, 1989). Due to the low volatilities of the quats these methods generally required extensive sample preparation and complex derivatisation steps prior to analysis by GC. The products of derivatisation steps need to be specific to the analyte of interest so as to avoid the possibilities of false positive results. One of the GC methods for CQ involved derivatisation with pentafluorophenol (Allender, 1992). Mortimer and Weber (1994) criticised this method because the product obtained after derivatisation does not arise from the CQ backbone, and so other substrates can produce the same derivatisation product. Hence, Allender's method (1992) could not reliably be applied to the analysis of real samples.

It can be seen that due to their polar nature and low volatilities (Section 1.2) the quats are not amenable to analysis by GC without employing time-consuming derivatisation procedures. Liquid chromatography (LC) is generally the most appropriate separation method for polar, involatile compounds, such as the quats (Slobodnik *et al.*, 1995; Hogendoorn and van Zoonen, 2000). LC can be carried out using different stationary phase materials in combination with appropriate mobile phases. Due to the variety of stationary phases available, chromatographic separation can be effected through several different mechanisms (Hamilton and Sewell, 1982).

Since the quats are cationic, ion exchange chromatography has been used for their determination. Ion exchange separates analytes on the basis of their differing strengths of interaction with the exchange site (Hamilton and Sewell, 1982). It is typically performed using dedicated cation exchange columns, and mobile phases containing cations which have a greater affinity than the analyte cations for the exchange sites present on the stationary phase. In terms of quat analysis, the majority of ion exchange methods reported are for the determination of CQ (Brewin and Hill, 1996; Startin *et al.*, 1999; Lautie *et al.*, 2000; Zhao *et al.*, 2000), although a method has been described for PQ and DQ (Nakagiri *et al.*, 1989). One drawback of cation exchange methods is that difficulties in analysis can occur due to deactivation of the cation exchange resin over time (Hamilton and Sewell, 1982). If this happens, the ionic strength of the counterion in the mobile phase may need to be modified in order to achieve the same retention times. This phenomenon was reported for the analysis of CQ by Startin *et al.* (1999). They observed that the concentration of the ion-exchanger cation in the mobile phase needed to be reduced after a period of column use, presumably due to deactivation of some of the cation exchange sites.

A more common mode of LC used in many analytical methods is reversed phase LC. This mode separates analytes on the basis of their hydrophobic character; those of greater hydrophobicity are more strongly retained and hence elute later than analytes with less hydrophobic character. Because the quats are charged species, ion-paring

reagents are often added to the mobile phases used in reversed phase LC separations (Lawrence *et al.*, 1981; Worobey, 1987, 1993; Hodgeson *et al.*, 1992; Carneiro *et al.*, 1994, 2000; Ibanez *et al.*, 1997; Marr and King, 1997; NDDH, 1997; Lee *et al.*, 1998; Taguchi *et al.*, 1998; Castro *et al.*, 1999; 2000; Takino *et al.*, 2000). In addition, ion pair chromatography has also been performed on silica columns (Chichila and Walters, 1991; Chichila and Gilvydis, 1993; Ibanez *et al.*, 1996a,b; Itagaki *et al.*, 1997). The anion of the ion-pair reagent added to the mobile phase is thought to associate with the quat cation to form an ion-pair, minimising any possible ionic interactions between the quat cation and the stationary phase. Separation is achieved predominantly on the basis of hydrophobicity (Hamilton and Sewell, 1982). Other methods have been developed for the quats on reversed phase stationary phases without employing ion-pair reagents (Ahmad 1982a,b, 1983; Barcelo *et al.*, 1993;Yoshida *et al.*, 1993; Vahl *et al.*, 1998; Juhler and Vahl, 1999; Hau *et al.*, 2000; Mol *et al.*, 2000).

Often UV detection is employed for LC separations due to its wide application range, ease of use and low cost (Hogendoorn and van Zoonen, 2000). Several of the LC methods for the determination of the quats employ UV detection (Lawrence *et al.*, 1981; Ahmad, 1982a,b, 1983; Worobey, 1987, 1993; Chichila and Walters, 1991; Hodgeson *et al.*, 1992; Chichila and Gilvydis, 1993; Carneiro *et al.*, 1994, 2000; Ibanez *et al.*, 1996a,b; Itagaki *et al.*, 1997; NDDH, 1997; Lee *et al.*, 1998). Unfortunately, UV detection is not a universal detection method because not all analytes possess a UV chromophore, for example, CQ and MQ. Therefore, LC methods for the determination of these two quats have employed alternative detection systems, such as conductivity (Fegert *et al.*, 1991; Lautie *et al.*, 2000) or mass spectrometry (Brewin and Hill, 1996; Vahl *et al.*, 1998; Juhler and Vahl, 1999; Startin *et al.*, 1999; Hau *et al.*, 2000; Zhao *et al.*, 2000). Since UV detection lacks selectivity and specificity, confirmation of identity can be problematic for pesticides of the same class because of similarities between their UV spectra. For example, the two quats PQ and DF have similar UV absorption maxima, 257 and 255 nm, respectively. Consequently, further confirmatory analysis is often required (Hogendorn and van Zoonen, 2000).

To eliminate the need for additional confirmatory detection, LC can be coupled with mass spectrometry (MS; Stout *et al.*, 1998). LC-MS is a powerful analytical tool, which permits the efficient separation, unambiguous identification and quantification of a wide range of analytes (Hogendoorn and van Zoonen, 2000) including those lacking UV chromophores. Although the first coupling of LC to MS was reported over 25 years ago, the technique was not adopted as a routine analytical tool until *ca.* 10 years ago (Slobodnik *et al.*, 1995; Pico *et al.*, 2000a). Technical difficulties involved in combining an instrument that operates in the condensed phase with one that operates under vacuum have been overcome through the use of interfacing devices (Pico *et al.*, 2000a). For LC-MS, there are three principle interfacing techniques: thermospray (TSP), particle beam (PB) and atmospheric pressure ionisation (API: Slobodnik *et al.*, 1995; Pico *et al.*, 2000a; Hogendoorn and van Zoonen, 2000). Until the mid 1990s most LC-MS applications in pesticide residue analysis involved TSP or PB interfaces (Slobodnik *et al.*, 1995), and three methods were published for the LC-MS analysis of the quats using either a TSP interface (Barcelo *et al.*, 1993; Yoshida et al.,1993) or a PB interface (Kambhampati *et al.*, 1994). In the main, LC-MS methods using TSP or PB interfaces have not been adopted in regulatory monitoring due to high costs, poor compatibility with LC separations, in particular when using PB interfaces, and highly variable compound dependent responses, especially in TSP (Slobodnik *et al.*, 1995).

After the mid 1990s LC-MS analysis of pesticide residues has predominantly involved the use of API interfaces (Hogendoorn and van Zoonen, 2000). In API a distinction is made between electrospray (ES) and atmospheric pressure chemical ionisation (APCI). In an LC-MS system the ion source region is located outside the mass spectrometer at ambient pressure and is separated from the high vacuum mass analyser region by a small sampling orifice (Figure 1.2). The LC column eluent is sprayed in the vicinity of the orifice, and a spray is formed by applying heat, a coaxial nebuliser gas stream, an electrostatic potential or a combination of these (Slobodnik *et al.*, 1995). Ionisation is effected prior to transport of the ions through the sampling orifice into the mass analyser region. In the mass analyser region mass

analysis occurs and ions are detected on the basis of their mass to charge ratios (*m/z*). In ES, application of a high voltage over the spray effects ionisation for those analytes not already present in ionic form, whereas in APCI a combination of a heated capillary and a corona discharge causes ionisation of the analyte (Pico *et al.*, 2000a).

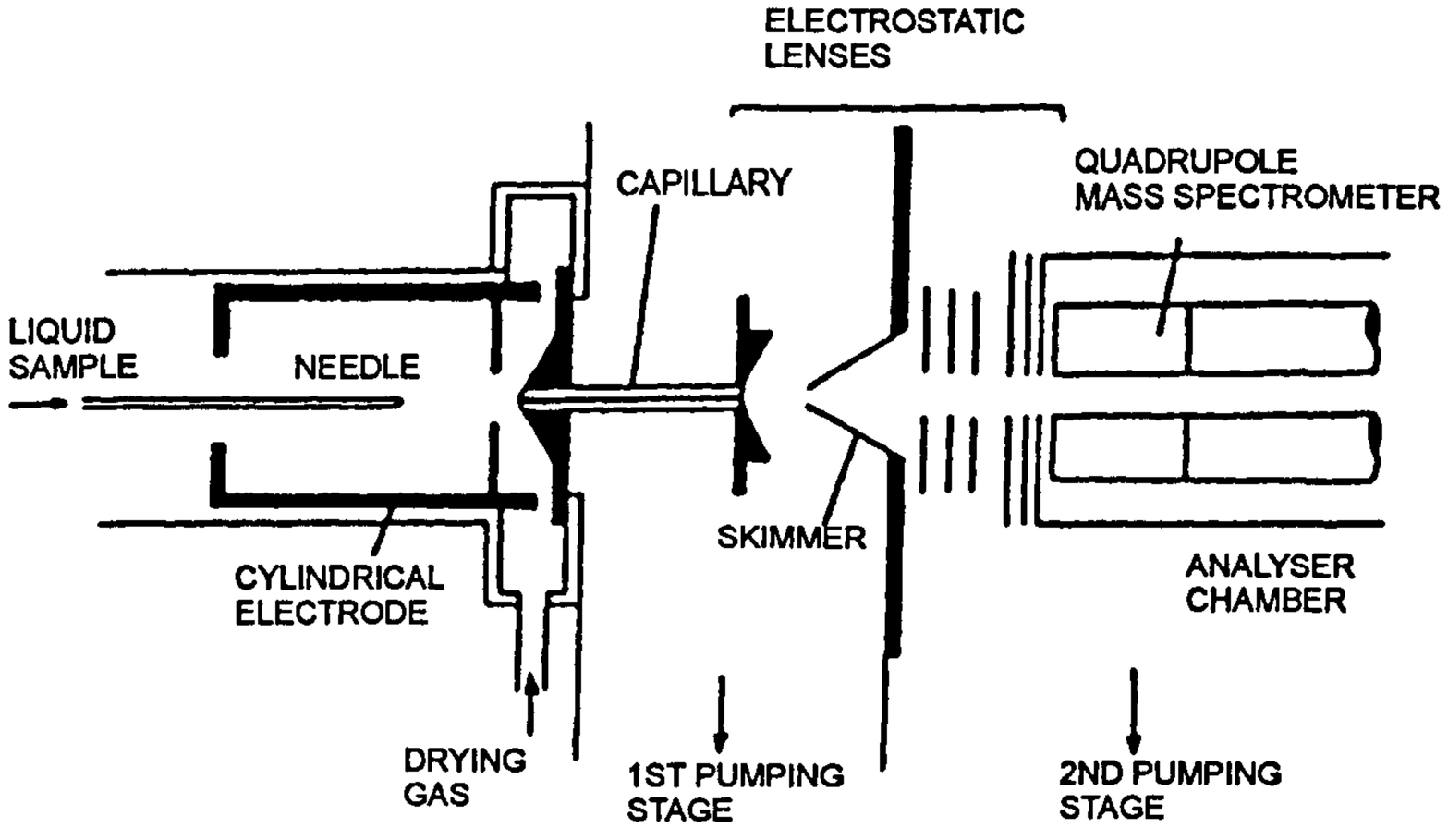


Figure 1.2: Schematic of an LC-API-MS system (from Slobodnik *et al.*, 1995).

Since API interfaces involve volatilisation of the LC eluent, additives present in the LC mobile phase need to be volatile, so as to prevent contamination of the ion source with involatile materials (Taguchi *et al.*, 1998;Takino *et al.*, 2000). Many of the LC-UV methods described earlier in this section used involatile buffer salts *e.g.* phosphate (Ahmad, 1982a,b, 1983) and/or involatile ion-pair reagents such as alkylsulfonate salts (Lawrence *et al.*, 1981; Worobey 1987; Hodgeson *et al.*, 1992; Carneiro *et al.*, 1994, 2000; Itagki *et al.*, 1997; NDDH. 1997; Lee *et al.*, 1998). These methods could not be directly applied to the LC-MS analysis of the quats (Takino *et al.*, 2000). Therefore, several LC-MS methods employing volatile mobile phase additives have since been developed for the analysis of the quats (Brewin and Hill, 1996; Marr and King, 1997; Taguchi *et al.*, 1998; Vahl *et al.*, 1998; Castro *et al.*, 1999, 2000; Juhler and Vahl, 1999; Hau *et al.*, 2000; Mol *et al.*, 2000; Startin *et al.*, 1999; Takino *et al.*, 2000; Zhao *et al.*, 2000). The majority of these methods have employed ES ionisation (Brewin and Hill, 1996; Marr and King, 1997; Taguchi

et al., 1998; Vahl *et al.*, 1998; Juhler and Vahl, 1999; Castro *et al.*, 2000; Hau *et al.*, 2000; Mol *et al.*, 2000; Startin *et al.*, 1999; Takino *et al.*, 2000; Zhao *et al.*, 2000), although one method did compare the mass spectral results obtained using the two types of API interface (Castro *et al.*, 1999).

API-MS is considered a soft ionisation technique that when used with basic or acidic species produces the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ions in positive or negative ionisation modes, respectively (Hogendoorn and van Zoonen, 2000). Usually no other ions, which could aid structural identification, are present within the mass spectrum. Since the quats are cationic, the predominant ion observed is usually the quat cation (Table 1.3), for example, *m/z* 114 for MQ (Juhler and Vahl, 1999). LC-API-MS methods provide improved selectivity and specificity over LC-UV methods. However, while monitoring only one ion per analyte is adequate for pesticide analysis difficulties could arise. Firstly, due to the lack of structural information available identification of unknown pesticide constituents within samples could be problematic. Secondly, false positive results could arise if another component within the sample has the same *m/z* value as the pesticide of interest *i.e.* so called isobaric interferences are present.

To obtain mass spectral data which could aid confirmation of identity, the molecular ion can be fragmented through collision induced dissociation (CID). CID can occur in either the interface region of the mass spectrometer, where it is known as pre-analyser CID, or in the mass analyser region, where it is known as multi-stage MS or tandem MS (MS/MS; Slobodnik *et al.*, 1995; Pico *et al.*, 2000a). As a result of pre-analyser CID, which is induced by varying the orifice voltage, additional confirmatory ions may be generated, although at the expense of the sensitivity obtained for the molecular ion (Pico *et al.*, 2000a). This approach has been used to enhance the certainty in detection of the five quats (CQ, MQ, DQ, PQ and DF) in drinking water (Castro *et al.*, 1999). During pre-analyser CID all ions in the interface undergo collisions, and some of the fragment ions observed may not arise from the pesticide ion of interest, resulting in potential difficulties in the interpretation of the mass spectral data.

By contrast, MS/MS involves the isolation of the analyte ion of interest prior to CID occurring within the mass analyser region. The product ions formed can only be derived from the precursor ion initially isolated. Detection is achieved through monitoring the formation of product ions from the precursor ion. For example, MS/MS of the CQ precursor ion at *m/z* 122 yields an ion at *m/z* 58, and so monitoring the transition *m/z* 122 to 58 confirms the presence of CQ within a sample (Brewin and Hill, 1996; Vahl *et al.*, 1998; Juhler and Vahl, 1999; Startin *et al.*, 1999; Hau *et al.*, 2000; Mol *et al.*, 2000). Thus, inclusion of MS/MS strategies within LC-API-MS methods enhances the specificity of the overall method. In addition to the LC-API-MS/MS methods developed for CQ, LC methods employing MS/MS detection have also been reported for MQ (Juhler and Vahl, 1999) and for PQ and DQ (Marr and King, 1997). It should be noted, however, that not all mass spectrometers are capable of performing MS/MS analyses. MS/MS can only be performed with instruments containing triple quadrupole and ion trap mass analysers.

1.3.3 Capillary electrophoretic separations

A complementary technique to LC is capillary electrophoresis (CE). CE is often regarded as being capable of separating analytes quicker and with greater efficiency than LC (Li, 1992). CE separates analytes due to differences in their rates of migration through a buffer-filled capillary, across which an electric potential is applied (Heiger, 1997). The rate of migration of an ion through the capillary is related to its charge-to-size ratio. As with LC a wide range of detectors is used in CE, with UV being the most common (Heiger, 1997). UV detection is usually performed on-column. The polyimide coating protecting the fused-silica capillary is removed by burning, creating a window (Foret *et al.*, 1993); this part of the capillary then serves as the detection cell. As the analytes pass across the window region the quantity of UV light absorbed is monitored, enabling detection of the peak. Several pesticides have been analysed by CE-UV (Menzinger *et al.*, 2000), including the quats (Wigfield *et al.*, 1993; Carneiro *et al.*, 1994, 2000; Galceran *et al.*, 1994; Kaniansky *et al.*, 1994; Perez-Ruiz *et al.*, 1996; Mallat *et al.*, 2001; Nunez *et al.*,

2001). If analytes lack a UV chromophore *e.g.* CQ and MQ, then indirect UV detection can be utilised. Indirect UV detection involves an ionic chromophore being placed in the CE buffer; the UV detector then receives a constant signal due to this substance. As the analyte passes through the window region, displacement of the chromophore ions occurs and a decrease in the UV signal is observed (Heiger, 1997), allowing determination of the analyte. This indirect UV approach has been used in the CE determination of the five quats, PQ, DQ, CQ, MQ and DF, in water (Galceran *et al.*, 1997) and of CQ and other quaternary ammonium ions present within a pesticide formulation (Wycherley *et al.*, 1996). Similar to LC separations enhanced specificity in CE separations can be achieved with MS detection. To date, two CE-MS methods have been reported for the analysis of the quats in water (Moyano *et al.*, 1996; Song and Budde, 1996).

1.3.4 Other methods for the determination of the quats

Although separation methods such as LC, GC and CE constitute a large proportion of all analytical methods developed for the quats, other techniques have been used. These include spectrophotometry (Cessna, 1991; Shivhare and Gupta, 1991; Jain *et al.*, 1993; Kesari *et al.*, 1997; Rai *et al.*, 1997), ion selective electrodes (Moody *et al.*, 1988; Kolecek *et al.*, 1993; Bianco and Aghourd, 1997; Saad *et al.*, 1998), electron spin resonsnce spectroscopy (Minakata *et al.*, 1988), mass spectrometry (without on-line separation) by fast atom bombardment (FAB) MS (Tondeur *et al.*, 1987), or by matrix assisted laser desorption ionisation (MALDI) MS (Horak *et al.*, 2001) and immunochemical methods (Niewola *et al.*, 1983; Vanemon *et al.*, 1983, 1985a,b, 1986, 1987; Nagao *et al.*, 1989; Spittler and Snook, 1993, 1996; Wu *et al.*, 1993; Selisker *et al.*, 1995; LarssonKovach *et al.*, 1996; Yeung *et al.*, 1996; Mallat *et al.*, 2001). These methods are limited in their application to the analysis of real samples. The spectrophotometric methods involve extensive sample pre-treatment and are often time-consuming to perform. In addition, not all the quats are chromophoric and so indirect spectrophotometric detection would be required for those analytes. Ion selective electrodes are not universal techniques as they are generally suitable for one analyte only. Immunochemical methods are only semi-quantitative and are

usually specific for one analyte only. They are, however, often used in initial pesticide screening programmes, due to their simplicity, low cost, high sensitivity, rapidity and ability to process large numbers of samples at the same time (Torres *et al.*, 1996). It can be seen that the principal disadvantage of these non-separation methods is their inability to allow simultaneous determination of the quats in a single sample.

1.3.5 Application of methods in regulatory monitoring of the quats

Most of the analytical methods mentioned above (Sections 1.3.2 to 1.3.4) are not sensitive enough to monitor compliance with the maximum permitted levels of quats in food and water matrices. Generally the GC methods developed lack the sensitivity or specificity needed for regulatory monitoring, for example Allender's method for CQ (Allender, 1992), and only the GC-MS method for monitoring DF in cereal samples (Tsukioka *et al.*, 1998) is routinely employed.

Of the CE-UV methods developed, only two methods have been applied to the analysis of crop samples (Wigfield *et al.*, 1993; Perez-Ruiz *et al.*, 1996). Both achieved limits of detection below those required for monitoring PQ and DQ in crop samples. The other CE-UV methods (Carneiro *et al.*, 1994, 2000; Galceran *et al.*, 1994, 1997; Kaniansky *et al.*, 1994; Mallat *et al.*, 2001; Nunez *et al.*, 2001) were applied to the analysis of water samples. Only one of them (Kaniansky *et al.*, 1994) achieved the necessary limits of detection required for regulatory monitoring of water. This method included a sample preconcentration step prior to CE-UV analysis (Kaniansky *et al.*, 1994). Although the two CE-MS methods (Moyano *et al.*, 1996; Song and Budde, 1996) both achieved detection limits in the low $\mu g\ L^{-1}$ range for pure water, the detection limits achieved for drinking water samples were two orders of magnitude greater than the limit required for regulatory monitoring in the UK.

The majority of regulatory methods currently used for analysis of the quats in food and water samples employ LC separation. Not all of the LC methods that have been

developed are suitable due to their high detection limits. LC-UV methods, which possess the sensitivity levels required, have been applied to the analysis of DF in wheat (Lawrence *et al.*, 1989) and of PQ and DQ in crops with high moisture contents such as corn and potato (Chichila and Walters, 1991; Worobey, 1987, 1993) and low-moisture crops such as barley (Chichila and Gilvydis, 1993). For the LC-UV analysis of PQ, DQ and DF in drinking water (Hodgeson *et al.*, 1992; Ibanez *et al.*, 1996a,b; NDDH, 1997; Carneiro *et al.*, 2000), preconcentration of the sample is needed, in order to achieve the low limit of detection required for regulatory monitoring *i.e.* 0.1 $\mu g\ mL^{-1}$ (Council Directive, 1998). For the analysis of MQ and CQ in foods, LC with conductivity detection is applicable to regulatory monitoring (Fegert *et al.*, 1991; Lautie *et al.*, 2000).

Although LC-MS(/MS) instruments are much more expensive than other LC detector systems (e.g. UV and conductivity detectors), their use in regulatory pesticide residue analysis is increasing. Thus, LC-MS methods have been developed for the analysis of the quats in both food and water matrices. All of the reported LC-MS methods for the analysis of the quats in food matrices can be applied to regulatory monitoring as they easily surpass the limits of detection required (Brewin and Hill, 1996; Vahl *et al.*, 1998; Juhler and Vahl, 1999; Startin *et al.*, 1999; Hau *et al.*, 2000; Mol *et al.*, 2000; Zhao *et al.*, 2000). For the analysis of water samples LC-MS methods often require preconcentration steps similar to those employed in LC-UV in order to achieve the limits of detection required to ensure compliance with legislation (Taguchi *et al.*, 1998; Castro *et al.*, 1999, 2000).

1.4 SUMMARY AND AIMS

Many methods have been used to analyse the quats but only a few offer the sensitivity and specificity required for regulatory analysis in food or water. To date, the methods best suited to regulatory analysis are those employing separation by either LC or CE and confirmation by MS. Enhanced specificity in detection can be achieved through the incorporation of MS/MS strategies into the analytical methodology. Although several of the quat MS/MS fragmentation pathways have been utilised in LC-MS/MS and CE-MS/MS methods, several pathways have yet to be examined.

Despite offering high sensitivity and specificity, mass spectrometers are not as widely used in pesticide analysis as their potential might suggest. This is due in part to their high cost. Therefore, laboratories investing in this instrumentation demand an economic pay-off, usually in terms of increased sample throughput.

This study aims to extend the knowledge of the MS/MS fragmentation pathways of the quats, so that selected transitions can be incorporated into suitable analytical methods for their rapid and highly specific determination. The scope of the thesis covers:

- Development of LC and CE separation methods
- Investigating and optimising the conditions for MS/MS fragmentation
- Identifying highly specific MS/MS transitions
- Development of a rapid flow injection-MS^n analysis method.

Chapter 1: Introduction

Chapter 2

Liquid Chromatographic and Capillary Electrophoretic Separations of the Quats

2.1 INTRODUCTION

2.1.1 Multi-residue methods for the analysis of the quats

Although the analysis of the quats is made difficult by their high aqueous solubility, cationic character, low volatility and for CQ and MQ lack of a UV chromophore, a number of analytical methods have been developed for their determination (see Chapter 1). Gas chromatographic (GC) and mass spectrometric (MS) methods have been used for their analysis, but often require laborious and time consuming sample clean-up and preparation, sometimes involving derivatisation (Marr and King, 1997; Lee *et al.*, 1998; Startin *et al.*, 1999). Increasingly, the methods employ liquid chromatography (LC) and capillary electrophoresis (CE). This move toward condensed-phase separations is due in part to the physical properties of the quats, namely their high aqueous solubility and low volatility (Hau *et al.*, 2000), and also to their greater compatibility with potential sample matrices resulting in less sample manipulation. Furthermore, advances in instrument technology enable both LC and CE to be coupled with MS, providing high sensitivity and enhanced specificity for detection (Zhao *et al.*, 1997).

Analytical methods capable of detecting the five quats, chlormequat (CQ), difenzoquat (DF), diquat (DQ), mepiquat (MQ) and paraquat (PQ), are known as multi-residue methods, and possess several advantages over those capable of detecting one or two target compounds. In particular, the capacity for simultaneous determination in one acquisition, or consecutive analyses of a range of different samples without the need for switching between methods, results in enhanced sample throughput.

A small number of multi-residue methods for the analysis of the quats have been developed (Barcelo *et al.*, 1993; Moyano *et al.*, 1996; Bianco, 1997; Galceran *et al.*, 1997; Castro *et al.*, 1999, 2000). Although one of these used electrochemical detection (Bianco, 1997), the remainder are based either on LC (Barcelo *et al.*, 1993; Castro *et al.*, 1999, 2000) or CE (Moyano *et al.*, 1996; Galceran *et al.*, 1997) coupled with UV or MS detection. Castro *et al.*, (1999, 2000) recently developed LC-MS

methods for the analysis of the quats in drinking water that involve preconcentration by solid phase extraction. CE-MS (Moyano *et al.*, 1996) has also been applied to the determination of the quats in water; the technique gave higher limits of detection (LODs) than the LC-MS methods (Castro *et al.*, 1999, 2000). CE separation with indirect UV detection (Galceran *et al.*, 1997) was proposed as a simpler, more economic solution than CE-MS for the determination of the five quats in water. All of these multi-residue methods have been applied to residue analysis in water. Most multi-residue methods are developed to simultaneously detect all target analytes in one acquisition. Due to their different uses it is unlikely that the five quats will all be present within a food sample, and this may in part be the reason why multi-residue methods have not previously been applied to the analysis of the quats in food matrices. Since multi-residue methods eliminate the need for method switching, their application in the analysis of the quats in food matrices would be advantageous.

2.1.2 This study

Recently, the analysis of CQ in pears (Brewin and Hill, 1996; Startin *et al.*, 1999; Evans *et al.*, 2000a) and cereals (Vahl *et al.*, 1998; Juhler and Vahl, 1999) has been achieved by direct methods employing liquid chromatography tandem mass spectrometry (LC-MS/MS). The methods for the analysis of pears utilise a strong cation exchange (SCX) column (Brewin and Hill, 1996; Evans *et al.*, 2000a), whereas the analysis of cereal samples is based on C_{18} phases (Vahl *et al.*, 1998). The methods interface LC and MS with electrospray (ES), which produces a simple mass spectrum that shows no fragment ions (Vahl *et al.*, 1998; Startin *et al.*, 1999).

Given that the signal intensity obtained in electrospray depends not only on the analyte concentration but also on the concentration of other electrolytes, for example buffer salts present in the mobile phase (Kerbale and Ho, 1997), chromatographic separations using different mobile phase compositions might be expected to exhibit different LODs under ES-MS conditions.

This chapter reports a comparison of the chromatographic behaviour of CQ on SCX and C_{18} stationary phases and examines the basis of the separation in the latter. The influence of the chromatographic system on the LODs and overall sensitivities for the quantification of CQ by LC-ES-MS will also be compared. Subsequently, a generic LC-MS method for the separation and determination of all five quats will be developed.

Due to the differences in their modes of operation, CE is a complementary separation technique to LC (Cai and Henion, 1995). In LC, separation is due to differential partitioning of the analyte between the mobile and stationary phases (Cai and Henion, 1995), whereas separation in CE is based on the differential rates of migration of ionic species in an electric field and depends on differences in their charge to size ratios (Tsai and Her, 1996). Because of their permanent charge and high aqueous solubilities, the quats are amenable to analysis by CE (Hau *et al.*, 2000). Potentially, CE could offer faster analysis times with higher separation efficiency and the possibility of trace analysis using very small amounts of sample (Cai and Henion, 1995; Belder and Stockigt, 1996; Zhao *et al.*, 1997). Thus, a CE-MS method for the analysis of the quats will be developed and compared with the generic LC-MS method.

2.2 RESULTS AND DISCUSSION

2.2.1 Liquid chromatographic analysis of CQ

2.2.1.1 Retention mechanisms of CQ on various stationary phases

To examine and compare the retention mechanisms of CQ in the two published methods, CQ was analysed using both methods and was monitored by ES-MS; data for selected ion monitoring (SIM) of the M_q^+ ion at *m/z* 122 corresponding to the species containing the ^{35}Cl isotope are presented. Isocratic elution of CQ from the Spherisorb S5 ODS1 C_{18} and Partisil 10 SCX phases was achieved using mobile phases comprising 50 mM ammonium acetate in 53/21/25/1 acetonitrile/methanol/ water/acetic acid and 50 mM ammonium acetate in 50/50 methanol/water,

respectively. Representative chromatograms obtained for CQ at a concentration of *ca.* 600 ng mL^{-1} in pear matrix (equivalent to the UK maximum residue level (MRL) for pears of 3 mg kg^{-1}; Statutory Instrument, 1997) on the SCX and C_{18} ODS1 columns show CQ eluting at 9.4 and 6.4 min respectively (Figure 2.1a,b). The SCX column employed was shorter than the ODS1 column (15 cm *vs.* 25cm), but allowed CQ to elute later than from the ODS1 column, thus indicating stronger retention of CQ with the SCX column. The retention of CQ on C_{18} ODS1 has been suggested to result from interaction of the cation with residual silanol groups in the stationary phase (Vahl *et al.*, 1998). To examine this, CQ was analysed using the same isocratic solvent programme on fully end-capped Hypersil ODS and base deactivated silica (BDS) C_{18} columns (Figure 2.1c,d).

During manufacture, the silica support for Hypersil BDS is pretreated to limit the number of active silanol groups present. Following attachment of the C_{18} chain the stationary phase is end-capped to reduce further the number of active silanol groups. The Hypersil ODS and Spherisorb ODS1 stationary phases are both based on untreated silica. After attachment of the C_{18} chain, the majority of the residual silanol groups in the Hypersil ODS phase are end-capped using trimethylchlorosilane. By contrast, the ODS1 phase is not end-capped and will contain more residual silanol groups on the surface of the silica. Thus, all three stationary phases have similar hydrophobicities but exhibit markedly differing degrees of silanophilic character, Hypersil BDS being the lowest and ODS1 the greatest (Waters, 2000). The term silanophilic character is used to refer to the potential of the column to retain analytes *via* silanol interactions (Cox, 1993; Nawrocki, 1997). On both Hypersil ODS and BDS, CQ exhibited less retention than on the ODS1 column (Figure 2.1), suggesting less interaction between CQ and the stationary phase in the case of the Hypersil ODS and BDS phases.

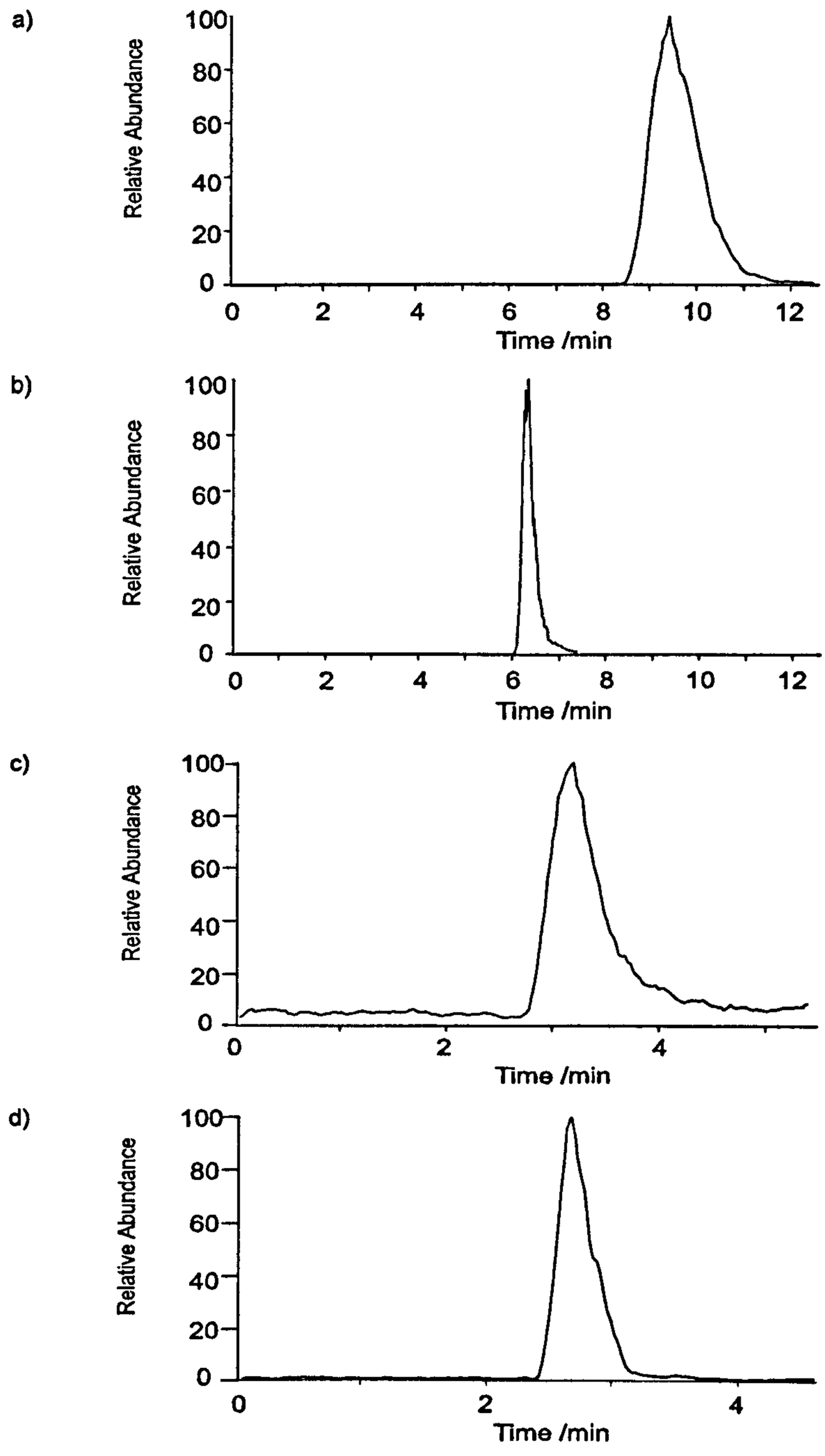


Figure 2.1: LC-MS total ion chromatogram obtained monitoring SIM *m/z* 122 during analysis of CQ (600 ng mL^{-1}) on a) the Partisil 10 SCX column b) the PhaseSep S5 ODS1 column c) the Hypersil 5 μm ODS column endcapped and d) the Hypersil BDS C_{18} column.

The column dead times were determined using sodium nitrite (Poole and Schuette, 1984) and β-carotene as unretained species on the C_{18} and SCX phases, respectively.

Retention factors, *k*, for CQ were calculated from the retention times and measured dead times of all three columns, the highest value being for the ODS1 phase (Table 2.1). Notably, the *k* values for CQ show that retention order correlates with the relative degrees of silanophilic character of the stationary phases (Waters, 2000), CQ being retained on ODS1, only slightly retained on the Hypersil ODS column and unretained on Hypersil BDS (Table 2.1). It follows that the high silanol content of ODS1 is responsible for retention of CQ on this stationary phase.

Column	Particle size / μm	Surface area / $m^2 g^{-1}$	Dead time (t_M) / min	Retention time (t_R) / min	Retention factor (k)[a]
Hypersil BDS C_{18}	5	170	2.62	2.63	0
Hypersil ODS	5	70	2.58	2.84	0.1
PhaseSep ODS1	5	220	2.47	6.52	1.64
Partisil SCX	10	500	2.67	9.42	2.53

a $k = (t_R - t_M)/t_M$

Table 2.1: Column properties and characteristics for the columns used in the RP-HPLC and IEC methods.

In ion exchange chromatography (IEC), retention of the analyte is strongly dependent on the ionic strength and pH of the mobile phase (Hamilton and Sewell, 1982; Poole and Schuette, 1984; Cox, 1993; Nawrocki, 1997). To ascertain if, as suggested by Vahl *et al.* (1998), the silanol sites on ODS1 act *via* cation exchange, the composition of the mobile phase was varied. Reduction in ionic strength, resulting from a tenfold decrease in the salt concentration at a constant buffer ratio $[CH_3COO^-]/[CH_3COOH] = 0.29$, led to a threefold increase in *k* ($k = 4.9$) from that obtained for CQ using the original conditions ($k = 1.64$; Table 2.2). This large increase is consistent with ODS1 acting *via* cation exchange under the conditions employed (Hamilton and Sewell, 1982; Poole and Schuette, 1984). At an ammonium

acetate concentration of 5 mM, a decrease in the buffer ratio to 0.029, achieved by increasing acetic acid to 1% (equivalent to 1.75 mM), caused a decrease in k by 40% (k = 3.41; Table 2.2). Conversely, increasing the buffer ratio to 2.9 caused an increase in k by 40% (k = 2.32), from the original conditions. An increase in pH will favour dissociation of the silanol groups, thereby increasing the practical specific capacity (Nawrocki, 1997). Thus, the increases in retention of CQ on increasing pH and on decreasing ionic strength are consistent with ODS1 exhibiting ion exchange capacity (Poole and Schuette, 1984). Clearly, the ODS1 column, operated under the conditions described by Vahl *et al.* (1998), exhibits sufficient ion exchange character to retain CQ to a value of t_R in excess of 2.5 times the column dead time (Table 2.1). Although ODS1 is not designed as a stationary phase for IEC the results presented show that the free silanol sites, which may represent up to 6.5 μmol m^{-2} (Cox, 1993; Nawrocki, 1997; Myers, 2000), cause it to be an effective cation exchange medium. Notably, under the conditions published for separation of CQ on the SCX and ODS1 phases, the ODS1 phase exhibits a similar practical specific capacity to that of SCX phase and gave reproducible retention times over *ca.* 300 analyses.

$[CH_3COO^-]$ / mM[a]	CH_3COOH / %	$[CH_3COOH]$ / mM	buffer ratio = $[CH_3COO^-]$ /$[CH_3COOH]$	t_R / min	k
50[b]	1[b]	175	0.29	6.52	1.64
5	0.1	17.5	0.29	14.58	4.90
5	1	175	0.029	10.89	3.41
50	0.1	17.5	2.9	8.21	2.32

[a] ammonium acetate concentration in total mobile phase

[b] salt and acid concentrations as used in the method described by Vahl *et al.*(1998)

Table 2.2: Effect of salt concentration and acid content on the retention of CQ on the ODS1 stationary phase.

In addition to differences in retention, the ODS1 and SCX columns exhibit significant differences in the peak profiles for CQ, with the peak width being significantly less on ODS1 than on SCX (width at half height (W_h) 0.3 *vs* 1.2 min respectively). Visual inspection revealed that both methods gave peaks exhibiting marked asymmetry at all concentrations. Peak asymmetry is described by the peak asymmetry factor (A_s), given by Equation 2.1, determined at 10% peak height (Figure 2.2; Poole and Schuette, 1984).

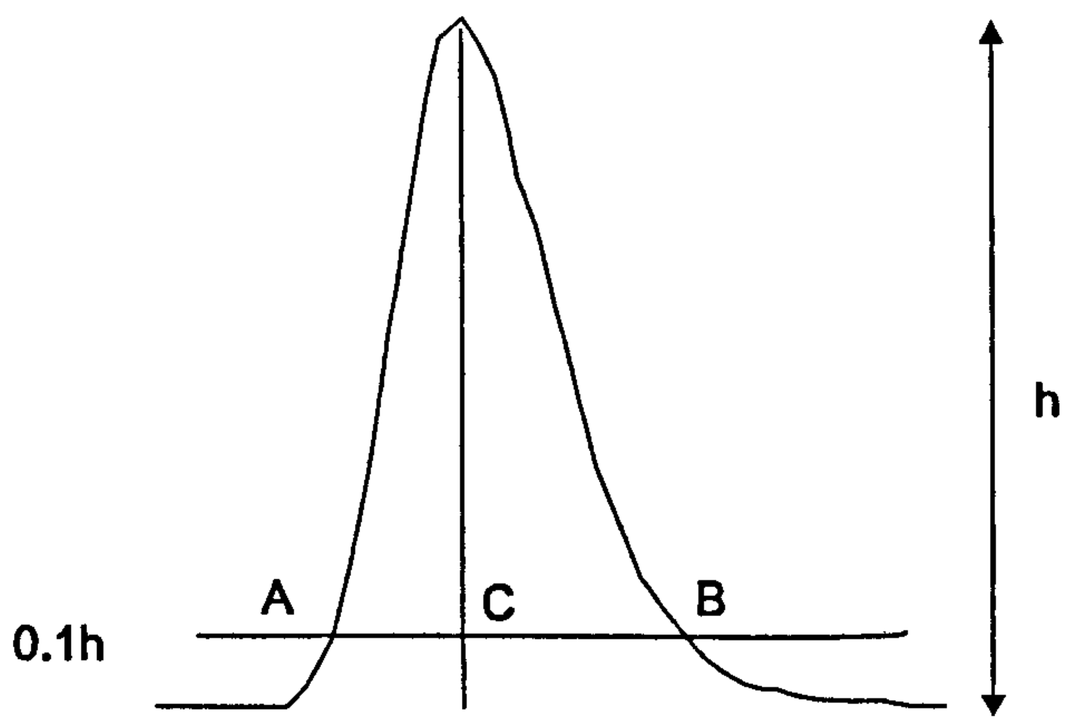


Figure 2.2: Diagram describing the calculation of the peak asymmetry factor, A_s.

$$A_s = \frac{CB}{AC}$$

Equation 2.1

Peak asymmetry factors for CQ on SCX (A_s = 2.14) and on ODS1 (A_s = 1.83) show that both are asymmetric tailing peaks (for a symmetrical peak A_s = 1). The higher value obtained on SCX indicates a greater degree of tailing and, along with the increased peak width (W_h= 1.2 min) observed with this method, reveals chromatography that is less than ideal. A concentrated solution of the polycyclic aromatic hydrocarbon pyrene (100 μg mL^{-1}) gave a more symmetrical peak on the ODS1 column (A_s = 1.36) but with the same peak width as CQ (W_h = 0.3 min). The

lower asymmetry factor obtained for pyrene illustrates that lower asymmetry results from hydrophobic interactions than from silanol interactions and reveals good homogeneity in the particle size distribution of the ODS1 stationary phase. Accordingly, the higher A_s value obtained for CQ indicates inhomogeneity within the cation exchange sites and may reflect the presence of different types of silanol sites: both very acidic and less acidic (*cf.* Cox, 1993; Nawrocki, 1997; Claessens *et al.*, 1998). The SCX stationary phase comprises irregular porous silica particles with sulfonate functionalities chemically bonded as strong cation exchange sites. Consequently, comparison of the A_s values for CQ on the ODS1 and SCX stationary phases suggests greater inhomogeneity within cation exchange sites in the latter.

The acidity of silanol groups depends on their local environment within the silica matrix (Cox, 1993; Nawrocki, 1997; Claessens *et al.*, 1998). The presence of metal ions within the matrix may cause neighbouring silanol groups to be more acidic due to electron withdrawal (Cox, 1993). The ODS1 stationary phase contains a high sodium content (1500 ppm; Myers, 2000) and so is likely to contain some highly acidic silanol sites, though these are estimated to represent less than 1% (Claessens *et al.*, 1998). Both types of silanol site will contribute to the retention of CQ on ODS1, and it appears likely that peak tailing is due mainly to the interaction of CQ with the more acidic silanol sites. CQ also exhibited tailing on Hypersil ODS (Figure 2.1c), which has fewer residual silanol groups than ODS1 due to endcapping. The peak tailing indicates some silanol activity, most likely caused by very acidic silanol groups. By contrast, CQ did not exhibit tailing on Hypersil BDS (Figure 2.1d). There was, however, a slight degree of peak broadening compared with the unretained species, indicating partial activity. The lower peak width on ODS1 compared with SCX is probably attributed to a combination of the smaller particle size and more spherical nature of the ODS1 stationary phase. In both cases, the columns were eluted at, or close to, their maximum flow rates (and optimal ES-MS conditions were used for the respective flow rates). Given the narrower peak width and lower asymmetry on ODS1 than on SCX, detection of lower concentrations of CQ should be possible due to the concentration dependence of the response in ES-MS (Kerbale and Ho, 1997). Furthermore, as A_s increases, it becomes more difficult

to detect the end of the peak, leading to increased uncertainty in peak area determination.

2.2.1.2 Limits of detection and quantification of CQ

Limits of detection (LODs) for CQ in pear matrix for both methods were measured using serial dilution from the standard of lowest concentration, 0.0016 μg mL^{-1}. LODs, based on 3 times signal to noise, were found to be *ca.* 20 times lower using ODS1 (0.04 ng mL^{-1}) than using SCX (1.0 ng mL^{-1}). This improvement is attributed mainly to the better peak shape, *i.e.* the narrower peak width on ODS1 than on SCX, and consequent increase in concentration at peak centre. The results compare favourably with the LOD (0.5 mg kg^{-1} ≡ 0.1 ng mL^{-1}) obtained independently on an SCX column following sample pre-treatment by solid phase extraction (SPE; Hau *et al.*, 2000). The LOD obtained on ODS1 is an order of magnitude better than that obtained previously using the same separation (Vahl *et al.*, 1998). This difference could be attributed to the use of different mass analysers for the detection of CQ: present results were obtained on an ion trap instrument, whereas the previous work (Vahl *et al.*, 1998) used a triple quadrupole instrument.

In this study the LODs for CQ in pear matrix on ODS1 for SIM are lower than the limit (0.1 ng mL^{-1}) for individual pesticides in drinking water set by a recent European Directive (Council Directive, 1998). Although other methods have achieved the required LOD they all involved a preconcentration step (Castro *et al.*, 1999, 2000). Due to the poor LOD achievable using existing methods, the analytical method for CQ is among those recommended for review by the UK Drinking Water Inspectorate (Rouse, 1997). The results presented here suggest that the ion trap LC-MS method operated under SIM conditions and using the ion exchange capacity of the Spherisorb ODS1 would be suitable for direct determination of CQ in water matrices including tap water. However, quantification should be performed using matrix matched calibration standards or by the method of standard additions. Measurements of LODs in tap water and deionised water confirm this to be the case (Table 2.3).

Matrix	Slope / mL μg^{-1}	LOD / pg mL^{-1}
Deionised water	9.6×10^{10}	0.1
Tap water	6.50×10^{10}	1.0
Pear extract	1.42×10^{7}	40

Table 2.3: LODs and slopes of SIM calibration graphs obtained for the analysis of CQ from various matrices on the ODS1 column using the method developed by Vahl *et al.* (1998).

The precision of the two LC methods was examined using solutions (50/50 methanol/water) containing 20 ng mL^{-1} of CQ. Better precision, reflected in lower relative standard deviations (RSDs), was obtained for SIM analysis on ODS1 (RSD = 5.2%; $n = 20$) than on SCX (RSD = 10%; $n = 20$).

Standard solutions of CQ in pear matrix (methanolic pear extract; see Startin *et al.*, 1999 for preparation of matrix) were analysed in random order using both separation methods. The calibration plot derived from SIM *m/z* 122 obtained using ODS1 (Figure 2.3a) reveals a greater linear dynamic range than with the SCX stationary phase (Figure 2.3b). This is due both to the lower limit of quantification and improved linearity at high concentrations on ODS1. The coefficient of determination (R^2) in the SIM linear regression calibration plot on the SCX column over the range 0.008 to 1.04 $\mu g\ mL^{-1}$ was found to be greater ($R^2 = 0.995$) than over the range 0.008 to 1.62 $\mu g\ mL^{-1}$ ($R^2 = 0.987$), indicating curvature in the calibration at high CQ concentrations. In contrast, the corresponding values obtained using the ODS1 column remained constant over the two ranges 0.008 to 1.04 $\mu g\ mL^{-1}$ and 0.008 to 1.62 $\mu g\ mL^{-1}$ ($R^2 = 0.997$ and 0.998 respectively). The calibration plots for standards of CQ prepared in deionised and tap waters obtained using the ODS1 column both revealed significantly greater slopes than the corresponding plot from pear matrix (Table 2.3), suggesting an influence from the matrix. This is borne out by the lower

LODs achievable in "cleaner" matrices such as water (Table 2.3). Thus, for routine monitoring of CQ in food matrices using the ODS1 method calibration standards should be matrix matched, so as to avoid errors in determining the actual CQ concentration. Further certainty could be achieved through the incorporation of an isotopically-labelled internal standard (*cf.* Hau *et al.*, 2000). For the purposes of routine monitoring, it is advantageous for calibration plots to be linear over a wide range and to extend to high concentrations. For example, quantification of CQ in foodstuffs may require accurate measurements over a wide dynamic range. Calibration that is linear over a wide range and extends to high concentrations removes the need for dilution of such samples and so eliminates one source of uncertainty in the measurement.

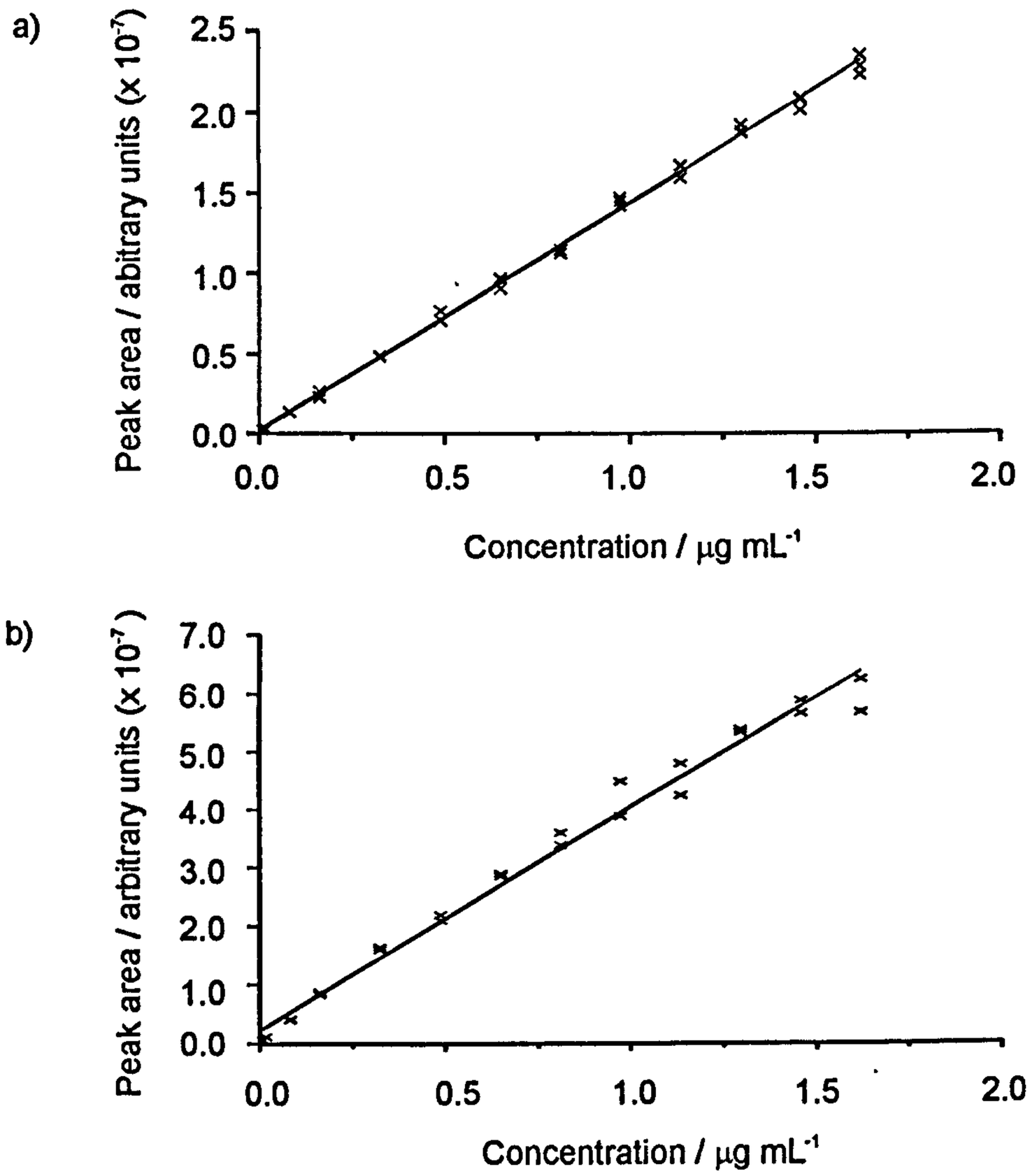


Figure 2.3: Calibration curves for the analysis of CQ in pear matrix using a) SIM *m/z* 122 on the ODS1 column [intercept (c) = 2.1 ± 0.7 x 10^5; slope (m, mL μg^{-1}) = 1.42 ± 0.01 x 10^7; R^2 = 0.998] and b) SIM *m/z* 122 on the SCX column [c = 2.3 ± 0.8 x 10^6; m, mL μg^{-1} = 3.8 ± 0.1 x 10^7; R^2 = 0.987].

2.2.2 Development of a generic LC-MS method for the analysis of the quats

2.2.2.1 Selection of stationary phase and preliminary development of a solvent programme

Previously, it was shown that CQ is retained on Spherisorb ODS1 through cation exchange interactions with free silanol sites present on the non-endcapped stationary phase (Section 2.2.1.1), as suggested by Vahl *et al.* (1998). Under the same conditions MQ and DF eluted together, later than CQ (t_R = 10 *vs.* 6.5 min). The dications PQ and DQ did not elute. Since the retention mechanism of the quats involves ion exchange, PQ and DQ would be expected to elute if the ionic strength of the mobile phase was increased. This approach was not employed here as earlier results (Section 2.2.1.1) indicate that even a small increase in ionic strength would cause CQ to elute at the dead time, as well as reducing retention of MQ and DF. To determine if ion exchange alone could be employed to develop a separation of all five quats, a Spherisorb silica column was operated using a mobile phase containing an appreciable aqueous content. The monocations CQ, MQ and DF eluted in under 12 min (t_R = 8.3, 6.1, 11.3 min, respectively) and PQ eluted as a broad tailing peak with t_R in excess of 40 min. DQ did not elute. Although doubling the ionic strength of the mobile phase caused PQ to elute earlier, peak shape was still poor and DQ was still retained. As expected the three monocations eluted earlier, close to the solvent front. Thus, under the conditions employed the five quats could not be separated on a normal silica column. In studies where PQ and DQ have been eluted from silica (Simon and Taylor, 1989; Chichila and Walters, 1991; Chichila and Gilvydis, 1993; Ibanez *et al.*, 1996a,b, 1997; Itagaki *et al.*, 1997) and endcapped C_{18} stationary phases (Yoshida *et al.*, 1993; Lee *et al.*, 1998; Takino *et al.*, 2000), ion-pairing reagents have been included in mobile phases with high aqueous contents. The results presented here were obtained using a four component mobile phase with 74% organic content (53 % acetonitrile and 21 % methanol), and support a previous study (Simon and Taylor, 1989) that reported that neither PQ nor DQ were significantly eluted from silica by either methanol or acetonitrile. Formation of ion-pairs between quat cations and added anions reduces the interaction of quat cations with residual

silanol groups present on the stationary phase. Thus, separation of the quats on the silica and endcapped C_{18} phases when using ion-pair reagents did not result from a cation exchange mechanism but from differences in the hydrophobicities of the ion-pairs. One of the aims of the present work was to reduce the dependence of the separation upon additives in the mobile phase, which can have a deleterious effect on ES-MS detection (Mirza and Chait, 1994).

The results above show that interactions of the dications with the silanol groups on Spherisorb silica are too strong to permit effective use of the stationary phase for separation of the quats. Modification of Spherisorb by attachment of cyanopropyl produces a stationary phase that is intermediate between ODS1 and silica. One of the reasons why this phase was chosen is that there are the possibilities of hydrogen bond interactions between protonated nitrile functionalities and free silanol anions or electron donation from nitrile functionalities to the sodium cations that cause silanol groups to become highly acidic (Section 2.2.1.1).

All five quats eluted from a non-endcapped cyano stationary phase in under 15 min (Table 2.4). The earlier elution of PQ and DQ from the cyano phase than from the silica phase is attributed to the reduced ion exchange capacity of this phase as a result of chemical modification of the surface silica, reducing the number and/or nature of available silanol sites. The peaks observed for the five quats were broad and tailing, as seen from W_h and the high A_s values (Table 2.4). Changing the salt and acid in the mobile phase to ammonium formate and formic acid reduced t_R, and generally resulted in narrower peak widths and better peak symmetry for the five quats (Table 2.4). Subsequent analyses all employed ammonium formate/formic acid as modifiers. Using this isocratic system, however, several of the quats coeluted (Table 2.4).

Salt/acid pairing	Quat	t_R / min	W_h / min	A_s
50 mM ammonium acetate / 1% acetic acid				
	MQ	4.3	0.2	1.1
	CQ	4.6	1.4	2.2
	DQ	13.5	1.5	4.3
	PQ	10.1	1.1	5.1
	DF	4.6	0.1	1.4
50 mM ammonium formate/ 1% formic acid				
	MQ	3.9	0.2	0.7
	CQ	3.7	0.4	1.2
	DQ	6.5	0.6	1.3
	PQ	5.9	0.7	1.2
	DF	4.0	0.2	0.7

Table 2.4: Retention times (t_R), peak width at half height (W_h) and peak asymmetry (A_s) for the five quats analysed on a cyano column (S5 cyano, 250 x 4.6 mm) using either ammonium acetate/ acetic acid or ammonium formate/ formic acid as mobile phase modifiers.

2.2.2.2 Application of a gradient programme

A solvent programme involving an initial isocratic stage, a salt gradient and another isocratic step (Method A, Table 2.5) was developed and allowed separation of the quats in under 20 min (Figure 2.4). Although the peaks are broad and tailing, separation of the principal pairs of analytes is observed. CQ (5.5 min) and MQ (8.3 min) are baseline resolved, whilst PQ (6.5 min) and DQ (6.9 min) are resolved to >

20% peak height (Figure 2.4). DF exhibits better peak width and greater retention than the other four analytes.

Method A				
Time / min	Flow rate / mL min^{-1}	Acetonitrile / %	B* / %	Water / %
0.0	1.0	5	6	89
5.8	1.0	5	6	89
6.0	1.0	10	6	84
12.0	1.0	10	10	80
12.5	1.0	70	20	10
19.0	1.0	70	20	10
19.5	1.0	5	6	89
23.0	1.0	5	6	89
Method B				
0.0	0.6	5	6	59
5.8	0.6	5	6	89
6.0	0.6	10	6	84
10.0	0.6	10	10	80
10.5	0.6	80	20	0
18.0	0.6	80	20	0
18.5	0.6	5	6	89
20.0	0.6	5	6	89

Table 2.5: Solvent programmes used for the elution of the quats from cyano phase HPLC columns; * Solvent B is 200 mM ammonium formate in water containing 5% formic acid, which has a pH of ~3.5.

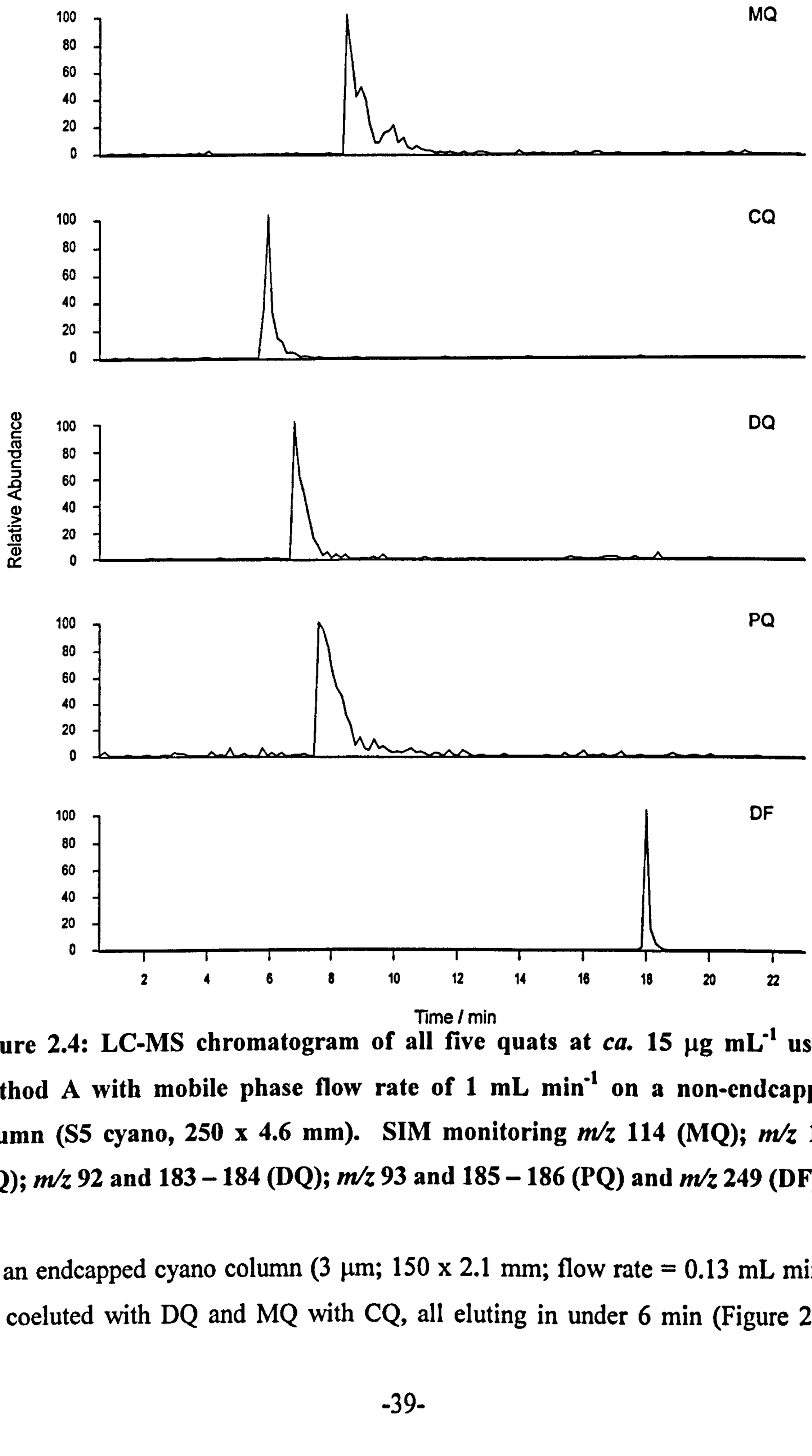


Figure 2.4: LC-MS chromatogram of all five quats at *ca.* 15 μg mL^{-1} using Method A with mobile phase flow rate of 1 mL min^{-1} on a non-endcapped column (S5 cyano, 250 x 4.6 mm). SIM monitoring *m/z* 114 (MQ); *m/z* 122 (CQ); *m/z* 92 and 183 – 184 (DQ); *m/z* 93 and 185 – 186 (PQ) and *m/z* 249 (DF).

On an endcapped cyano column (3 μm; 150 x 2.1 mm; flow rate = 0.13 mL min^{-1}) PQ coeluted with DQ and MQ with CQ, all eluting in under 6 min (Figure 2.5),

demonstrating the role of silanol sites in the retention mechanism (*cf.* ODS1). The peaks observed for CQ and MQ are flat-topped and suggest overloading. The t_R of DF (*ca.* 20 min) is similar to that achieved on the non-endcapped cyano phase, suggesting that DF associates with the cyano phase mainly through hydrophobic interactions. Improvement in chromatographic peak shape for all of the quats, except DF, was achieved using a non-endcapped cyano phase with 3 μm particle size (flow rate = 0.6 mL min^{-1}) and this column (S3 cyano, 150 x 4.6 mm) was used for all subsequent analyses.

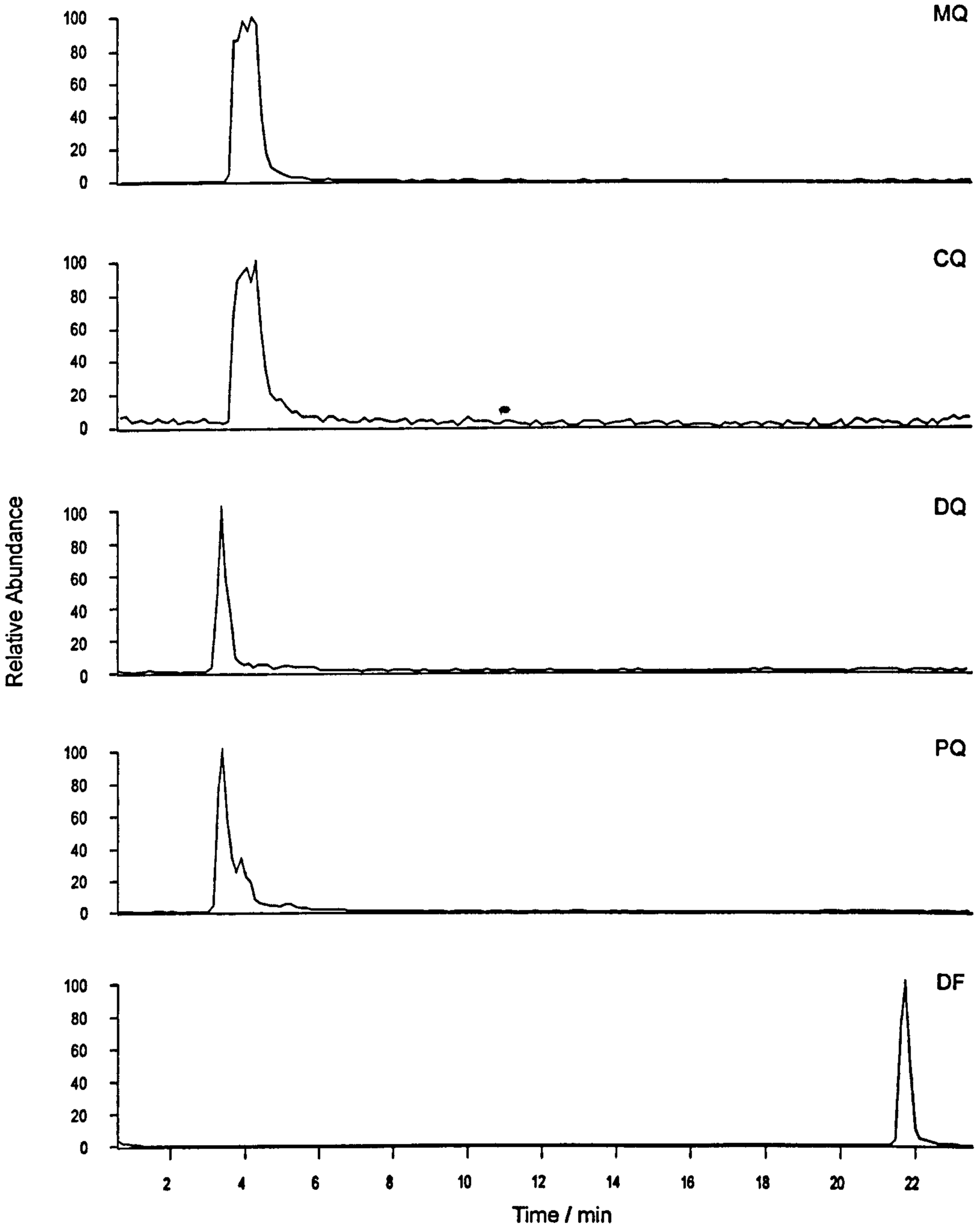


Figure 2.5: LC-MS chromatogram of all five quats at ca. 25 μg mL^{-1} using Method A with a mobile phase flow rate of 0.13 mL min^{-1} on an endcapped cyano column (ACE 3 CN, 150 x 4.6 mm). Mass spectrometric detection as described in Figure 2.4.

In order to elute DF at a t_R closer to the other four quats the mobile phase was modified. The initial isocratic step, in which 5% acetonitrile had been found to give

the best peak shape for CQ, DQ and PQ (t_R = 5.2, 5.5 and 6.0 min, respectively; data not presented) was maintained. Following elution of PQ the effective salt concentration was increased linearly from 12 to 20 mM to effect earlier elution of MQ. The acetonitrile content was then increased in a step to 80% to effect earlier elution of DF with improved peak shape. This is in good agreement with a recent study (Castro *et al.*, 1999), which reported that DF required a higher elution strength than the other four quats, *i.e.* a higher percentage of organic modifier in the mobile phase.

Method B utilises the ion exchange capacity of the residual silanol sites available on the non-endcapped cyano phase and achieves separation of the principal pairs of analytes, CQ and MQ and PQ and DQ. Because DF associates with the cyano phase predominantly through hydrophobic interactions, it can be well separated from he other four quats (Figure 2.6). Although peak tailing is evident, especially for PQ and DQ, this method can be used to monitor all five quats simultaneously or individually.

2.2.2.3 Quantification of the five quats

Calibration standards of CQ, MQ, DQ, PQ and DF in deionised and tap water were analysed in a random order using Method B. The column and method provided reproducible retention times in over 600 analytical runs with no obvious degradation in performance. The calibration plots obtained for the SIM analyses of standards prepared in deionised water had similar slopes to those obtained for the standards prepared in tap water (Table 2.6). As a result, similar LODs for the five quats were achievable in both deionised and tap water, reflecting the limited influence of matrix on the calibration. The higher LODs reported for PQ and DQ are attributed to the higher peak asymmetry observed for these peaks, which probably arises through interaction of the quat dication with highly acidic silanol sites. By contrast, the much lower LOD achieved for DF, compared with the other quats, is probably a result of its better chromatographic peak shape and narrower peak width (Figure 2.6).

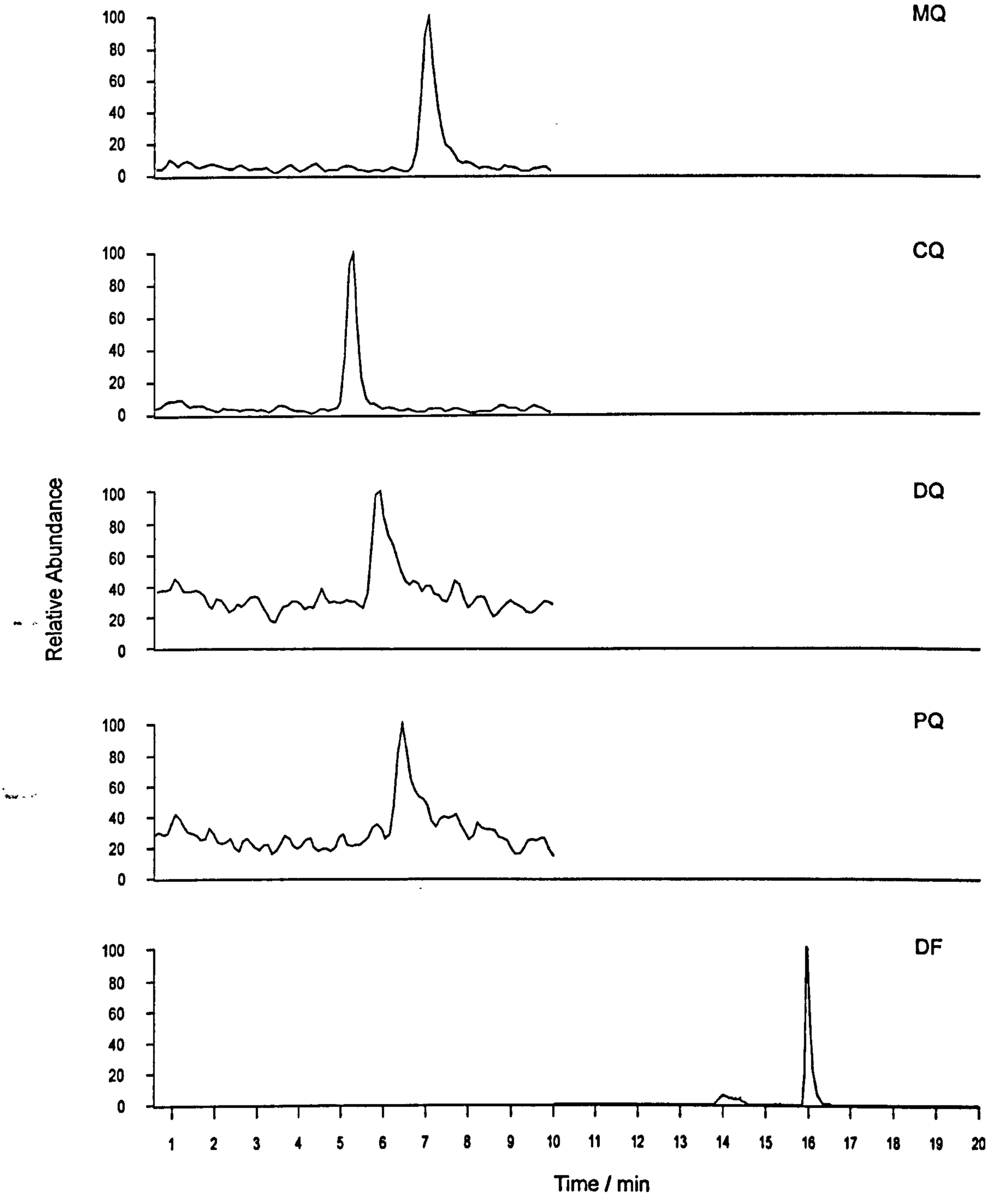


Figure 2.6: LC-MS chromatogram for the five quats at various concentrations [50 ng mL^{-1} (CQ, MQ); 100 ng mL^{-1} (DF); 500 ng mL^{-1} (DQ, PQ)] obtained using Method B on a non-endcapped cyano column (S3 cyano, 150 x 4.6 mm). Mass spectrometric detection as described in Figure 2.4.

While this work was in progress, two ion-pair LC-MS multi-residue methods for the analysis of the quats were published (Castro *et al.*, 1999, 2000). Although these methods have similar run times (*ca.* 20 min), separation and peak shapes to those observed with Method B, their LODs without preconcentration for the dictations DQ and PQ were lower (6 ng mL^{-1} and 11 ng mL^{-1} respectively; Castro *et al.*, 2000) than those obtained in Method B (87 and 2450 ng mL^{-1}, respectively). Similarly, lower LODs were obtained previously (Castro *et al.*, 2000) for the monocations, CQ and MQ, 0.9 and 0.4 ng mL^{-1}, respectively, than obtained with Method B, 2.3 and 4.7 ng mL^{-1}, respectively (Table 2.6). However, the LOD obtained for DF using Method B (0.02 ng mL^{-1}) was significantly lower than that obtained by Castro *et al.* (2000; 1 ng ml^{-1}) probably as a result of the higher organic content in the mobile phase at the time of elution of DF (80% Method B *vs.* ~ 45% Castro *et al.*, 2000). Nevertheless the LODs achieved with Method B are likely to be suitable for the routine monitoring of the quats in foodstuffs or in formulation products.

Analysis of matrix-matched calibration standards for the determination of CQ in pear revealed a linear calibration over three orders of magnitude. The slope of the calibration plot in pear matrix is similar to the slopes obtained for CQ in deionised and tap water (Table 2.6), confirming the limited influence of matrix upon the calibration. This contrasts with earlier work performed on an ODS1 column (Section 2.2.1.2), where the matrix was seen to exert a large influence on the slope, and hence, the LOD achievable. As a result of the limited influence of matrix on the calibrations, the LOD for the analysis for CQ in pear (1.7 ng mL^{-1}) is comparable to values in both deionised and tap water (Table 2.6). It is, however, 40 fold greater than that obtained previously on an ODS1 column (0.04 ng mL^{-1}) even though similar peak widths (W_h = 0.3 min for both ODS1 and cyano columns) and peak asymmetry values (A_s = 1.83 on ODS1 *cf.* 1.7 on cyano) were obtained for both columns. The principal difference between the two methods is believed to be the eluent composition at the ES needle. As CQ elutes from the ODS1 column the eluent contains 74% organic solvent, whereas that from the cyano column contains only 5% organic solvent. The surface tension of the eluent droplets formed at the end of the ES needle is reduced by increasing the organic content of the eluent

relative to the aqueous phase, resulting in increased eluent volatilisation. Therefore, transmission of the analyte into the gas phase occurs more readily with eluent compositions containing a greater percentage of organic phase. This mass spectrometric dependence on the eluent composition could be responsible for the observed differences in the LODs achievable for these two methods. Although the LOD (1.7 ng mL^{-1} equivalent to 0.009 mg kg^{-1}) obtained on the cyano column is greater than that reported previously on an ODS1 column, it is below the UK maximum residue level set at 3 mg kg^{-1} of CQ in pears (Statutory Instrument, 1997). Thus, the method achieves LODs sufficient for monitoring CQ in food matrices and could be applied for regulatory purposes.

Quat	Deionised water				Tap water				Pear matrix			
	Slope / 10^{-4} mL ng^{-1}	Intercept / 10^{-6}	R^2	LOD / ng mL^{-1}	Slope / 10^{-4} mL ng^{-1}	Intercept / 10^{-6}	R^2	LOD / ng mL^{-1}	Slope/ 10^{-4} mL ng^{-1}	Intercept / 10^{-6}	R^2	LOD / ng mL^{-1}
MQ	14.1 ± 0.6	-4.8 ± 6.2	0.968	4.7	17.6 ± 0.4	0.2 ± 1.8	0.990	2.8	-	-	-	-
CQ	9.6 ± 0.3	1.0 ± 3.5	0.978	2.3	11.2 ± 0.3	2.6 ± 1.6	0.989	1.7	8.8 ± 0.4	9.9 ± 4.0	0.972	1.7
DQ	2.8 ± 0.3	-2.6 ± 3.6	0.912	87	4.3 ± 0.4	3.5 ± 1.7	0.944	84	-	-	-	-
PQ	3.3 ± 0.5	-10.6 ± 6.4	0.894	240	4.6 ± 0.4	4.0 ± 1.9	0.947	100	-	-	-	-
DF	32.5 ± 0.4	-3.0 ± 3.0	0.994	0.02	32.9 ± 0.7	10.6 ± 4.9	0.984	0.02	-	-	-	-

Table 2.6: Calibration data for the quats obtained using Method B on a non-endcapped cyano column (S3 cyano, 150 x 4.6 mm).

2.2.3 Development of a CE-MS method for the analysis of the quats

2.2.3.1 Analytical set-up for CE-MS

Unlike CE-UV, where detection is performed on-column, CE with detection by MS requires the transport, vaporisation and ionisation of the analytes as they migrate from the end of the fused silica CE capillary (Belder and Stockigt, 1995). Hence, modification of the standard CE apparatus was required in order to develop a CE-MS method for the analysis of the quats.

The arrangement used in this work was an electrospray source with a coaxial sheath liquid flow interface (Figure 2.7). The end of the fused silica CE capillary was inserted into a stainless steel needle with an inner diameter that closely matched the external diameter of the CE capillary. A liquid sheath is supplied through the needle around the last section of the CE capillary, where it mixes with the capillary eluent and an electrospray is generated through application of the ES spray voltage to the stainless steel needle (Figure 2.7; McClean *et al.*, 2000). The sheath liquid or make-up flow performs two main tasks: firstly, it provides electrical contact at the end of the CE capillary, thus allowing electrophoresis to occur (Tsai and Her, 1996; McClean *et al.*, 2000); secondly, it supplements the flow leaving the CE capillary (nL min^{-1}) to a level suitable for electrospray generation (μL min^{-1}; Tsai and Her, 1996; Wycherley *et al.*, 1996).

It is known that the relative positions of the fused silica capillary and the stainless steel ES needle greatly affect detection sensitivity using the CE-ES-MS interface (Tsai and Her, 1996). Often, best sensitivity is obtained with the CE capillary protruding beyond the tip of the ES needle (Tsai and Her, 1996; Lazar and Lee, 1999). If the CE capillary protrudes too far electrical contact will be lost (Tsai and Her, 1996), whilst if the CE capillary is withdrawn inside the ES needle separation efficiency can deteriorate or signal may be lost completely (Lazar and Lee, 1999). Since the position of the CE capillary relative to the ES needle is critical, its position can be adjusted with the help of a micrometer attached to the ES source (Figure 2.7).

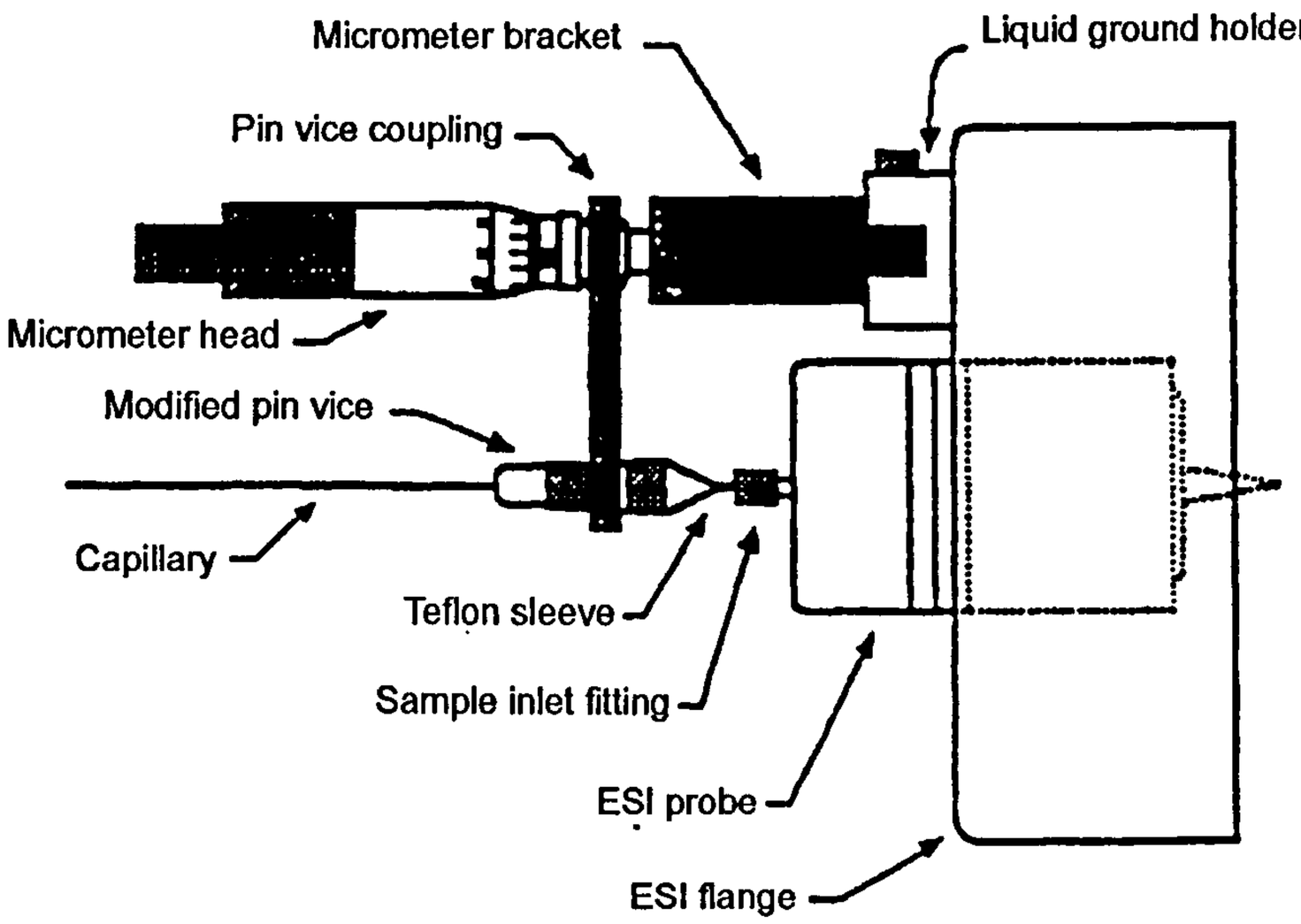


Figure 2.7:Modified ES probe similar to that on the LCQ mass spectrometer showing the micrometer attachment for capillary position adjustment [From McClean *et al.*, 2000].

An increase in the sensitivity achievable for the analysis of 1,4 benzodiazepines by CE-MS has recently been reported (McClean *et al.*, 2000). This improvement was, in part, attributed to tapering the end of the fused silica CE capillary. Since the analysis of the quats in food, environmental and water matrices requires high sensitivities in detection, the capillary used in this work was tapered 1 cm from the end that enters the ES source. Tapering the capillary has been described previously (McClean *et al.*, 2000), and was carried out by drawing out the end of the capillary in a Bunsen burner flame. The end was then trimmed to remove any blockages which may have occurred due to heat-sealing of the tip. Although this tapering procedure is quick and simple, it should be noted that the dimensions of the orifice at the end of the capillary might vary slightly from capillary to capillary (McClean et al., 2000). Positioning the tapered CE capillary relative to the ES needle, with the aid of the micrometer, was optimised using a procedure similar to that described previously by McClean *et al.*, (2000). The optimum position (0.5 mm protruding) resulted in a stable mass spectrometric signal. It should be noted that when a flat-ended capillary

(untapered) was used periodic loss of the mass spectrometric signal occurred, and so prevented continuous MS detection during the CE separation.

Most of the commonly used buffer systems for CE involve aqueous electrolytes with non-volatile buffer components, *e.g.* sodium phosphate and sodium borate (Belder and Stockigt, 1996). These buffers cannot be used in CE-MS systems, since buffer components must be volatile as in the case of LC-MS. Involatile buffers, such as phosphate and borate salts, can cause an accumulation of residue on the MS orifice and ion optics. If accumulation occurs, increased background signals and a decrease in sensitivity are likely to result (Belder and Stockigt, 1996). In the present study the run buffer comprised 50 mM aqueous ammonium acetate/acetic acid at pH 4.7. As recommended previously by Moyano *et al.*, (1996) a low pH less than 5.5 is required to minimise the ionisation of the silanol groups and hence reduce the electrostatic interactions between the quat cations and the negatively charged surface of the fused silica capillary wall.

A sheath liquid was used in order to complete the electrical contact and supplement the CE flow to transport the migrating quats from the end of the capillary into the electrospray (Johnson *et al.*, 1996; McClean *et al.*, 2000). Since the run buffer was prepared in water the surface tension of the eluent at the end of the capillary was reduced by infusion of a sheath liquid make-up flow comprising 1% acetic acid in methanol. Since both the make-up flow and the CE run buffer have the same counterion, problems associated with moving ionic boundaries, *e.g.* variations in migration time and fluctuating capillary current, were not evident (Lazar and Lee, 1999). A liquid sheath flow of 2.5 $\mu L\ min^{-1}$ was found to be optimal for maintaining stable mass spectrometric signal. To minimise any possible distortion of the low liquid flow, and hence the electrospray (McClean *et al.*, 2000), a low sheath gas flow (5 arbitrary units) was used.

In these studies hydrodynamic pressure injection was employed, since this injection mode is expected to introduce an aliquot of sample representative of the original sample (Foret *et al.*, 1993). Unlike electrokinetic injection, hydrodynamic injection

does not suffer from injection bias caused by highly-charged analytes entering the capillary faster than weakly-charged analytes (Wycherley *et al.*, 1996). After injection of the sample the separation potential (30 kV) was applied. It should be noted that for the first 30 s of the analytical run the ES spray voltage was set to zero, preventing reverse electroosmosis in the direction of the CE electrode in the input vial. If reverse electroosmosis were to occur the integrity of the sample injection could be affected (McCLean *et al.*, 2000) resulting in misrepresentation of the sample. After 30 s the ES spray was set to 3.6 kV, resulting in an overall separation potential of 26.4 kV (30.0-3.6 kV).

2.2.3.2 CE-MS of the quats

An example of an electropherogram (Figure 2.8) obtained using the method described above shows that the quats migrate from the capillary in under 10 min. The separation achieved is very similar to that observed previously for CE-MS (Moyano *et al.*, 1996), but the analysis time was shorter, suggesting the potential for higher sample throughput. As expected, the dications (PQ and DQ) migrate before the monocations (CQ, MQ and DF). Because of the very small differences in their charge to size ratios very little discrimination in the migration times is observed between PQ and DQ. A slight separation of MQ and CQ is apparent with CQ migrating before MQ, as observed previously (Moyano *et al.*, 1996; Galceran *et al.*, 1997). DF migrates later than the other four quats due to its smaller charge to size ratio. Good peak shape is observed for both CQ and MQ, whereas peak tailing is evident for DQ, PQ and DF. The extensive tailing observed for PQ, DQ and DF is attributed to interaction between the quat dications with silanol groups on the capillary wall.

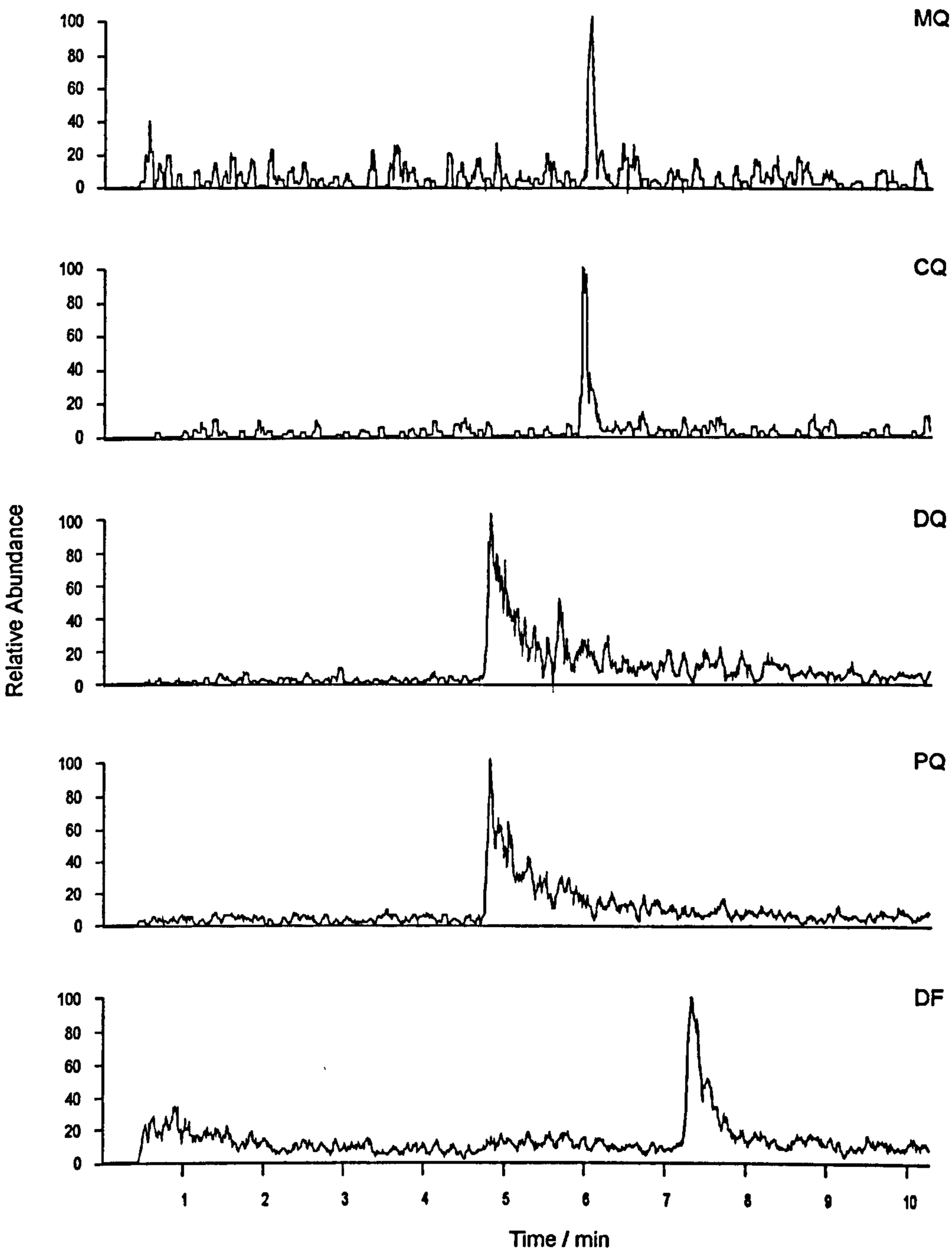


Figure 2.8: A CE-MS electropherogram for the five quats at various concentrations [0.25 μg mL^{-1} (CQ, MQ); 0.5 μg mL^{-1} (DF); 40 μg mL^{-1} (DQ, PQ)] (see text for experimental conditions).

Small linear dynamic ranges were observed for CE-MS analysis of the quats (Table 2.7). The poor linearity observed for PQ and DQ is attributed to the high peak

asymmetry observed for these analytes, leading to difficulties in peak area determination. In addition, the poor peak symmetry results in higher LODs for these dications (Table 2.8). Conversely, lower LODs and wider linear dynamic ranges, up to two orders of magnitude, were observed for MQ and CQ, and result from the near symmetrical peak shape observed for these analytes. The LOD achieved in the present study for CQ (61 ng mL^{-1}) was less than that achieved in a previous study (500 ng mL^{-1}; Wycherley *et al.*, 1996). In another multi-residue CE-MS method for the analysis of the five quats in water (Moyano *et al.*, 1996), poor peak shape and narrow linear dynamic ranges were also observed. Even though similar electrophoretic-mass spectrometric conditions were employed in both methods, the earlier method achieved lower detection limits, for example, for CQ 0.8 ng mL^{-1} (Moyano *et al.*, 1996) *vs.* 61 ng mL^{-1} (Table 2.8).

Quat	Calibration level/ $\mu g\ mL^{-1}$	Slope / 10^{-6} mL μg^{-1}	Intercept / 10^{-5}	R^2
MQ	0.02 to 3.8	2.8 ± 0.16	-8.1 ± 3.2	0.968
CQ	0.08 to 8.1	1.0 ± 0.07	7.7 ± 2.4	0.926
DQ	13 to 50	3.6 ± 0.13	110± 45	0.505
PQ	3.6 to 36	0.33 ± 0.01	38 ± 24	0.717
DF	0.17 to 3.4	36 ± 0.42	5.7 ± 7.8	0.999

Table 2.7: CE-MS calibration data for the five quats.

2.2.4 Comparison of the LC-MS method with a CE-MS method

Comparison of the CE-MS method with the LC-MS method described earlier reveals that although the run time in the LC-MS method is twice as long as that in the CE-MS method (20 min *vs.* 10 min), LC-MS achieves better separation of the principle pairs of analytes, *i.e.* of DQ/PQ and CQ/MQ. In addition, better peak shape is generally observed for the quats using the LC-MS method. Calibrations performed

for the five quats revealed that small linear dynamic ranges were obtained with CE-MS contrasting with the wide linear dynamic ranges exhibited in LC-MS. Concentration LODs in CE-MS are much higher than those in the LC-MS (Table 2.8). However, lower LODs were obtained for CE-MS than LC-MS in terms of number of moles injected on column. Thus, although lower mass detection limits are achievable with CE-MS, the method detection limit is greater than LC-MS because more concentrated samples have to be analysed.

Quat	CE-MS		LC-MS	
	Concentration LOD / ng mL^{-1}	Mass LOD / 10^{-15} mol	Concentration LOD / ng mL^{-1}	Mass LOD / 10^{-15} mol
MQ	130	0.06	4.7	820
CQ	61	0.03	2.3	380
DQ	18000	4.8	87	9400
PQ	25000	6.8	240	26100
DF	220	0.04	0.02	1.6

Table 2.8: Comparison of LODs obtained for the five quats in deionised water using CE-MS and LC-MS.

2.3 CONCLUSIONS

The work presented here has established that separation of CQ on a non-endcapped C_{18} stationary phase (Spherisorb ODS1) results from ion exchange processes involving residual silanol sites. Use of an ODS1 column resulted in improvements in the chromatographic analysis of CQ over that obtained on a standard cation exchange column. In particular, shorter analysis time, narrower peak width, better peak symmetry, lower LOD and wider linear dynamic range were achieved. The method using an ODS1 column was shown to achieve the low detection limits required for direct analysis of CQ in drinking water on an ion trap MS instrument. Thus,

separation of CQ on ODS1 combined with MS detection on an ion trap instrument allows direct quantification at levels lower than those required for residue analysis in food and drinking water.

Knowledge of the importance of ion exchange processes on the ODS1 stationary phase has proved to be of enormous value for developing a generic LC method for the separation of all five quats. The retention of the quats was investigated on a variety of silica-based stationary phases, and a non-endcapped cyano phase was found to permit elution of all five quats under isocratic conditions. A gradient elution programme for the five quats was subsequently developed for use on the cyano column. Four of the quats (CQ, MQ, DQ and PQ) were found to be retained *via* ionic interactions with residual silanol groups and were eluted *via* cation exchange with ammonium ions. The fifth quat, DF, was retained with a significantly greater t_R than the other four quats through hydrophobic interactions with the cyanopropyl chain, and elution was effected through an increase in eluent strength by increasing the organic modifier concentration.

Linear calibrations over several orders of magnitude were achieved for standards prepared in deionised water, tap water and pear matrix (CQ only) using the full LC-MS method. Similar LODs were achieved in all three matrices, showing that the method was not affected by the nature of the matrix.

A CE-MS method was also developed. This gave a shorter run time, but allowed linear calibrations only over a narrow concentration range. Although, lower mass detection limits were achieved for CE-MS, *e.g.* for CQ 0.03 x 10^{-15} moles *vs.* 380 x 10^{-15} moles for LC-MS, overall method LODs were greater, *e.g.* for CQ 0.61 *vs.* 2.3 for LC-MS. A preconcentration step would be required in order to apply the CE-MS method for routine monitoring purposes.

Application of the LC-MS method for the analysis of foodstuffs was performed for the determination of CQ in pear. A wide linear dynamic range and an LOD (0.009 mg kg^{-1}) well below the UK maximum residue level (3 mg kg^{-1}) were achieved.

Thus, this LC-MS method using a non-endcapped cyano phase column could be used for monitoring quat pesticide levels in foodstuffs with increased specificity achievable with MS/MS detection. In addition, the generic LC method can be used for the analysis of all five quats within one acquisition. Alternatively, since DQ, PQ and CQ elute under isocratic conditions, adaptation of the method (using only the isocratic stage) would allow analysis of these three quats in a straightforward manner. This would allow the time taken to analyse several samples to be substantially reduced.

Chapter 3

Optimisation of the MS Response in an Ion Trap Mass Spectrometer

Chapter 3: Optimisation of the MS Response in an Ion Trap Mass Spectrometer

3.1 INTRODUCTION

3.1.1 Ion trap mass spectrometry

3.1.1.1 Development of ion trap mass spectrometry

Increased specificity in detection can be achieved through incorporation of tandem mass spectrometric (MS/MS) detection within a liquid chromatography-mass spectrometry (LC-MS) method. One mass analyser capable of performing MS/MS experiments is the ion trap.

The ion trap was first described by Paul and Steinwedel (1956a,b; 1960) in the 1950s and the first commercial instrument was launched in 1983 (Kelley *et al.*, 1985; 1986). During that same year a new mode of operation called mass selective instability mode (Section 3.1.1.3) was developed (Stafford *et al.*, 1984; Kelley *et al.*, 1985), enabling trapped ions to be sequentially ejected toward the detector in increasing *m/z* value (Creaser and Stygall, 1998). The use of mass selective instability mode allowed the acquisition of the entire mass spectrum (Creaser and Stygall, 1998). The development of mass selective instability mode was followed by further enhancements to the instrumentation. These included high resolution (Kaiser, 1990; Schwartz *et al.*, 1991; Londry *et al.*, 1993), extended mass range (Kaiser *et al.*, 1991) and injection of ions formed externally (Louris *et al.*, 1989) from ion sources including APCI (Taylor *et al.*, 1995) and ES (Van Berkel, 1990; Mordehai *et al.*, 1992). These advances, coupled with sophisticated computer control (Bier and Schwartz, 1997) and the ability of the ion trap mass spectrometer to perform multiple stages of mass spectrometry (MS^n), have seen an increase in the use of ion trap mass spectrometers within analytical laboratories over recent years. Furthermore, the performance characteristics of ion trap mass spectrometers, combined with their low cost compared to triple quadrupole instruments and widespread availability, makes them an attractive option for the routine detection and determination of organic residues including components in environmental and food matrices.

3.1.1.2 Structure of an ion trap

An ion trap consists of three cylindrically symmetrical electrodes, two end caps and a ring (Figure 3.1). The three electrodes form a cavity in which mass spectrometric analysis occurs (Heather, 1997). The ring electrode is sandwiched between the two end-cap electrodes at a precise distance, typically maintained by ceramic or quartz spacers (Bier and Schwartz, 1997; Heather, 1997). In order to produce an ideal quadrupolar field r_o^2 is equal to $2z_o^2$, where r_o is the radius of the ring electrode and $2z_o$ is the distance between the end-cap electrodes (March, 1992, 1997, 1998; Jonscher and Yates, 1997). In theory, the electrodes (Figure 3.1) extend to infinity to meet the asymptotes shown. For practical purposes, however, the electrodes are truncated (March, 1997).

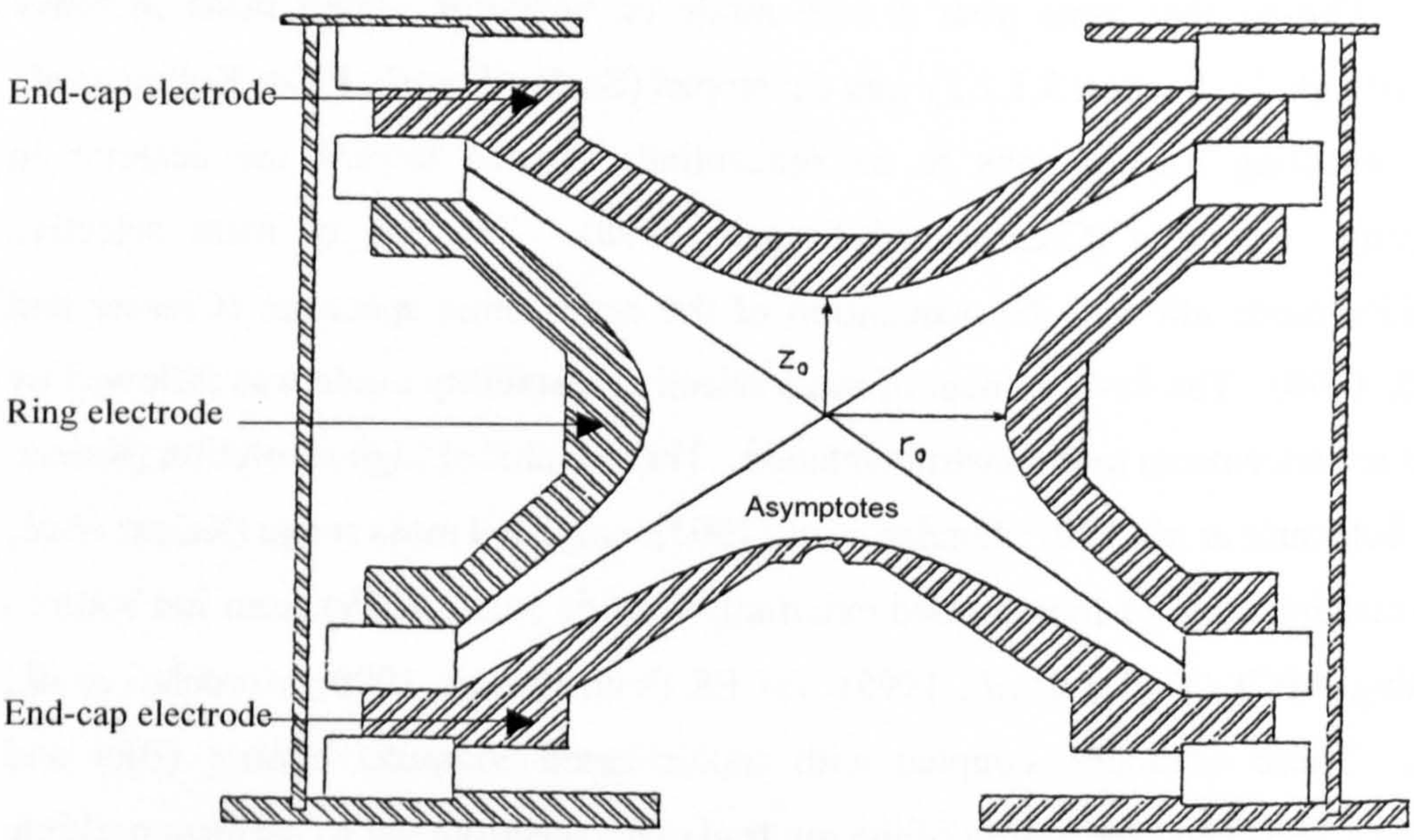


Figure 3.1: Schematic of the ion trap (modified from March, 1997).

Truncating the end-cap electrodes in a trap of normal geometry affects the field, which traps the ions (March, 1997, 1998, 2000). In addition to the pure quadrupole field, higher multipole fields are also present and their presence is attributed to formation of field imperfections resulting from the entrance and exit holes in the end-cap electrodes (Bier and Schwartz, 1997). These higher multipole fields result in

poor mass accuracy and so deviations between the expected and recorded *m/z* values of ions are observed (Bier and Schwartz, 1997). In order to overcome the problem of mass shifts the stretched geometry ion trap was developed by Finnigan (Syka, 1995). Subsequent instruments developed by Finnigan have utilised this technology, for example the LCQ (Figure 3.2). The geometry of the LCQ has been stretched by ~ 57%, with z_o increased to 0.785 cm while r_o remains at 0.707 cm (March, 1997). It should be noted that because the shape of the electrodes were not modified, the system does not achieve pure quadrupolar geometry.

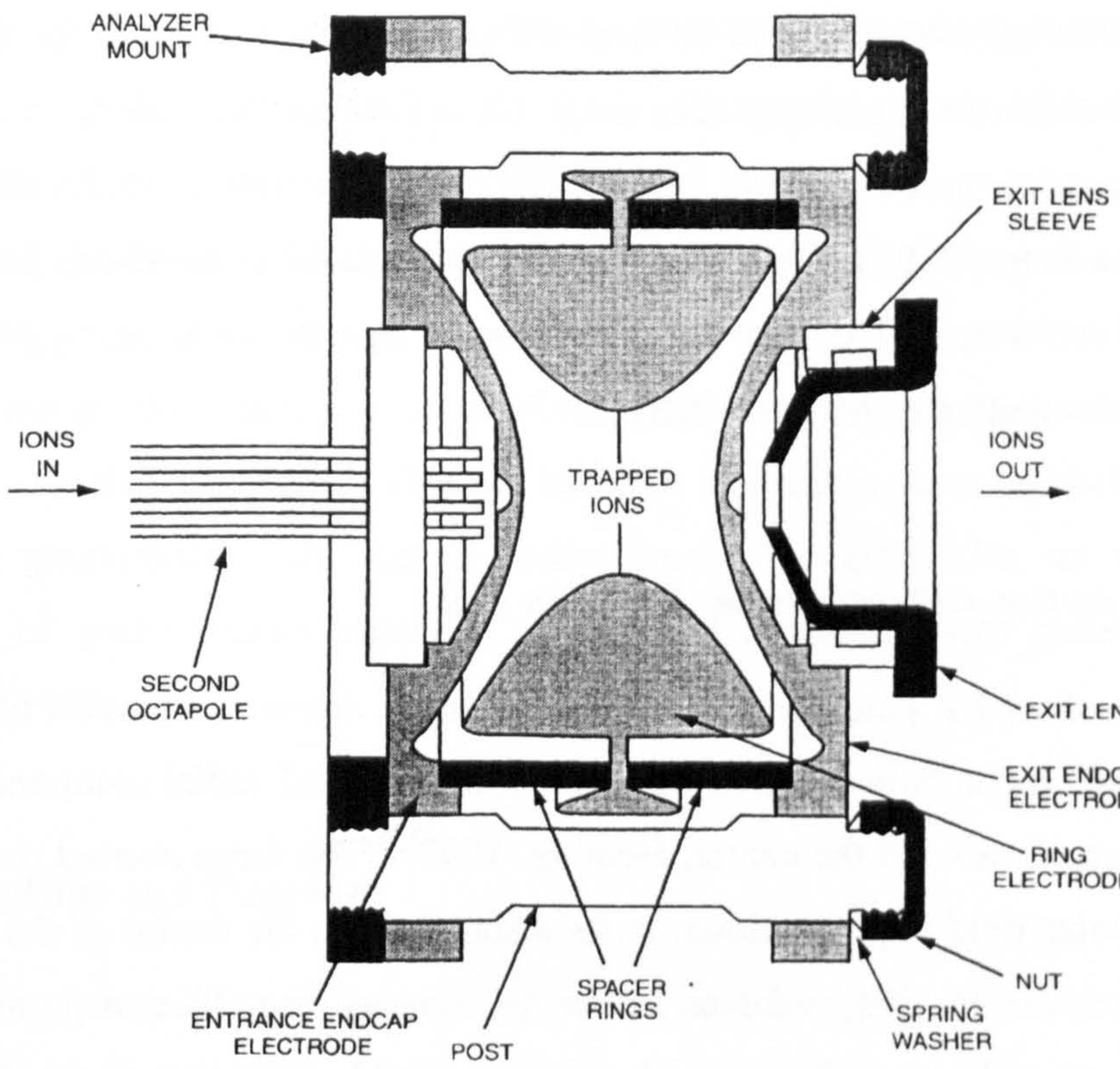


Figure 3.2: Schematic of the stretched geometry Finnigan LCQ ion trap (taken from Heather, 1997).

3.1.1.2 Basic operation of an ion trap mass spectrometer

Application of a small DC offset (-10 V) to all three electrodes allows externally produced ions to be introduced into the trap through perforations in an end-cap

electrode (Bier and Schwartz, 1997; Heather, 1997). Application of an oscillating RF potential (an AC voltage of constant frequency, 760 kHz, and variable amplitude) to the ring electrode produces the quadrupolar field. The field traps ions in stable orbits by continuously forcing them toward the centre of the trap (Heather, 1997). This trapping is aided by the presence of a small amount of helium buffer gas, typically at a pressure of 1 mTorr (Allison and Stepnowski, 1987; Jonscher and Yates, 1997; Heather, 1997). On collision with buffer gas, the kinetic energy (KE) of the ions is reduced to 0.1 eV (collisional focussing) and their deviation from the centre of the trap is reduced (March, 1997, 1998, 2000). Ramping of the amplitude of the RF potential causes ions to be ejected from the trap in order of increasing *m/z* value (mass selective instability scanning). The ions can be scanned out through either end-cap electrode (Louris *et al.*, 1987; March, 1992, 1997, 1998, 2000; Jonscher and Yates, 1997). Only those leaving through the exit end-cap are focussed toward the detector, and the subsequent detector signals are used to generate the resulting mass spectrum (March, 1998).

3.1.1.3 Ion motion and trajectories in an ion trap

Application of an RF potential to the ring electrode drives the motion of ions in a trajectory which has both axial (toward the end-caps) and radial components (from the ring electrode toward the centre; Heather, 1997). The force exerted on an ion by the quadrupolar field is proportional to its distance from the centre of the trap (Bier and Schwartz, 1997). Trapped ions follow trajectories resembling a figure of eight. Their motions can be described according to their axial and radial secular frequencies which are inversely proportional to their *m/z* values (March, 1997). Thus, ions with high *m/z* values follow larger trajectories than ions with smaller *m/z* values. Their trajectories can be expressed mathematically by second order linear differential equations described originally by Mathieu in 1868. Solutions to the Mathieu equations, a_z and q_z (Equations 3.1 and 3.2) are known as the trapping parameters (March, 1998).

$$a_z = -\frac{16eU}{m(r_o^2 + 2z_o^2)\Omega^2}$$

Equation 3.1

$$q_z = \frac{8eV}{m(r_o^2 + 2z_o^2)\Omega^2}$$

Equation 3.2

The equations show that the stability of the trajectory depends upon the *m/z* value (*m*), the size of the ion trap (r_o and z_o), the oscillating frequency of the RF voltage (Ω) and the amplitude of the applied DC (*U*) and RF (*V*) voltages. Since Ω, r_o, and z_o are fixed, the values of a_z and q_z for a given ion (*m*) are governed mainly by the DC and RF potentials, U and *V*, respectively (Equations 3.1 and 3.2). It should be noted, however, that there is a slight divergence in the trajectory predicted by the equations and that observed. This deviation in ion motion can be attributed to an imperfect quadrupolar field due to truncation of the electrodes, as well as the influence of space charge repulsion effects and collisions with gases (Bier and Schwartz, 1997).

3.1.1.4 Stability and Trapping

The trapping parameters define regions of ion trajectory that are stable or unstable (Jonscher and Yates, 1997; March, 1997). These regions can be portrayed by a stability diagram (Figure 3.3), which is formed by plotting and overlapping the solutions to the Mathieu equations in *a-q* space for the *r* and *z* dimensions (Bier and Schwartz, 1997). The coordinates of the stability diagram are the trapping parameters a_z and q_z. Ions can be stored within the trap provided that their radial and axial trajectories are stable simultaneously, *i.e.* their (a_z-q_z) pairing falls within the stability region shown (March, 1997). Outside this region the trajectory is unstable regardless of the initial velocity and position. The boundaries of the stability

diagram represent the conditions at which the trajectory of an ion becomes unstable and the ion is lost from the trap (March and Londry, 1995).

Since q_z is inversely proportional to the *m/z* ratio (*m* in Equation 3.2), high *m/z* ratios have low q_z values and low *m/z* ratios have high q_z values (Figure 3.3). Thus, ions of relatively high *m/z* values have q_z values near the origin. In most mass spectrometers the operating scan line is at $a_z = 0$. This represents the (a_z-q_z) pairings in the stability diagram that ions pass through until they are scanned out of the trap at $q_z = 0.908$, by mass selective instability scanning (Bier and Schwartz, 1997). Increasing the RF amplitude (*V*) applied to the ring electrode at a constant rate, corresponding to approximately 5,500 Th s^{-1} (Heather, 1997), causes the ions positioned along the operating line (Figure 3.3) to move to higher q_z values. Thus their trajectories become successively unstable in the axial direction. On reaching the stability boundary the ions are ejected from the trap. The voltage at which an ion is ejected from the ion trap is known as its resonance excitation voltage (Heather, 1997).

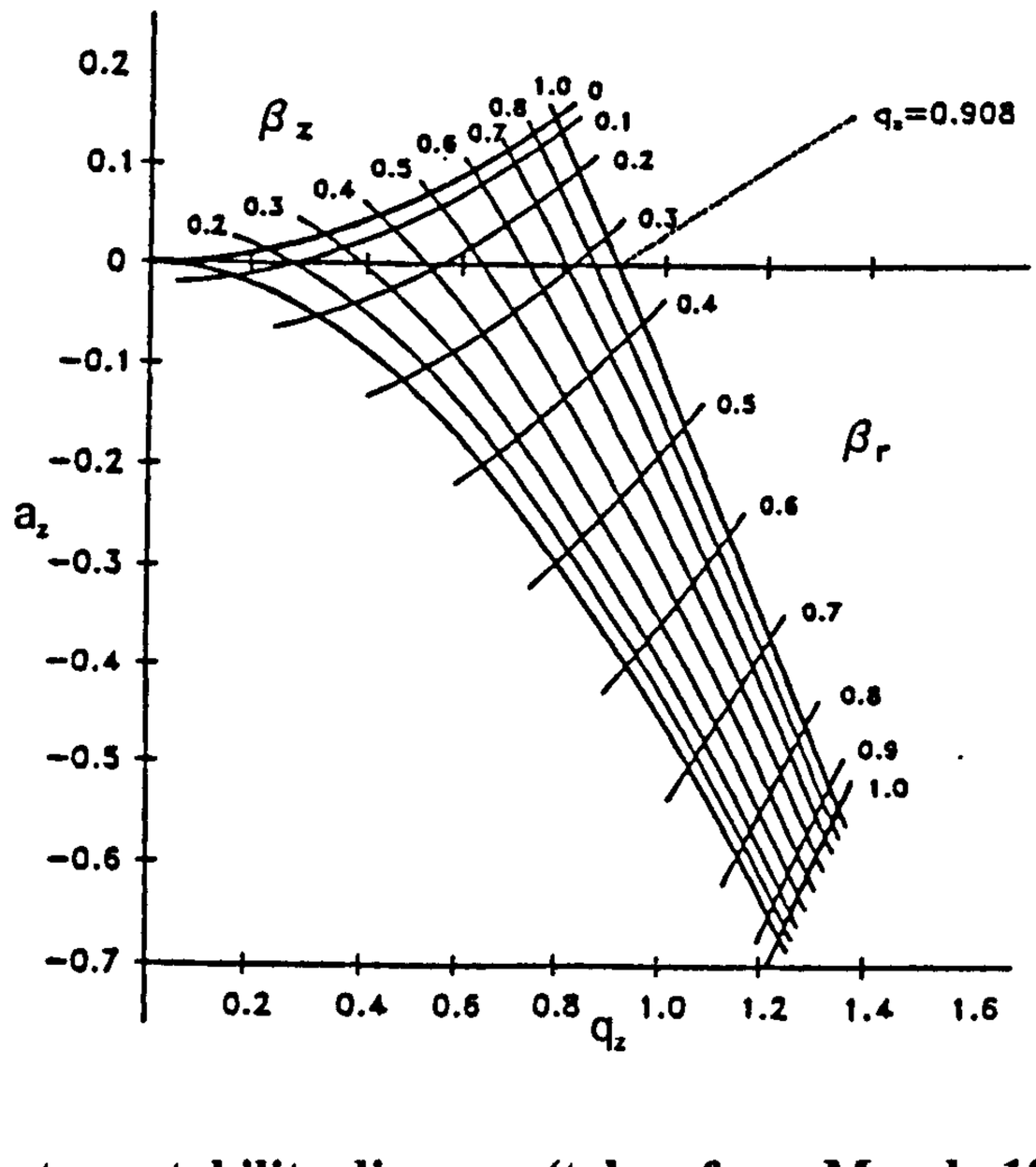


Figure 3.3: Ion trap stability diagram (taken from March, 1997).

3.1.1.5 Ion isolation

Knowledge of the stability diagram can also be used to set the conditions for isolation of an ion of interest prior to a tandem MS experiment. This can be illustrated by considering the case in which three ions, x, y and z, of similar *m/z* ratios are present in the trap, where x is of higher *m/z* ratio and z is of lower *m/z* ratio than y, and isolation of y is required (Figure 3.4). Because the ions have similar *m/z* values, the initial (a_z-q_z) pairings of all three ions lie close to point A on the q_z axis. The (a_z-q_z) pairings can be moved along the q_z axis by increasing the RF voltage on the ring electrode until that for y lies directly under the upper apex in the stability diagram (point B). A dc potential is applied, increasing a_z (Equation 3.1), and the (a_z-q_z) pairing for this ion is located just under the upper apex (point C) so that the y ion has a stable trajectory. The (a_z-q_z) pairings for x and z have also been modified and now their ion trajectories lie outside the stability region, causing the ions x and z to be lost radially and axially, respectively, and therefore they do not reach the detector. The RF and DC voltages are then reset and the (a_z-q_z) pairing of y is restored to a low q_z value on the q_z axis. The ion species y has been isolated (March, 1992; Jonscher and Yates, 1997) and can now undergo further mass analysis.

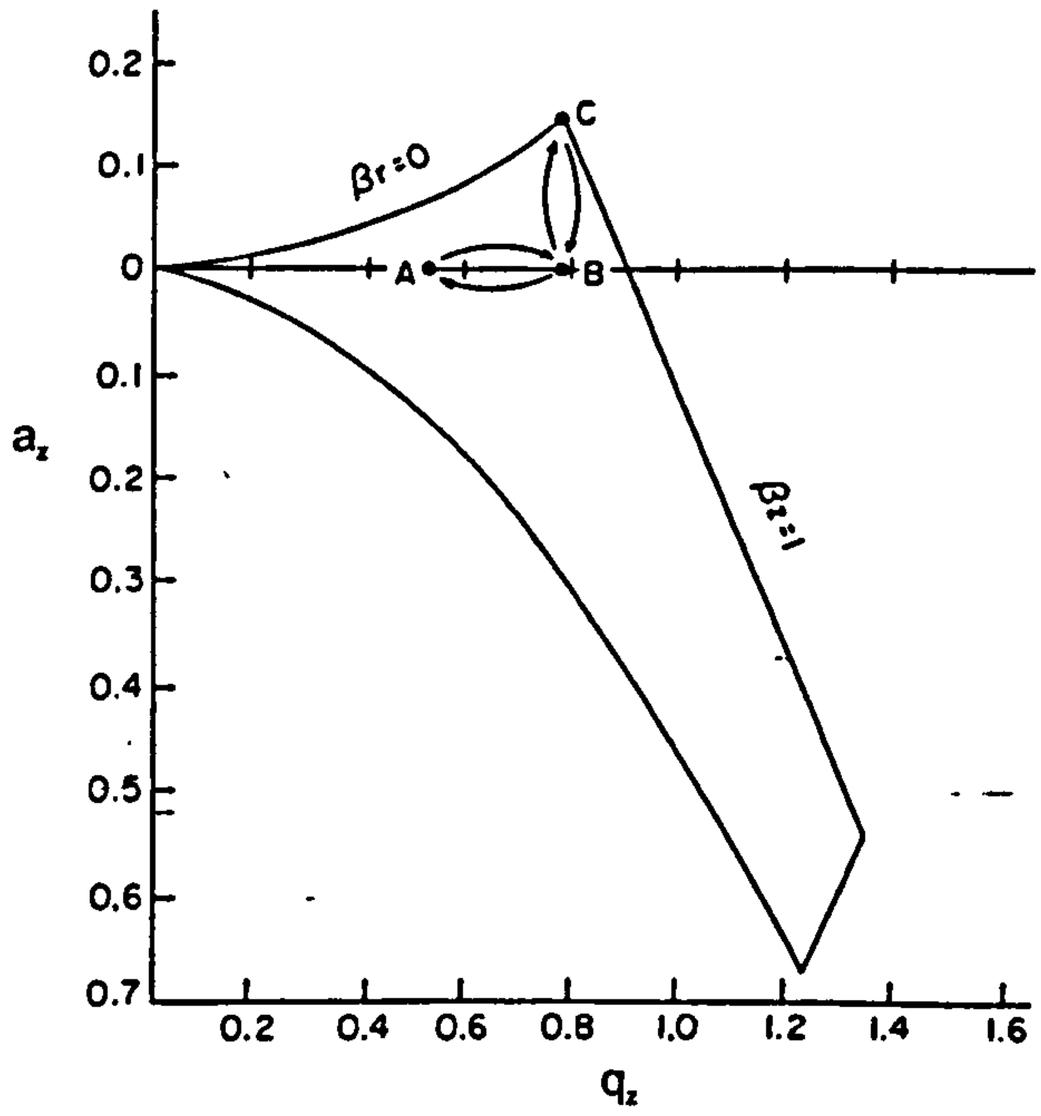


Figure 3.4: Schematic showing ion isolation (from March, 1992).

3.1.1.6 Tandem MS on an ion trap

The ion trap is capable of performing MS^n experiments on a preselected precursor ion, *i.e.* performing one mass selective operation after another (March, 2000). The first stage of mass spectrometry is the isolation step of an ion species, designated the precursor ion, while the second stage is the mass analysis of the fragment or product ions formed by the collision induced dissociation (CID) of the precursor (Bier and Schwartz, 1997; March 2000). In CID the process involves excitation of the isolated precursor ion so that it increases in KE followed by collision with the helium buffer gas, where KE is converted into internal energy and fragmentation then occurs to give product ions (Allison and Stepnowski, 1987). The product ions can undergo further excitation and fragmentation (MS^n) or can be scanned out of the ion trap and detected (MS^2). In subsequent discussions MS/MS refers to fragmentation of the precursor ion, while MS^n denotes the number of stages of fragmentation.

After the precursor ion is isolated (Section 3.1.1.5), it is brought into resonance by a resonant excitation voltage (< 5 V) applied for a selected period of time across the end-cap electrodes, causing the KE of the ions to increase and energetic collisions to be induced (Schwartz, 1999). Generally, no CID is observed unless a resonance excitation voltage is applied. The time for which the voltage is applied needs to be long enough for the precursor ion to reach sufficient KE for CID to occur; values are typically 5 - 30 ms and inversely related to the excitation voltage (Louris *et al.*, 1987; Bier and Schwartz, 1997; March 1997). In addition to the frequency and magnitude of the resonance excitation voltage and the time for which it is applied, the trapping RF voltage used during resonance excitation is important and all four parameters are interrelated (Louris *et al.*, 1987; March, 1997).

Since the q_z value of the trapped precursor ions is related to the trapping RF voltage (Equation 3.2), the q_z value at which the ions are excited ($q_{z\text{-}excite}$) will affect the MS/MS spectra obtained. At very low $q_{z\text{-}excite}$ there is an increased probability that ions will be lost from the trap due to forming unstable trajectories (Paradisi *et al.*, 1992). The KE of precursor ions is related to $q_{z\text{-}excite}$. Thus, at intermediate $q_{z\text{-}excite}$

values only the least energetic fragmentation processes occur. The fragments arising through the most energetic processes begin to appear at slightly higher $q_{z\text{-}excite}$ values (Louris *et al.*, 1987). However, more energetic collisions are obtained at the expense of the mass range over which fragment ions can be trapped (Louris *et al.*, 1987; Schwartz, 1999) since the $q_{z\text{-}excite}$ value of the precursor ion determines the minimum mass of the resulting product ions that can be trapped (Equation 3.3; Bier and Schwartz, 1997; March, 1997). Since fragment ions generated from the precursor ion will have higher associated q_z values at the same trapping voltage (Equation 3.2), product ions will only be trapped and subsequently detected if their *m/z* values fall above the low mass cut off (LMCO) of the trap. Thus, use of a low $q_{z\text{-}excite}$ value, for example 0.2 to 0.3, will allow the precursor to gain enough internal energy to fragment (Bier and Schwartz, 1997), as well as allowing the majority of the product ions to lie below the stability boundary at $q_{z\text{-}edge} = 0.908$ (Figure 3.3). Notably, however, low mass fragments may be lost if their associated q_z values exceeds 0.908 (Paradisi *et al.*, 1992).

$$LMCO = \left(\frac{q_{z-excite}}{q_{z-edge}} \right) \times \text{precursor} \quad m/z$$

Equation 3.3

The four parameters controlling CID in the Finnigan LCQ ion trap (Schwartz, 1999) are termed tickle frequency, activation amplitude (AA), activation time (AT) and activation Q (AQ). Tickle frequency is the frequency of the resonance excitation voltage applied to the ring electrode. It is predetermined by the *m/z* value of the precursor ion and so cannot be adjusted manually. AA is the magnitude of the resonance excitation RF voltage used in fragmentation, AT is the time for which AA is applied and AQ is the value to which Q is set during fragmentation. Q represents the Mathieu parameter $q_{z\text{-}excite}$ (Schwartz, 1999). The basic LCQ Navigator software only allows adjustment of the isolation window (IW), *i.e.* the mass range isolated prior to fragmentation, and of a single setting termed collision energy (Pettijohn and Mason, 1997). By contrast, the advanced software controls available through a

software patch allow more detailed control of the MS/MS conditions through independent adjustment of each of the parameters (AA, AT and AQ) that contribute to the setting termed collision energy (Schwartz, 1999).

3.1.2 Optimisation of instrumental parameters

As discussed above (Section 3.1.1.6) the operation of an ion trap mass spectrometer in MS/MS mode involves four interdependent parameters; tickle frequency, AA, AT and AQ. However, only AA, AT and AQ can be adjusted manually. In order to achieve maximum signal response for MS/MS product ions, the parameters governing MS/MS in the trap need to be optimised. Simplex optimisation has proved to be a simple and effective technique for optimising systems with several variables (Miller and Miller, 1993), for example, flow injection systems (Vereda *et al.*, 1997). Compared with methods based on factorial design, simplex requires fewer experiments to reach the maximum response (Nelder and Mead, 1965; Deming, 1984).

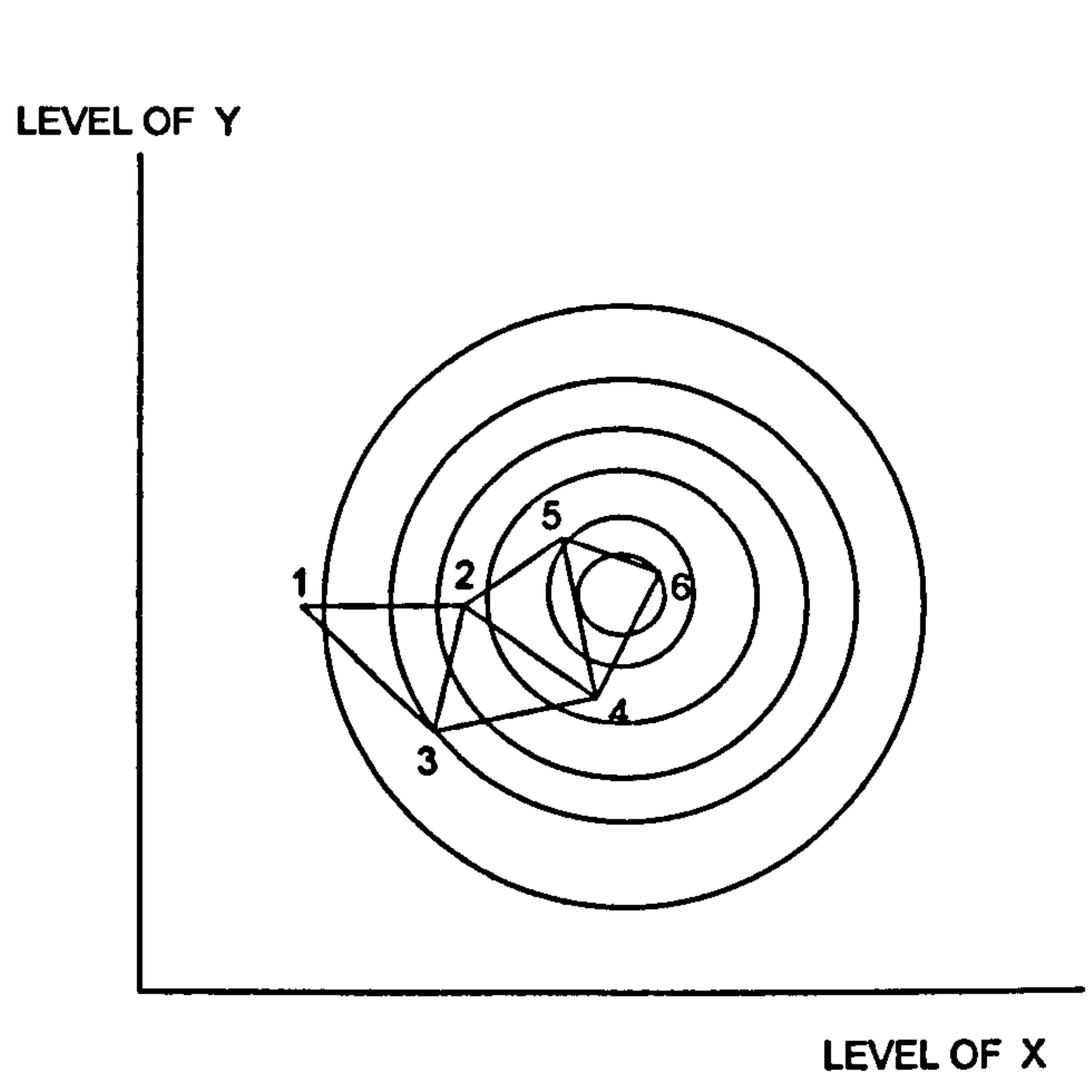


Figure 3.5: Diagram illustrating simplex optimisation for two factors.

A simplex is a geometric figure, with n+1 vertices, where n is the number of variables (factors) explored in the optimisation. For example, for two factors the

simplex will be a triangle. Simplex optimisation can be illustrated with the aid of a contour plot (Figure 3.5). The initial simplex is chosen and the responses at the vertices are determined experimentally. The lowest response (here vertex 1) is reflected in the line joining the other two vertices to produce vertex 4. Thus, the new simplex is defined by the vertices 2, 3 and 4. The response is determined experimentally at vertex 4 and the process continued until no further improvement is measured. For more than two variables, optimisation is achieved by calculation of the simplex (Miller and Miller, 1993). In this work, with three variables (AA, AT and AQ), a four factor simplex was used.

In the example illustrated (Figure 3.5) near-optimum response is achieved (vertex 6). Continuing the simplex optimisation would only result in circling around the true optimum. Therefore, the basic method of simplex optimisation is not entirely satisfactory and modifications, which include variable size simplex, have been developed (Deming, 1984; Betteridge *et al.*, 1985 a,b). Variable size simplex optimisation allows contraction and expansion of the simplex depending on the relative responses obtained at each of the vertices (Deming, 1984). It enables faster ascent toward the optimum and more accurate location of the optimum. In addition, the use of variable size simplex can reduce the number of experiments needed and hence the time taken to optimise the response (Deming, 1984; Betteridge *et al.*, 1985 a,b). The algorithms governing the application of variable size simplex (Betteridge *et al.*, 1985a,b) are best understood using a flow chart (Figure 3.6; from MultiSimplex, 1999). Both normal and variable size simplex were used in this work.

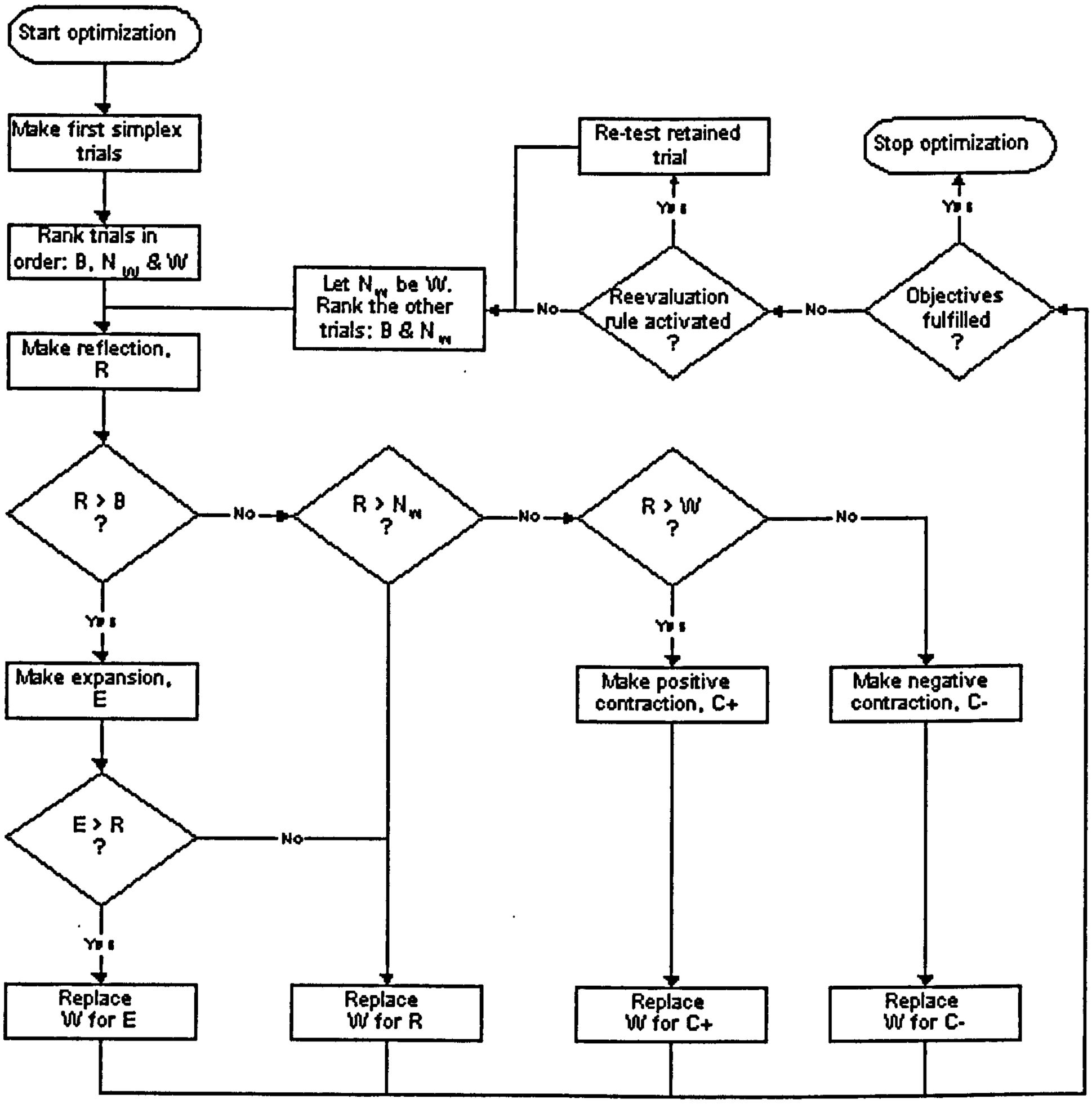


Figure 3.6: Flow chart illustrating the variable size simplex algorithms where B is the vertex that gives the best response, W is the vertex that gives the lowest response, N_w is the vertex that gives the next to lowest response. R is the vertex corresponding to a reflection from the lowest vertex. E is an expansion of the vertex by a factor 2, C_+ and C. are contractions by a factor 0.5 either away from or toward the best vertex (from MultiSimplex, 1999).

3.1.3 This study

Recently, relatively simple methods employing liquid chromatography ES electrospray (ES) tandem mass spectrometry (LC-ES-MS/MS) have been developed and applied for the analysis of chlormequat in pears (Brewin and Hill, 1996; Startin

et al., 1999) and cereals (Vahl *et al.*, 1998; Juhler and Vahl, 1999). ES produces a mass spectrum of chlormequat that is simple and totally devoid of fragments (Startin *et al.*, 1999). Consequently, MS^2 with CID using triple quadrupole instruments has been exploited to enhance specificity and selectivity. A similar approach could be applied using ion trap instruments. However, because the chlormequat cation is of low mass and the conditions used for trapping low mass ions are critical to the performance of the ion trap in MS/MS mode (Paradisi *et al.*, 1992), fragmentation under MS/MS conditions in the ion trap may be expected to present specific challenges (Section 3.1.1.6). Consequently, CID trapping parameters need to be optimised to achieve maximum sensitivity. In this study the ion trap parameters will be optimised for specific transitions under MS^2 mode so as to give enhanced specificity in the detection of chlormequat. Using the optimised conditions, the performance of the ion trap will be compared with that of a triple quadrupole mass spectrometer for the analysis of chlormequat in pear extracts.

3.2 RESULTS AND DISCUSSION

3.2.1 Preliminary MS/MS results

The full scan ES-MS spectrum of chlormequat is devoid of fragment ions (Brewin and Hill, 1996; Startin *et al.*, 1999), and is characterised by M_q^+ ions at *m/z* 122 and *m/z* 124 corresponding to the species containing the ^{35}Cl and ^{37}Cl isotopes respectively (Figure 3.7). Under MS^2 conditions on a triple quadrupole instrument the M_q^+ ions both fragment to give a product at *m/z* 58, the dimethylimmonium cation, which aids the identification of chlormequat (Startin *et al.*, 1999).

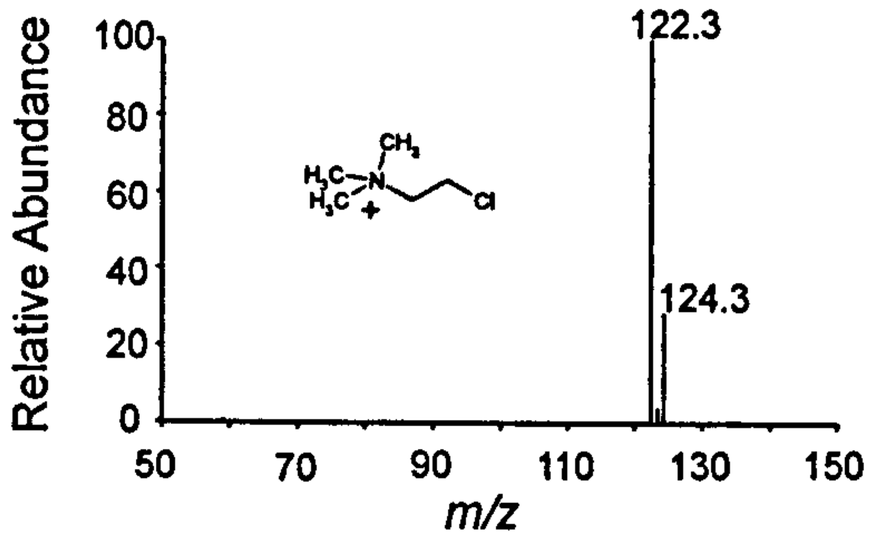


Figure 3.7: Full scan ES-MS mass spectrum of CQ.

In an attempt to obtain MS^2 spectra an aqueous solution of chlormequat (10 μg mL^{-1}) was introduced into the LCQ ion trap mass spectrometer by infusion. An isolation window of 1.85 Th caused the ^{13}C M_q^+ precursor to be trapped and fragmented in addition to the ^{35}Cl M_q^+ at *m/z* 122. Reducing the isolation window to 1.5 Th trapped only the *m/z* 122 precursor while retaining the efficiency of trapping. Reduction beyond 1.5 Th resulted in a decrease in the observed signal for the precursor ion, indicating a low trapping efficiency. Accordingly, an isolation window of 1.5 Th was used in subsequent experiments (Evans *et al.* 2000a).

The major product ion observed in MS^2 experiments of CQ using a triple quadrupole is *m/z* 58. For that reason the transition *m/z* 122 to 58 was selected in order to set the operating conditions for MS^2 on the ion trap instrument. The basic Navigator operating software has a facility for automatic tuning either for total ion count (TIC) or for a particular fragmentation process. Automatic tuning for the transition *m/z* 122 to 58 set a collision energy of 20%. This setting corresponds to a $q_{z\text{-}excite}$ of 0.25 and unknown values for tickle frequency, AT and AA. Under these conditions the intensity of the major fragment (*m/z* 58) was 0.1% relative to the precursor ion in full scan MS. Since it has been shown that the isolation step for the precursor is efficient (see above), the low intensity of *m/z* 58 cannot be attributed to low abundance of the precursor prior to fragmentation. Thus, the fragment ion was either not produced efficiently, or its trajectory within the trap was unstable. The precise reasons for its low intensity are discussed below (see Section 3.2.3).

3.2.2 Preliminary optimisation of MS/MS parameters

The results discussed above show much lower intensity for the product ion *m/z* 58 than has been observed with a triple quadrupole (Brewin and Hill, 1996; Startin *et al.*, 1999). In order to determine whether this behaviour was due to poor fragmentation efficiency or to unstable trajectory the MS^2 fragmentation conditions were investigated using the advanced software controls. Using these controls the response of the *m/z* 58 product ion was monitored under various MS^2 conditions during loop injections into the mobile phase stream. Beginning with the default settings, AA = 0.1 V, AQ = 0.25 and AT = 30 ms, each parameter was varied to maximise the response. This process took approximately 10 minutes. The best response was observed with AA = 0.95 V, AQ = 0.35 and AT = 30 ms, and the MS^2 spectrum (Figure 3.8) shows the presence of several product ions, the most abundant being at *m/z* 58, assigned as the dimethylimmonium ion. The other ions are assigned formally (Evans *et al.*, 2000a, 2001a; see also Section 4.2.2.2) as arising through loss of methane (*m/z* 106), ethene (*m/z* 94), hydrogen chloride (*m/z* 86) and chloroethyl (*m/z* 59). At the optimised settings the signal intensity for *m/z* 58 was 1000-fold greater than with those set automatically using basic controls.

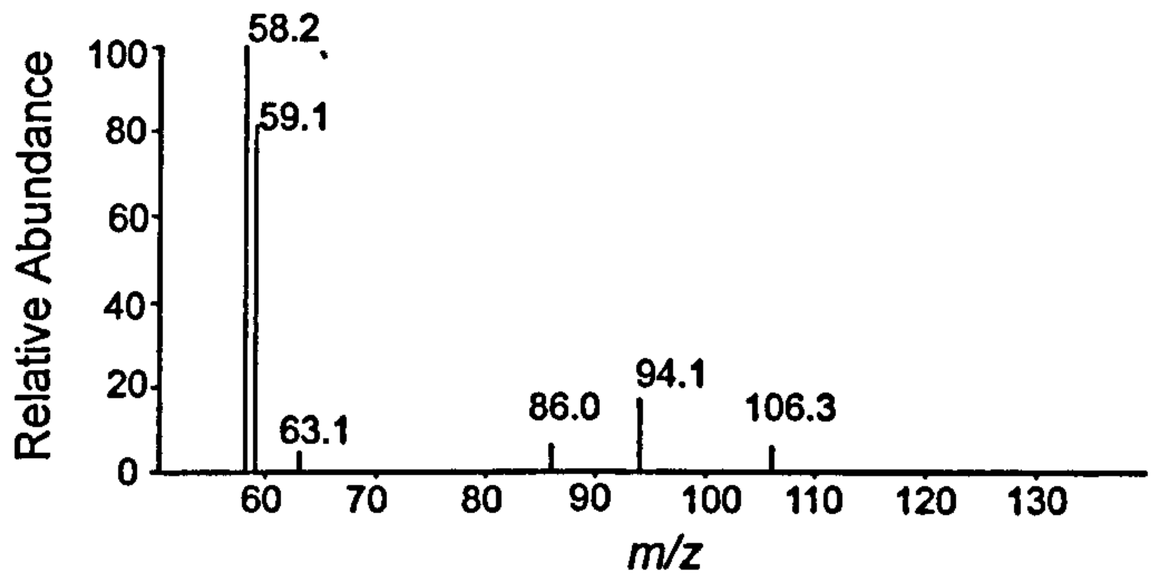


Figure 3.8: Full scan ES MS^2 spectrum of chlormequat (*m/z* 122) obtained on the LCQ ion trap mass spectrometer using an isolation window of 1.5 Th; AA = 0.95 V; AQ = 0.35; AT = 30 ms.

The preliminary optimisation described above showed that the AQ value exerts a strong influence on the yield of *m/z* 58. Under the basic controls AQ was set automatically to 0.25. Using the advanced controls, which allow manual adjustment of AQ, the best results were obtained at 0.35. During CID the average KE of

accumulated ions increases with increasing values of $q_{z\text{-}excite}$ (average KE $\propto q_{z\text{-}excite}^2$; March and Londry, 1995). Changing the AQ values from 0.25 to 0.35 corresponds to an increase of a factor of *ca.* 2 ($[0.35/0.25]^2$) in the KE of the precursor ions. Thus, a higher yield of product ions is expected at AQ=0.35.

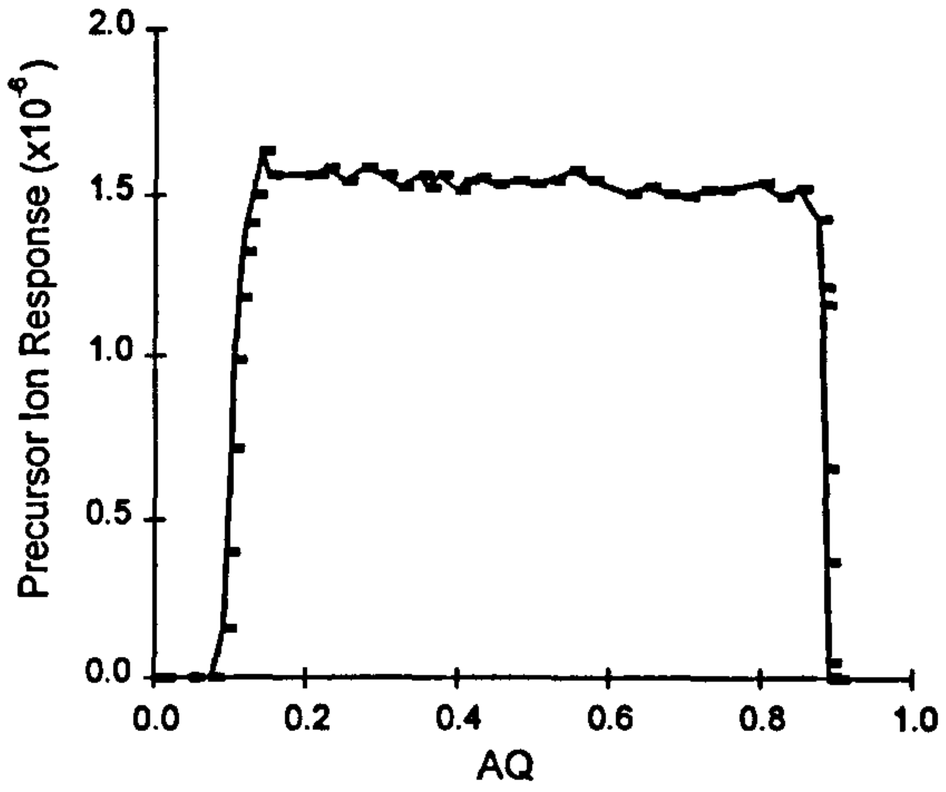
3.2.3 Investigation of ion intensity as a function of AQ ($q_{z\text{-}excite}$)

The previous results show the influence of AQ on the yield of *m/z* 58 and so the intensities of the precursor ion and *m/z* 58 were monitored as a function of AQ during two separate infusion experiments (Evans *et al.*, 2000a), during which automatic gain control (AGC) was operational. AGC controls the number of ions entering the trap, so that it is filled with approximately the same number of ions in each analytical scan. The number of ions admitted into the trap by the AGC is assessed *via* a prescan. In the first experiment, resonance excitation, which is required for CID, was disabled by setting AA to zero. In the second, AA was set to 0.96 V, a value shown previously to result in a good yield of *m/z* 58 (Section 3.2.2). In both experiments AT was held at 32 ms. Efficient trapping of the precursor ion was achieved at AQ values from approximately 0.115 to 0.9 (Figure 3.9a) and the plateau observed is due to the AGC. This wide stability range is consistent with ion trap theory (Figure 3.3; Section 3.1.1.4) and with experimental observations reported previously (Paradisi *et al.*, 1992).

By contrast, the product ion *m/z* 58 was observed in significant abundance over a narrow range of AQ values, *ca.* 0.3 to 0.4 (Figure 3.9b). At values below 0.26 *m/z* 58 is either not produced or has an unstable trajectory and is expelled from the trap. For reasons discussed above (Section 3.2.2) the absence or very low abundance of *m/z* 58 at values of AQ $\leq$ 0.26 is attributed to lack of fragmentation of the precursor. At AQ values above 0.26 more energetic collisions resulted and fragmentation occurred. The maximum abundance of *m/z* 58 was found at AQ = 0.342, and at AQ values above this value the signal level for *m/z* 58 decreased. The observed drop in response could result either from the fragment ion (*m/z* 58) being unstable as a result

of excess internal energy or from the change in AQ leading to *m/z* 58 having an unstable trajectory in the trap (Section 3.1.1.6). Thus, although collisions are more energetic with higher values of AQ, ions of low *m/z* may be lost from the trap if their *m/z* falls below the LMCO (Johnson *et al.*, 1990). At AQ values greater than 0.435 *m/z* 58 was not observed. At this AQ value the LMCO of the trap is calculated to be *m/z* 58.5 ([0.435/0.908] x 122.2; Equation 3.3) and ions below this value will not reach the detector (Figure 3.9b). Thus, the sharp decline in product ion intensity at AQ values between 0.410 and 0.435 is attributed to the proximity of the ion at *m/z* 58 to the stability/instability boundary, leading to loss from the trap. The value observed for the stability/instability boundary, between 0.410 and 0.435, is in good agreement with the range calculated using Equation 3.3 (between 0.423 and 0.438).

a)



b)

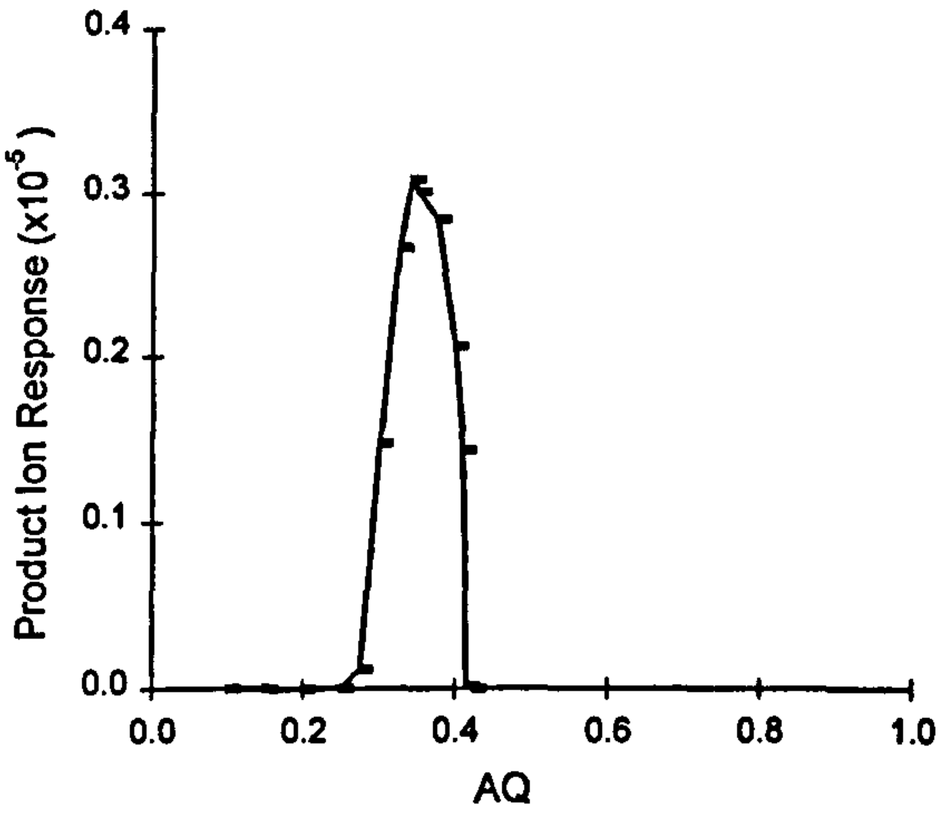


Figure 3.9: Response of a) precursor ion (*m/z* 122) as a function of AQ (AA = 0.0 V and AT = 32 ms) and b) product ion (*m/z* 58) as a function of AQ (AA = 0.96 V and AT = 32 ms).

3.2.4 Simplex optimisation of MS/MS parameters

Preliminary optimisation (Section 3.2.2) assumed all parameters to be independent. As discussed in Section 3.1.1.6, this is not the case since higher AA values have been shown to require lower activation times and vice versa (Johnson *et al.*, 1990). In addition, AA and AQ both affect the KE of the precursor ion and so the AQ value chosen has a large influence on the activation amplitude required to induce fragmentation. Simplex optimisation (Miller and Miller, 1993), based on the three factors AA, AT and AQ, was used as a systematic approach to define conditions for the optimum signal response for *m/z* 58.

To ensure that the global maximum and not a local maximum was reached, the simplex was initiated at various points contained within a predetermined region. AA values were varied between 0.1 and 1.5 V because preliminary experiments established that at values greater than 1.5 V no *m/z* 58 was observed. In addition a wide range of AQ values (0.2 to 0.9) was used, since the precursor has been shown to be trapped efficiently over this range (Section 3.2.3). AT values between 0 and 50 ms were allowable as this range encompasses those typically used in MS/MS experiments *i.e.* 5 – 30 ms. Each vertex co-ordinate in the simplex represents individual combinations of variable settings. An initial simplex with a relatively large step size was used to move rapidly over the response surface. Once the measured response at each vertex had been obtained, the levels for all four vertices were compared and the new vertex was calculated (Table 3.1). The vertex co-ordinates corresponding to the conditions which gave the lowest response were excluded; the other co-ordinates were summed, averaged and then doubled, and the co-ordinates from the excluded vertex subtracted giving the co-ordinates for the new vertex (Miller and Miller, 1993). The new vertex and the original three vertices form a new simplex. The procedure was continued until a broad region of optimum response was located. Subsequently, other simplex experiments with progressively smaller step sizes were started near this crude optimum and allowed to continue until no further improvement in signal was obtained.

The optimum response, twice that obtained using the parameters from the preliminary optimisation conditions described above (Section 3.2.2), was achieved with AA = 0.96 V, AQ = 0.342 and AT = 32 ms. Variable size simplex optimisation, performed according to the protocol described earlier (Figure 3.6), confirmed the global maximum response at AA = 0.96 V, AQ = 0.354 and AT = 35 ms (Evans *et al.*, 2000a). The mass spectrum (Figure 3.10) obtained at this maximum revealed an additional product ion at *m/z* 60 attributed to the trimethylammonium cation (Section 4.2.2.2; Evans *et al.*, 2001a).

Vertex	Values of the factors			Response (arbitrary scale)
	AA	AQ	AT	
V_1	0.98	0.370	25.0	3.2
V_2	0.99	0.350	40.0	4.0
$V_3 = W$	0.95	0.400	33.0	2.6
V_4	0.84	0.346	28.3	3.5
$V_5 = R = (2x - W)$	0.93	0.310	29.2	3.3
[a]Σ	2.81	1.066	93.3	
$x = \Sigma/3$	0.94	0.355	31.1	
[b]$x - V_3$	-0.01	-0.045	-1.9	

[a] Sum of all values excluding that which gave the lowest response

[b] Average minus the values which gave the lowest response

Table 3.1: Values of factors and response for initial simplex optimisation of ion trap settings.

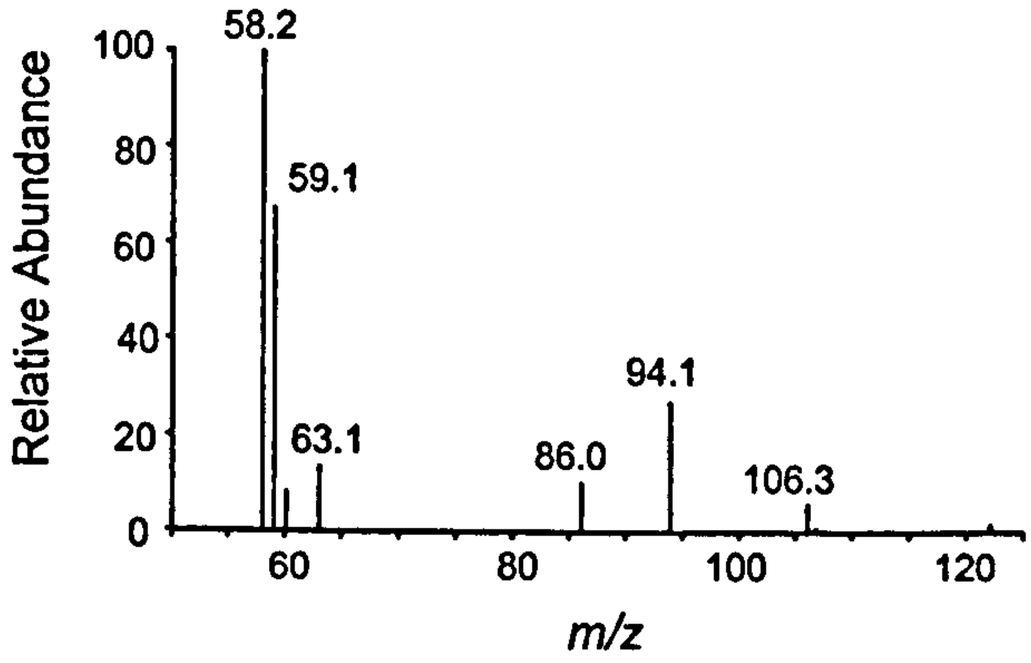


Figure 3.10: Full scan ES MS^2 spectrum of *m/z* 122 obtained at the optimum found by simplex optimisation (AA = 0.96 V; AQ = 0.354; AT = 35 ms).

The results from the sets of simplex calculations (vertex values and corresponding responses for *m/z* 58) were used to construct a scatter plot (Figure 3.11). The region of the response surface over which near optimum response (within 10% of the maximum) for *m/z* 58 is achieved spans the region AA between 0.96 and 0.90 with corresponding AQ values between 0.34 and 0.36, revealing stability for the transition *m/z* 122 to 58 within this working range.

The value of 35 ms for AT is slightly higher than those typically needed (5 – 30 ms) for fragmentation within an ion trap instrument (Bier and Schwartz, 1997). For example, in a previous study of ion trap activation parameters for diethylethylphosphonate (MH^+, *m/z* 166) an AT of 15 ms was used (Johnson *et al.*, 1990). However, the previous study was carried out on an older Finnigan instrument, which had a different stretched geometry to that of the LCQ. Nevertheless, our results are in agreement with the earlier observations, in that longer activation times favour production of low mass product ions over high mass ions formed from the same precursor. This is due to the increase in ion kinetic energy with increasing resonance excitation time, which promotes the formation of lower mass product ions.

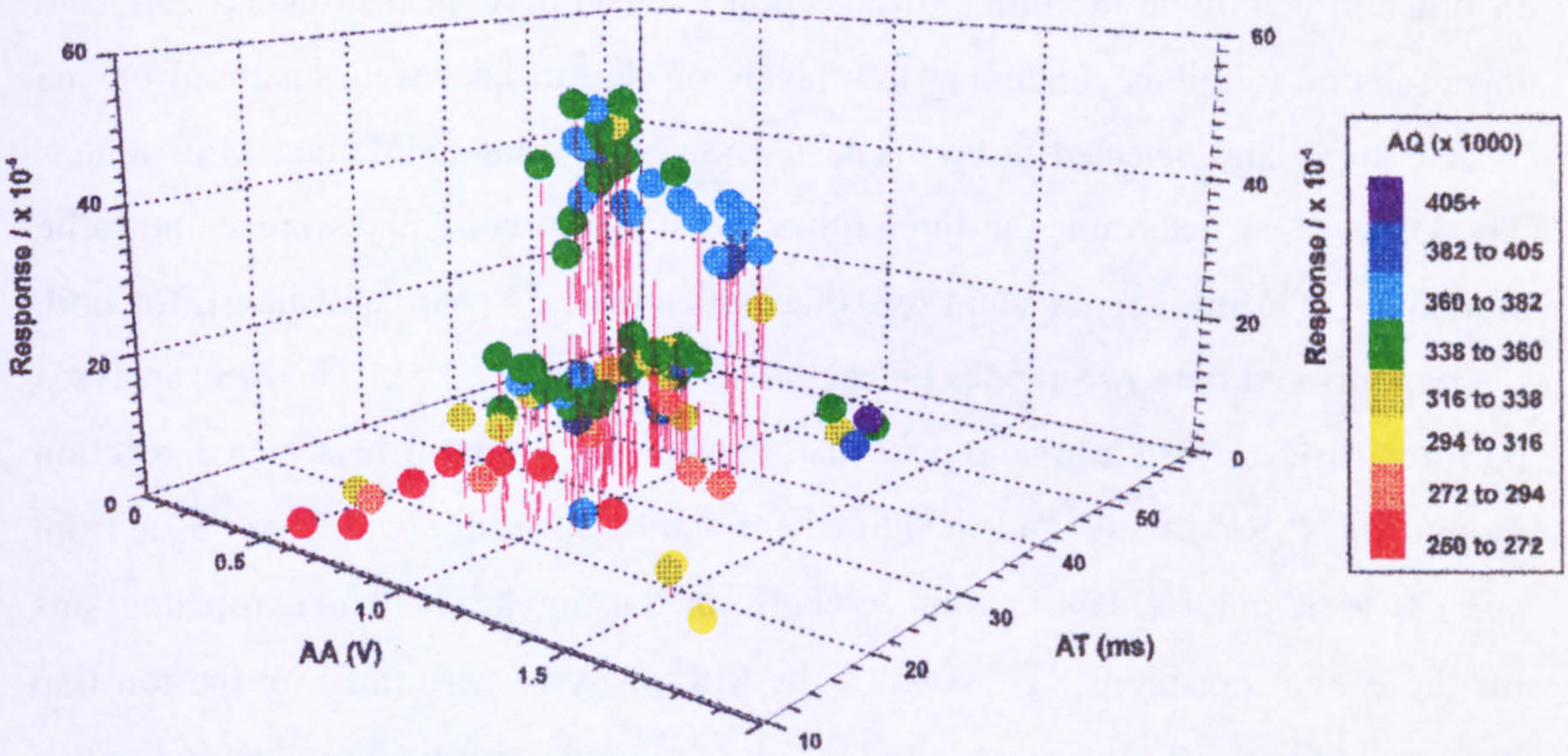


Figure 3.11: Scatter plot obtained from simplex optimisation of product ion response using three factors, AA, AT and AQ [original in colour].

3.2.5 Comparison between an ion trap mass spectrometer and a triple quadrupole mass spectrometer for the analysis of chlormequat in pears

The performance characteristics of ion trap and triple quadrupole instruments are similar. In particular, both offer the capability of MS/MS experiments and so allow increased specificity in detection. Comparing the overall performance of these two types of mass analyser for the routine detection of pesticides in environmental matrices would be beneficial since the cost of an ion trap instrument is less than a triple quadrupole instrument. In order to achieve such a comparison, standard solutions (four replicates) of chlormequat in pear matrix were analysed in random order on both the ion trap and triple quadrupole mass spectrometers. Under the LC conditions employed, ion exchange using a strong cation exchange column (SCX) chlormequat eluted at approximately 9 min (see Figure 2.1) and showed considerable peak asymmetry (Evans *et al.*, 2000a,b). The peak shape is a feature of the ion chromatographic separation; even though the peak shape is not ideal and limits the sensitivity of the overall method, the LC method is sufficient for food monitoring requirements (Chapter 2).

In order to determine the limits of detection (LODs) on both instruments replicate injections of solutions containing low levels of chlormequat were analysed on the SCX column and detected in both selected ion monitoring (SIM) and MS^2 modes. The LODs were determined at three times the signal to noise and were found to be similar, ~ 1 ng.mL^{-1} (equivalent to 0.005 mg cation kg^{-1} pear equivalent), for both instruments and both MS modes on each instrument (Table 3.2). The MS^2 analyses performed on the triple quadrupole instrument were acquired in selected reaction monitoring (SRM) mode and monitored the transition from *m/z* 122 to 58 or from *m/z* 124 to 58 only. Quantification was performed using the resultant chromatograms for these two transitions. By contrast the MS^2 analyses performed on the ion trap instrument for *m/z* 122 were acquired in full scan mode, so called product ion mode, and the extracted mass chromatogram for the transition *m/z* 122 to 58 was used for quantification. The LODs for SIM of *m/z* 124 (due to ^{37}Cl CQ) and MS/MS on the triple quadrupole were found to be ten times higher than for *m/z* 122. The LODs for SIM and MS^2 analysis of chlormequat in pear are all below the EU and UK levels (see Section 1.1; Statutory Instrument and EU directive) and so are adequate for monitoring purposes.

Instrument	SIM *m/z* 122 (ng mL^{-1})	SIM *m/z* 124 (ng mL^{-1})	MS^2 *m/z* 122 to 58 (ng mL^{-1})*	MS^2 *m/z* 124 to 58 (ng mL^{-1})*
Triple quadrupole	0.9	13	0.8	9.0
Ion trap	1.0	11	1.0	Not measured

Table 3.2: LODs for chlormequat cation using various mass spectrometric analyses. * MS^2 analyses on the triple quadrupole instrument were performed in SRM mode; MS^2 analyses on the ion trap instrument performed in full scan (product ion) mode.

Replicate injections of standards containing varying levels of chlormequat were analysed so as to derive calibrations plots both from SIM of *m/z* 122 and from MS^2 for the transition *m/z* 122 to 58 (Figure 3.12). Log-log plots (not shown) for both instruments gave calibrations that were linear over the range of concentrations measured (2 to 1000 ng mL^{-1}). Calibration plots for the analyses carried out on the ion trap (Figure 3.12) reveal a greater spread in the data for the MS^2 results than for SIM. In SIM and MS^2 modes the ion trap showed less scatter than the triple quadrupole (Figure 3.12). Better repeatability on the ion trap is reflected in lower relative standard deviations (RSDs) obtained for SIM analysis (10% *vs* 12%, $n = 20$) of calibration standards at 20 ng mL^{-1} in methanol/water (50/50). By comparison, RSD values for MS^2 (*m/z* 122 to 58) were 11% and 14% ($n = 20$) for the ion trap and triple quadrupole respectively.

An independent comparison of the analysis of chlormequat in pears on an LCQ ion trap mass spectrometer and a triple quadrupole instrument for the analysis of chlormequat in pears has been reported (Mol *et al.*, 2000). The LCQ used in that study was not equipped with the developmental software controls and so, for the reasons outlined in this chapter, was unable to perform MS/MS experiments. Thus, the advanced control software is essential for achieving optimal MS/MS performance of the LCQ in this application. It should also be noted that the advanced software does not permit concurrent MS and MS/MS, nor does it allow multiple reaction monitoring (Evans *et al.*, 2000a) or unattended operation of the autosampler. Although these limitations present major obstacles to routine use, there are still significant benefits to be gained in using the advanced software controls.

3.3 CONCLUSIONS

The work presented here has demonstrated the need for optimisation of tandem mass spectrometric conditions in studies of low mass ions on the ion trap mass spectrometer. Use of the advanced mass spectrometric controls on the LCQ has allowed significant improvement in sensitivity of MS^2 detection for chlormequat. The use of simplex optimisation, particularly variable size simplex, gave enhanced

response with rapid ascent toward the optimum. In particular, simplex optimisation enabled the product ion (*m/z* 58) response to be improved 1000 fold. The most critical activation parameters for MS^2 of chlormequat in the ion trap are activation amplitude and activation Q (when an appropriate isolation window is used). It has been found that low mass ions require a higher AQ value to promote fragmentation than that set automatically by the software.

Comparison of the performance of the optimised ion trap with that of a triple quadrupole instrument shows that very similar LODs can be achieved. For the MS^2 transition of the ^{35}Cl precursor to the most abundant product, LODs were 0.8 ng cation mL^{-1} (0.004 mg cation kg^{-1} pear equivalent) and 1.0 ng cation mL^{-1} (0.005 mg cation kg^{-1} pear equivalent) on the triple quadrupole and ion trap instruments respectively. Thus, both offer a sensitivity and precision that is suitable for the routine analysis of chlormequat. Access to the advanced controls of the LCQ is essential for enhancement of sensitivity for the MS^2 analysis of chlormequat, and access to these advanced controls, preferably implemented through routine operations software, would be of benefit in the development of methods for the routine analysis of pesticides.

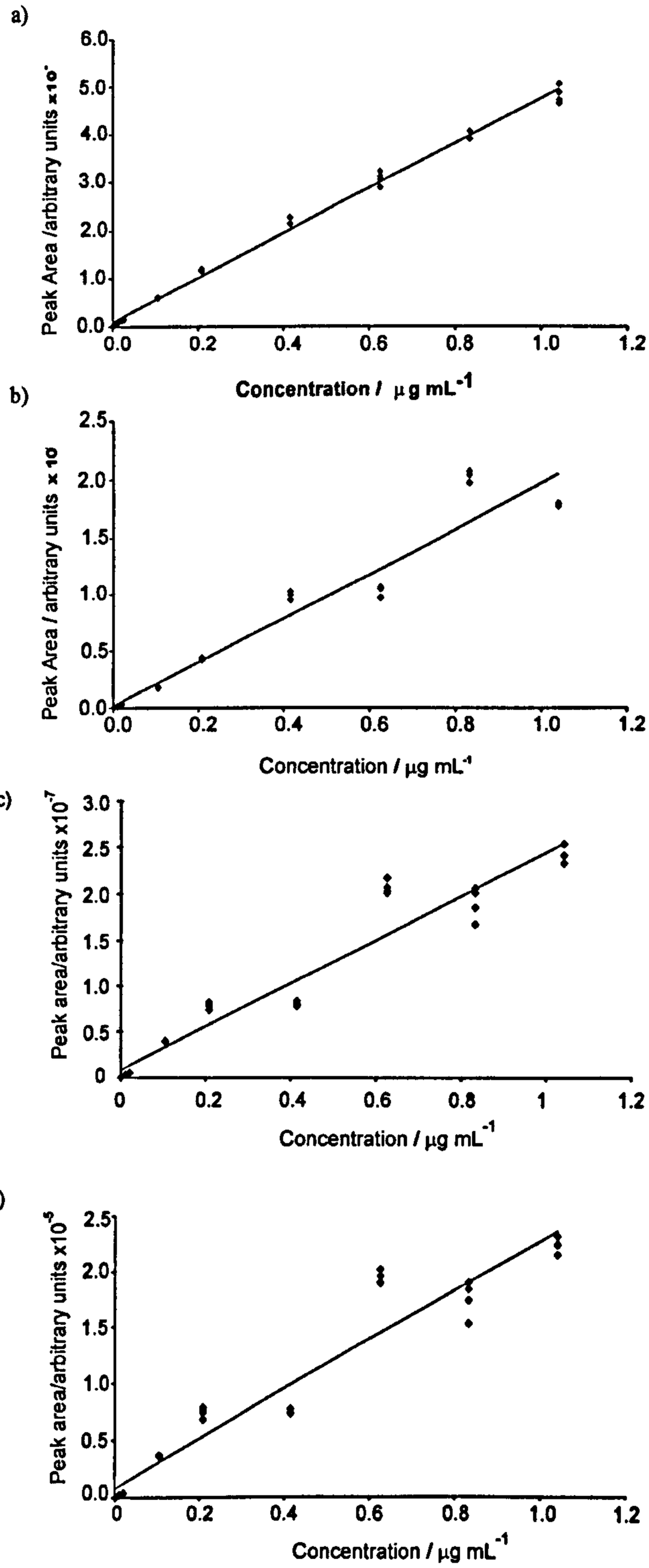


Figure 3.12: Calibration curves for the analysis of chlormequat in pear matrix measured using a) SIM *m/z* 122 on an ion trap and b) the transition *m/z* 122 to 58 on an ion trap c) SIM *m/z* 122 on a triple quadrupole d) the transition *m/z* 122 to 58 on a triple quadrupole.

Chapter 4

Mass Spectrometric Analysis of the Quaternary Ammonium Pesticides

Chapter 4: Mass Spectrometric Analysis of the Quaternary Ammonium Pesticides

4.1 INTRODUCTION

4.1.1 Previous mass spectrometric studies of the quats

Increasingly, analytical methods incorporating confirmatory detection by MS are required by regulatory bodies such as the United States Environmental Protection Agency, since they offer both high specificity and sensitivity. In the previous chapter, chlormequat was used as a model cation to demonstrate the benefit of optimisation of the tandem mass spectrometric conditions on an ion trap mass spectrometer. Incorporation of the optimised parameters into an LC-MS/MS method allowed chlormequat to be quantified with high specificity over a wide linear dynamic range.

Previous reports of MS analysis of quats have employed thermospray (Barcelo *et al.*, 1993), fast atom bombardment (FAB; Tondeur *et al.*, 1987) and atmospheric pressure ionisation (API) interfaces (Moyano *et al.*, 1996; Song and Budde, 1996; Wycherley *et al.*, 1996; Marr and King, 1997; Taguchi *et al.*, 1998; Castro *et al.*, 1999; Lazar and Lee, 1999; Evans *et al.*, 2000a,b; Hau *et al.*, 2000; Takino *et al.*, 2000; Zhao *et al.*, 2000), typically in combination with separation by either liquid chromatography (LC; Barcelo *et al.*, 1993; Marr and King, 1997; Taguchi *et al.*, 1998; Castro *et al.*, 1999; Evans *et al.*, 2000a,b; Hau *et al.*, 2000; Takino *et al.*, 2000; Zhao *et al.*, 2000) or capillary electrophoresis (CE; Song and Budde, 1996; Moyano *et al.*, 1996; Wycherley *et al.*, 1996; Lazar and Lee, 1999). Generally, these reports concentrate either on detection of the quaternary ammonium cation (M_q^+) or the use of tandem MS (MS/MS) for selected reaction monitoring (SRM) of characteristic transitions. Of the MS approaches available, API-MS(/MS) approaches are the most widely used for the determination of quats for regulatory purposes, since they are often coupled with condensed-phase separations (Marr and King, 1997; Taguchi *et al.*, 1998; Vahl *et al.*, 1998; Juhler and Vahl, 1999; Startin *et al.*, 1999; Hau *et al.*, 2000; Zhao *et al.*, 2000).

4.1.2 Electrospray mass spectrometry and cluster formation

The tendency of API spray conditions to favour formation of cluster species is well established. For example, gas phase metal halide (MX) cluster ions of the type $[M_{n-1}X_n]^+$ have been used to probe the electrospray (ES) process (Wang and Cole, 1998). Furthermore, due to the ease of formation and their subsequent stability, $Na^+(NaI)_n$ cluster ions allow calibration of a mass spectrometer over its full *m/z* range (Bruins, 1997). Cluster formation during the ES process has also been used to study biochemical interactions including antibody-antigen, receptor-ligand, enzyme–substrate pairings (Cole, 1997) and non-covalent protein polymers of bovine albumin and alcohol dehydrogenase (Wang *et al.*, 2000). Formation of clusters between (poly)peptides and anions present in the sample or solution was found to cause a reduction in the average net charge of the cations (Mirza and Chait, 1994). The nature of the interaction was shown to depend on the electronegativity of the anion and to reflect its ability to attach to the (poly)peptide (Mirza and Chait, 1994): the more electronegative the anion the greater the degree of charge neutralisation of the (poly)peptide. Thus, ES is sufficiently gentle to allow clusters to form between species within an electrosprayed sample.

Analysis of a range of quaternary ammonium chlorides $[M_q^+Cl^-]$ using positive ion mode liquid secondary ion mass spectrometry and ES-MS (Fisher *et al.*, 1994) demonstrated the formation of dimeric clusters of the type $[2M_q+A]^+$ and $[2M_q-H]^+$ with chloride, iodide and trifluoroacetate (TFA) counterions (A). In particular, it was noted that the cluster $[2M_q+Cl]^+$ gave the strongest signal and was useful in distinguishing species with quaternary ammonium functionality from protonated amines, which do not form dimeric species (Fisher *et al.*, 1994). Later analysis of two diquaternary ammonium chloride salts by ES-MS revealed that the analytes formed ion-pair complexes with indigenous chloride anion and also with TFA introduced into the system (Wang and Cole, 1996). Thus, both mono- and diquaternary ammonium cations have been shown to associate with anions to form cluster species detectable by ES-MS.

Zook and Bruins (1997) reported the formation in ES-MS of clusters of the type $TAA^+(TAAX)_n$ from a number of tetraalkylammonium halides (TAAX) over the concentration range 2×10^{-5} to 5×10^{-3} M. Given that many mobile phases used in the analysis of quaternary ammonium pesticides contain salts at concentrations around 50 mM (Vahl *et al.*, 1998; Juhler and Vahl, 1999; Startin *et al.*, 1999; Evans *et al.*, 2000a,b; Hau *et al.*, 2000), and quantification standards and samples often encompass cation concentrations where TAA^+ has been shown to form clusters, the formation of clusters might also be expected to be a significant process in these analyses. Should this be the case, the limit of linearity (LOL) of the quantification for the free pesticide cation (M_q^+) will be affected.

4.1.3 This study

The purpose of this study was twofold. Firstly, the ES-MS studies of ionic analytes performed previously indicate that the quats are likely to form cluster ions with anions present within an analytical system. Thus, quat cluster species formed during ES-MS will be identified and the conditions required for their formation will be characterised. Secondly, the previous MS/MS studies of the quats have concentrated only on the major product ions and many of the weaker transitions remain unassigned. Since the ion trap has the capability of performing MS^n analyses, the second objective of this study is to obtain very detailed information regarding fragmentation pathways for the quats, which could facilitate the choice of highly specific transitions for MS detection.

Earlier work has shown that the ion trap mass spectrometer is sensitive to variations in trapping and resonance excitation conditions and that simplex optimisation of MS^2 conditions for CQ significantly enhanced signal response for the product ion at *m/z* 58 (see Chapter 3). Since MQ^+ is of similar *m/z* value to CQ^+ (*m/z* 114 *cf.* 122), its fragments, like those of CQ^+, would be expected to exhibit unstable trajectories, necessitating the use of specific trapping conditions (Chapter 3). By contrast, the monocations of DF, DQ and PQ are of higher *m/z* value (*m/z* = 249, 184 and 186, respectively), and it is likely that most of the fragment ions reported previously

(Tondeur *et al.*, 1987; Moyano *et al.*, 1996) have sufficiently high *m/z* values to exhibit stable trajectories within a trap operated under standard conditions. Clearly, due to the nature of the trap, the MS/MS conditions for the analysis of the quats may not be optimal. Thus, simplex optimisation of the ion trap MS/MS parameters will be performed in order to establish optimum MS/MS response and also to find conditions capable of promoting specific transitions.

4.2 RESULTS AND DISCUSSION

4.2.1 Full Scan MS characterisation including cluster ion studies

4.2.1.1 Full scan MS

In order to characterise the full scan mass spectra for the quats, solutions of the quats (10 μg mL^{-1}) were introduced into the mass spectrometer by infusion into the mobile phase (50 mM ammonium acetate in 50/50 methanol/water). As reported previously, the full scan ES-MS spectrum of CQ (Figure 4.1a) is dominated by the M_q^+ ions at *m/z* 122 and *m/z* 124 (Brewin and Hill, 1996; Startin *et al.*, 1999). Similarly, the ES-MS full scan spectra of MQ and DF (Figure 4.1b,c) are devoid of fragment ions and are dominated by *m/z* 114 and *m/z* 249 respectively, corresponding to M_q^+ as seen in previous studies (Barcelo *et al.*, 1993; Moyano *et al.*, 1996; Castro *et al.*, 1999). The ES-MS full scan mass spectra of DQ and PQ (Figure 4.1d,e) are characterised by base peak ions at *m/z* 183 and *m/z* 185 respectively, assigned previously as $[M_q^{2+}\text{-}H^+]^+$ for both analytes (Song and Budde, 1996; Marr and King, 1997; Taguchi *et al.*, 1998; Castro *et al.*, 1999; Takino *et al.*, 2000). The DQ ion at *m/z* 183 has also been attributed to $[M_q\text{-}H]^{+\cdot}$ (Moyano *et al.*, 1996). It should be noted, however, that *m/z* 183 must be an even electron ion and so could not correspond to $[M_q\text{-}H]^{+\cdot}$ and the former assignment appears more likely. Ions at *m/z* 92 and 184 (DQ) and *m/z* 93 and 186 (PQ) correspond to the dications (Moyano *et al.*, 1996; Takino *et al.*, 2000) and $M_q^{+\cdot}$ ions respectively for both analytes (Moyano *et al.*, 1996; Song and Budde, 1996; Marr and King, 1997; Taguchi *et al.*, 1998; Castro *et al.*, 1999). The ion at *m/z* 157 for DQ was assigned previously as arising from the loss of HCN from $DQ^{+\cdot}$

(Tondeur *et al.*, 1987; Moyano *et al.*, 1996; Marr and King, 1997; Castro *et al.*, 1999) and that at *m/z* 171 for PQ to loss of CH_3^+ from the PQ dication (Tondeur *et al.*, 1987; Barcelo *et al.*, 1993; Moyano *et al.*, 1996; Marr and King, 1997; Castro *et al.*, 1999).

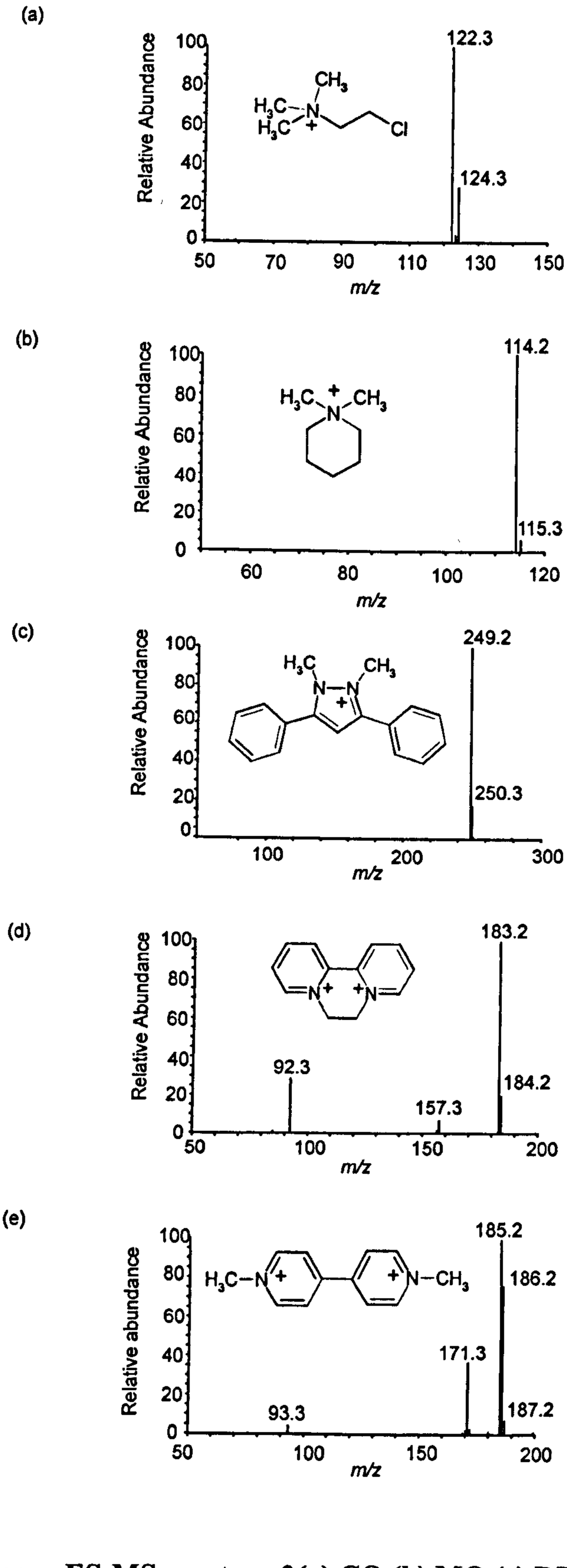


Figure 4.1: Full scan ES-MS spectra of (a) CQ (b) MQ (c) DF (d) DQ (e) PQ.

4.2.1.2 Studies over an extended mass range

A limited mass range (*m/z* 50 –300), typical of many analytical approaches, was used to obtain the spectra in the analyses described above (Section 4.2.1.1). Preliminary experiments over the range *m/z* 50 – 1000, with 50 mM ammonium acetate in the mobile phase and individual quat solutions (CQCl, MQCl, $DQCl_2$, DF methyl sulfate, $PQCl_2$) at 10 μg mL^{-1}, showed an absence of ions at *m/z* values greater than base peak (*m/z* 183) for DQ and (*m/z* 249) for DF. By contrast, ions at *m/z* values greater than the base peak were observed with significant intensity for PQ (*m/z* 203), MQ (*m/z* 263 and 287) and CQ (*m/z* 279 and 303; Figure 4.2).

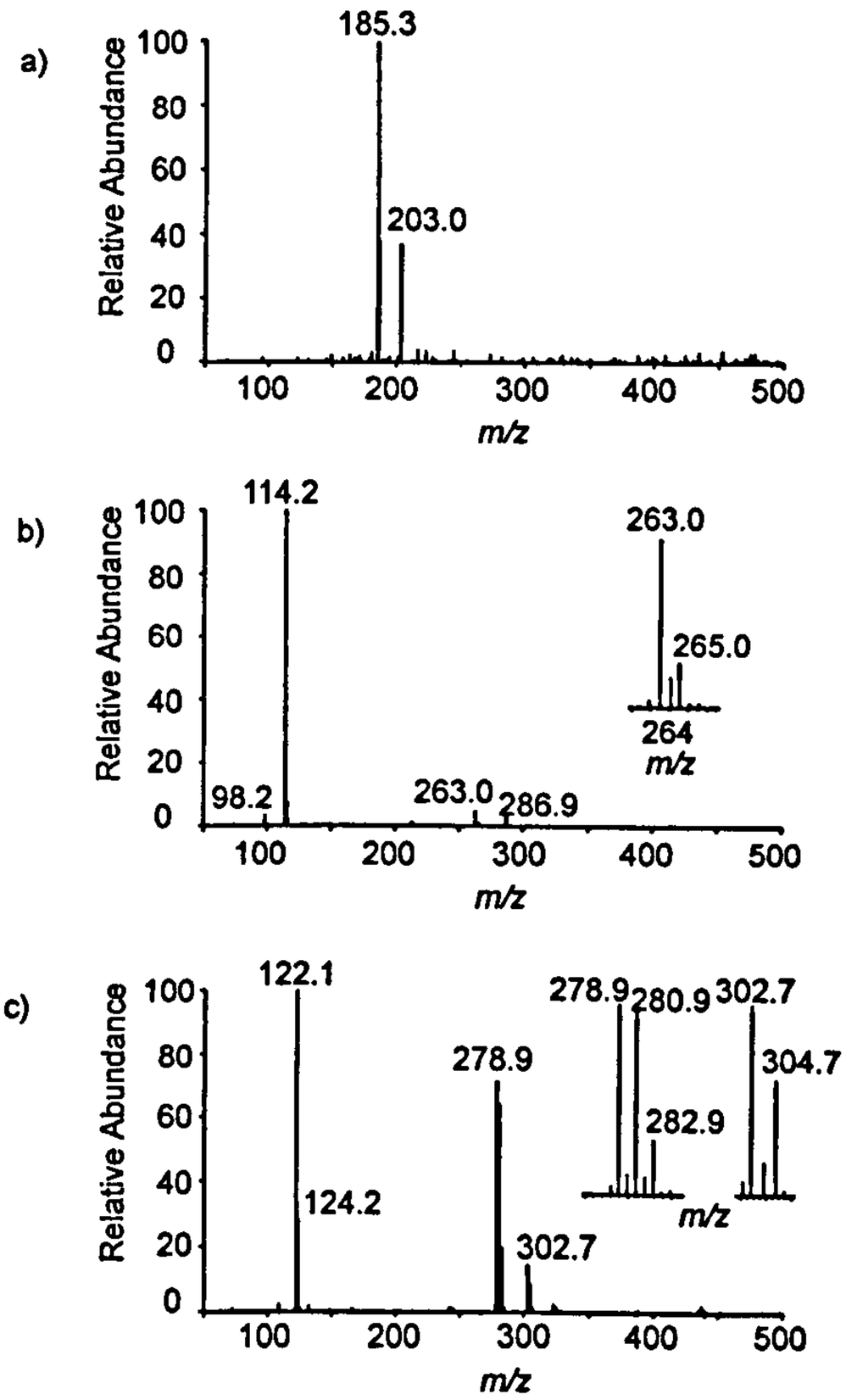


Figure 4.2: Full scan mass spectra of a) PQ b) MQ and c) CQ over the range *m/z* 50 – 500.

In order to examine formation of the ion at *m/z* 203, observed in the PQ mass spectrum, five sets of experiments were performed at various ammonium acetate concentrations. The ion at *m/z* 203 was observed at ammonium acetate concentrations above 10 mM and increased in abundance at higher concentrations (Figure 4.3). The ion at *m/z* 203 arises through addition of 17 Da to *m/z* 186 ($M_q^{+\cdot}$) and could be attributed to addition of either ammonia or $OH^{\cdot}$. To ascertain which, the mobile phase was replaced with ammonia solution and with potassium hydroxide solution. Replacement of the mobile phase by ammonia solution (10 mM) produced significantly more *m/z* 203 than was formed at the higher ammonium acetate concentrations (25 and 50 mM). With potassium hydroxide solution (10 mM) as the mobile phase *m/z* 203 was not observed, confirming that the adduct was not formed through addition of $OH^{\cdot}$. Thus, the ion at *m/z* 203 is attributed to $[PQ.NH_3]^{+\cdot}$. MS/MS of *m/z* 203 produced the monocation at *m/z* 185 corresponding to $[PQ.NH_3^{+\cdot} - NH_3 - H^{\cdot}]^{+}$.

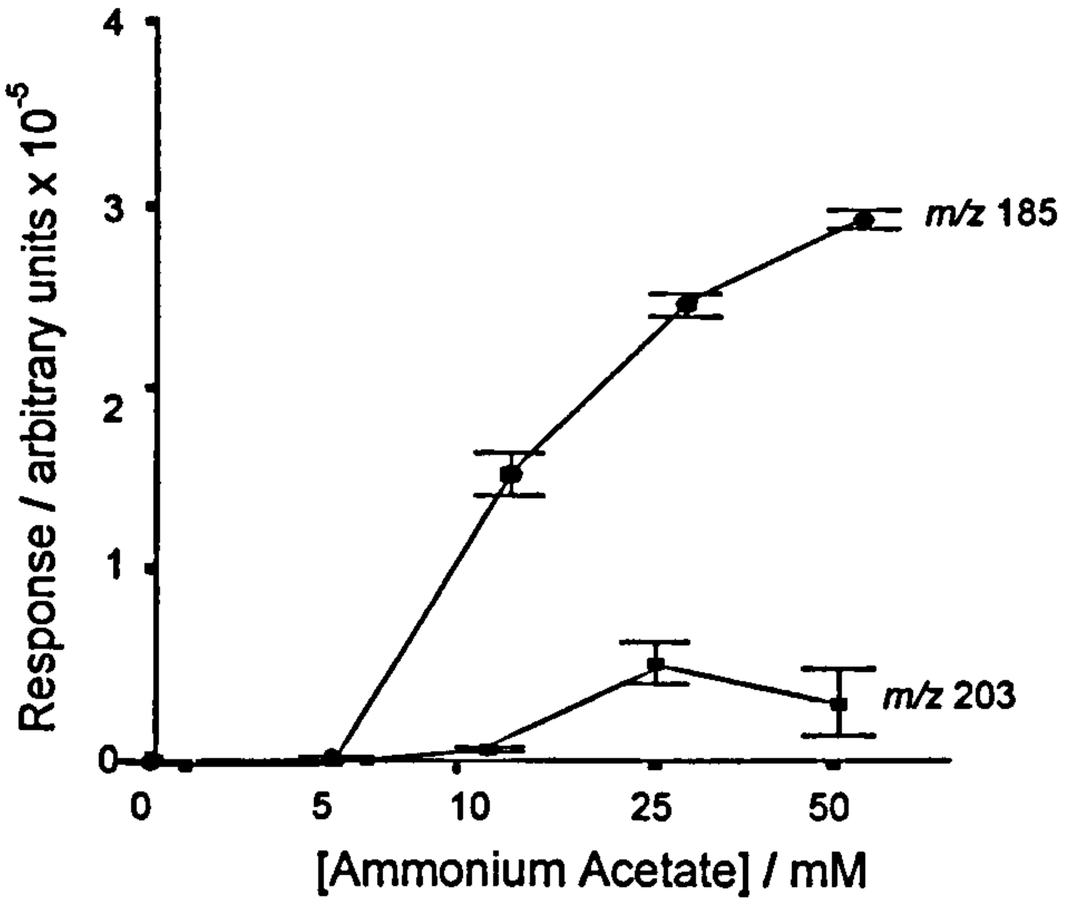


Figure 4.3: Response for *m/z* 203 and *m/z* 185 from PQ as a function of ammonium acetate concentration.

The response for the PQ monocation at *m/z* 185 also increased over the range of ammonium acetate concentrations (Figure 4.3), suggesting that formation of the adduct and the monocation are both dependent on the presence of ammonium acetate within the electrosprayed sample. Clearly this has implications for the sensitivity and LOD of methods based on determination of either the monocation or product

ions formed from it. Thus, appropriate mobile phase compositions should be chosen. A previous study showed the formation of ion pair complexes between PQ and acetate and between PQ and chloride (Taguchi *et al.*, 1998). In the present study, clusters with acetate were not observed over the range of ammonium acetate concentrations investigated (0 to 50 mM), and in a mobile phase containing equimolar concentrations of chloride and acetate, PQCl was the only ion-pair present in the spectrum, indicating preferential association of PQ with chloride.

In the analysis of MQ the MQ^+ ion at *m/z* 114 was accompanied by groups of ions centred on *m/z* 263 and 287 (Figure 4.2b). The ions around *m/z* 263 have an isotope pattern consistent with the presence of one chlorine atom within the cluster (Figure 4.2b inset) and are assigned formally as $[2MQ^+ + Cl^-]^+$. The cluster at *m/z* 287 lacks the chlorine isotope pattern and is consistent with a mepiquat-acetate cluster $[2MQ^+ + Ac^-]^+$. Thus, MQ forms mainly dimeric clusters of the type $[2M_q^+ + A^-]^+$, as has been observed for other quaternary ammonium compounds (Fisher *et al.*, 1994).

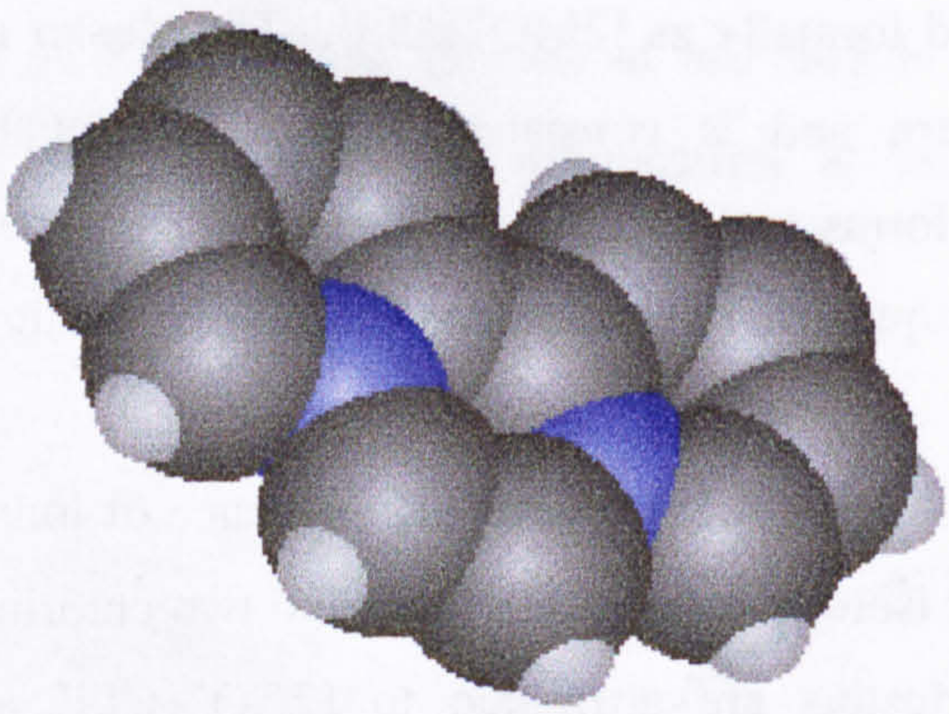
The mass spectrum of CQ (Figure 4.2c) reveals the presence of ions at *m/z* 279 and 303, showing characteristic isotope patterns for three and two chlorines, respectively. Accordingly, the major clusters are attributed to $[2CQ^+ + Cl^-]^+$ at *m/z* 279 and $[2CQ^+ + Ac^-]^+$ at *m/z* 303, revealing a similarity in the cluster-forming behaviour of CQ and MQ.

The clusters of MQ and CQ containing acetate and chloride were isolated (IW = 5 Th) in the ion trap and maintained good signal response over a range of AQ ($q_{z\text{-}excite}$) values while no activation was applied (AA = 0 V). Such behaviour has been observed previously for CQ^+ (Chapter 3, Figure 3.9a; Evans *et al.*, 2000a) and shows that the ions can be isolated and trapped over a wide range of AQ values, confirming them as cluster species and not artefacts.

Although a previous FAB-MS study of DQ (Tondeur *et al.*, 1987) reported the presence of a DQ-monobromo ion-pair complex (*m/z* 263/265), the mass spectrum obtained in this study did not contain ions at *m/z* values greater than $[M_q^{+\bullet} - H^\bullet]^+$ at *m/z*

183. Thus, DQ does not form detectable ion-pair complexes or cluster species under the ES-MS conditions used here. This is consistent with a previous report in which DQ ion-pair complexes were not detected by ES-MS, as a result of the facile loss of the counterions during the ES process (Taguchi *et al.*, 1998). Cluster species of DQ are probably not formed due to steric constraints (Figure 4.4a). Similarly, the mass spectrum for DF did not reveal ions greater than *m/z* 249, the M_q^+ ion. The lack of formation of DF cluster species is probably also due to steric constraints (Figure 4.4b).

a)



b)

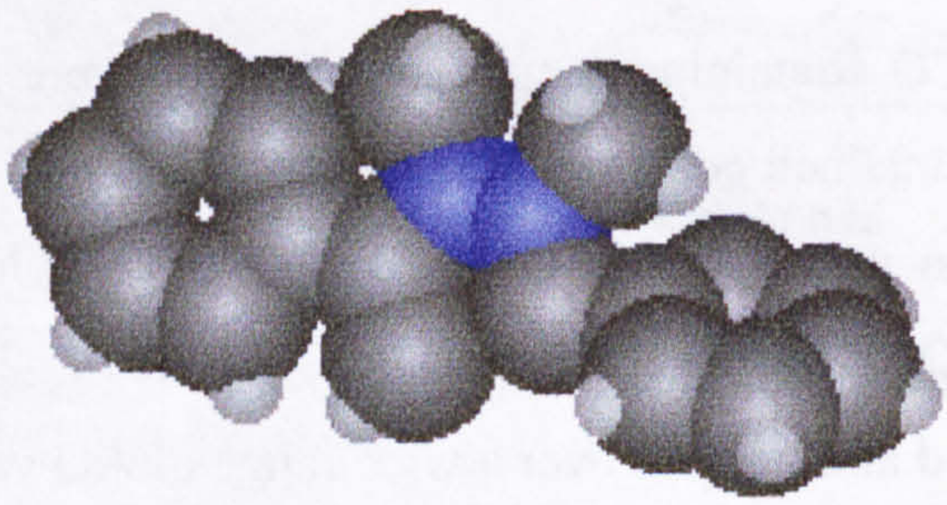


Figure 4.4: Space-filling representations of the structures of a) DQ and b) DF: dark grey represents carbon atoms; light grey hydrogen atoms and blue nitrogen atoms [original in colour].

Of the five quaternary ammonium pesticides studied, only MQ and CQ were found to form cluster species both with acetate and with chloride anions. Accordingly, further work performed in order to rationalise cluster formation only involved these two pesticide cations.

4.2.1.3 Formation of chloride-containing clusters

The experiments outlined above featured an analytical system containing both acetate and chloride anions. The acetate anions originated from the mobile phase while the chloride ions were the counterions for MQ^+ and CQ^+ in solution. In order to monitor only the formation of chloride-containing cluster species, direct infusion of the individual quat solutions into the mass spectrometer was performed. Direct infusion of MQCl, monitored over *m/z* 50 - 1000, revealed a single dimeric cluster $[2MQ^+ + Cl^-]^+$ at *m/z* 263, indicating that MQ does not form higher-order clusters of the type $[nMQ^+ + (n-1)Cl^-]^+$ where $n > 2$. By contrast, direct infusion of CQCl over *m/z* 50 – 1250 revealed that, in addition to the cluster corresponding to $[2CQ^+ + Cl^-]^+$ at *m/z* 279, other groups of ions centred on *m/z* 438, 595, 754, 911, 1070 and 1227 were present (Figure 4.5). Clusters were also observed over the entire *m/z* range 50 – 2000 though the operating conditions discriminate against the ions at lower *m/z* values. The cluster ions in the range *m/z* 438 – 1227 show characteristic patterns for increasing numbers of chlorine atoms. For example, the ion at *m/z* 754 corresponds to $[5CQ^+ + 4Cl^-]^+$, *i.e.* with 9 chlorines in total. Clearly, therefore, CQ^+ and free chloride anions readily form clusters of the type $[nCQ^+ + (n-1)Cl^-]^+$, where $n > 2$, extending across the whole *m/z* range.

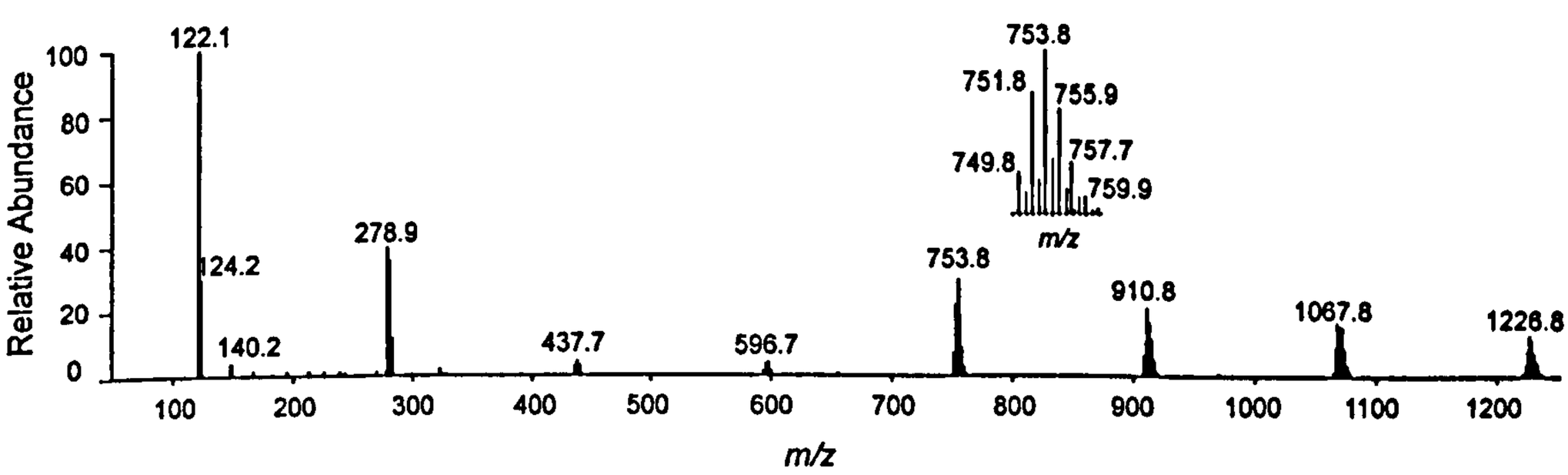


Figure 4.5: Full scan mass spectrum of CQ over the range *m/z* 50 – 1250.

The mass spectrum (Figure 4.5) reveals varying signal levels for the different cluster species, indicating differences in their stabilities or ease of formation. The species at *m/z* 754 $[5CQ^+ + 4Cl^-]^+$ (alternatively written $[CQ^+(CQCl)_x]$, where $x = 4$) exhibited a slight dominance. Stability of clusters where $x = 4$ has been observed previously for tetrabutylammonium bromide clusters monitored by ES-MS (Zook and Bruins, 1997). The low signal level for $x = 2$ and 3 at *m/z* 438 and 595 is probably due to non-ideal conformations and hence lack of stability associated with the cluster. The propensity for CQ to form clusters, with higher clusters being particularly prevalent, is in marked contrast to MQ which was only observed to form clusters of the type $[2MQ^+ + Cl^-]^+$. This difference may be related to steric factors associated with greater hindrance around the nitrogen in MQ due to the rigidity of the ring system.

MS/MS spectra were obtained in order to confirm the assignment of the cluster species based on isotope pattern recognition. Isolation of the mepiquat cluster $[2MQ^+ + Cl^-]^+$ within the ion trap (IW = 5 Th), and subsequent MS/MS fragmentation with a collision energy of 22% (based on tuning of the total ion current generated in MS/MS), yielded exclusively *m/z* 114, the free MQ^+ ion, with an overall efficiency of 81%. Similarly, MS/MS (AA = 0.96 V, AQ = 0.354, AT = 35 ms and IW = 5 Th) of the cluster at *m/z* 279 produced *m/z* 122, corresponding to CQ^+. MS/MS of the higher order CQ clusters revealed that the majority of MS/MS transitions from the cluster species involved the loss of 157 Da, corresponding to $[CQ^+Cl^-]$. Thus, the product ions show isotope clusters characteristic of two fewer chlorines, one from the counterion and one from within CQ. Clearly, however, this MS/MS strategy cannot be used as a means to improve the ion count for the free quat ion under full scan MS conditions. Thus, in an attempt to dissociate cluster species, $[2MQ^+ + Cl^-]^+$ and $[nCQ^+ + (n-1)Cl^-]^+$, a range of collision energies (between 0 and 100%) were examined for in-source collision induced dissociation (CID). The only effect observed was a decrease in the ion count for both clusters and for the free quat cation, indicating that in-source CID also cannot be used to increase the signal for the free quat cation.

4.2.1.4 Formation of acetate-containing clusters

The preliminary experiments indicate that both MQ^+ and CQ^+ form cluster species with acetate anions. The effect of acetate concentration on the formation of these cluster species was investigated. The ammonium acetate concentration within the mobile phase was varied while keeping the quat concentration constant at 10 μg mL^{-1}. The signal levels for the cluster species $[2M_q^+ + Ac^-]^+$ (*m/z* 287 for MQ and *m/z* 303 for CQ) increased to a constant value between 25 and 50 mM ammonium acetate (Figure 4.6). The plateau in signal response indicates a saturation point for cluster formation for both species. In addition to the expected increases in acetate cluster formation, decreases in the signal levels for the corresponding chloride clusters, $[2M_q^+ + Cl^-]^+$ at *m/z* 263 and 279, are apparent (Figure 4.6). This is attributed to partitioning according to the relative concentrations of counter ions present. Thus, acetate clusters are more prevalent at high concentrations of cation and acetate counterion.

Previously, it was reported that formation of cluster species had a negligible effect on the signal for the free tetraalkylammonium ion (Zook and Bruins, 1997). In our studies, the signal level obtained for the free MQ^+ and CQ^+ cations was found to decrease with increasing ammonium acetate (Figure 4.6). The decreases in signal level for the free quat ions at high ammonium acetate concentration could be due either to signal suppression within the electrospray or to incorporation of the quat cation within a cluster species; the latter is the more likely. Thus, it is clear that the sensitivity for the free quat ions may be limited at high concentrations, and will affect the LOL of a calibration.

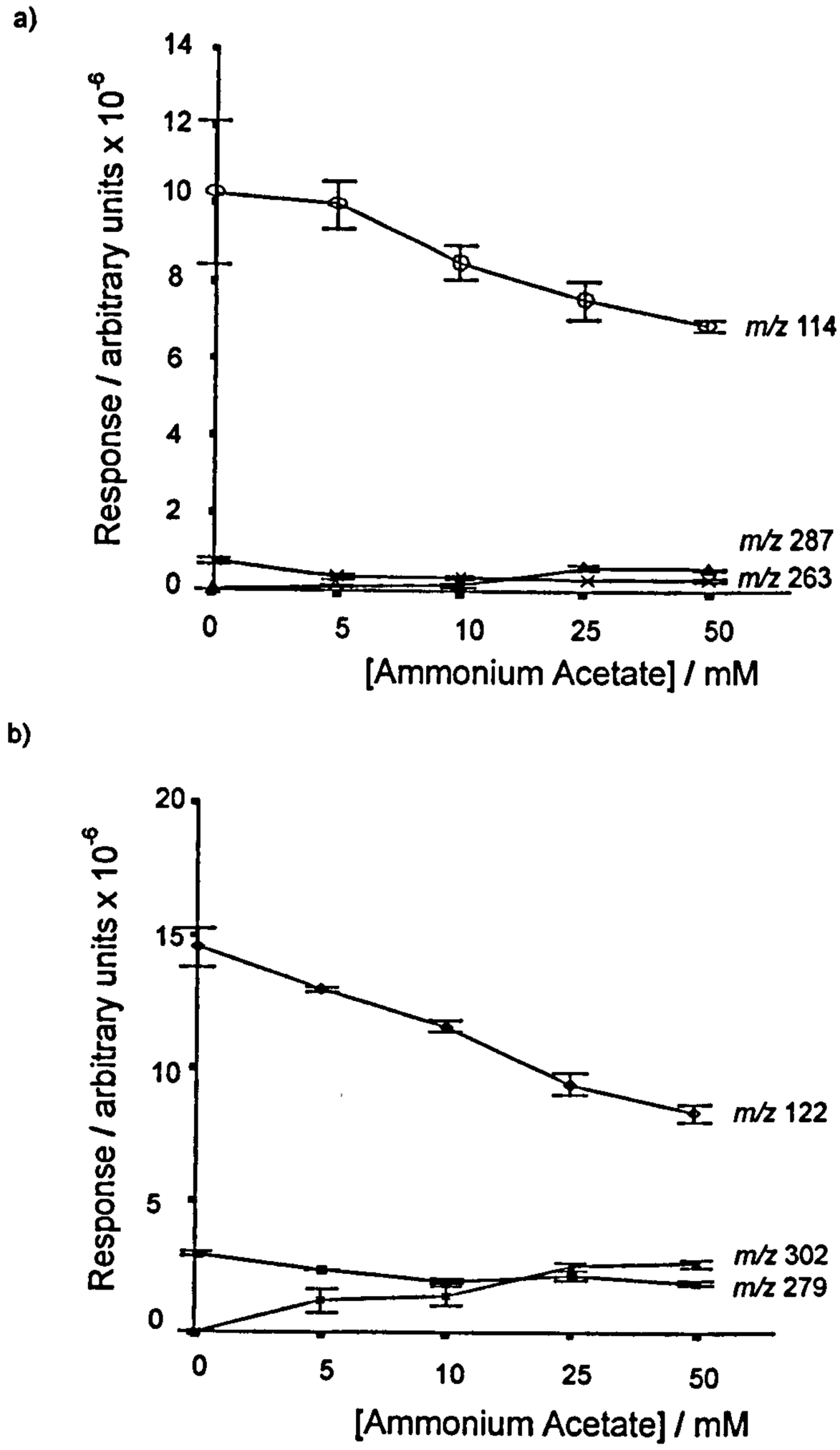


Figure 4.6: Response for the free quat cation (M_q^+), acetate cluster $[2M_q^+ + Ac^-]^+$ and chloride cluster $[2M_q^+ + Cl^-]^+$ as a function of ammonium acetate concentration for a) MQ and b) CQ.

Acetate cluster formation was examined over a range of MQ and CQ concentrations in a mobile phase containing 50 mM ammonium acetate, as is routinely employed in analytical methods reported for determination of MQ and CQ (Vahl *et al.*, 1998; Juhler and Vahl, 1999; Evans *et al.*, 2000a,b; Hau *et al.*, 2000; Startin *et al.*, 2000). Given that there is an excess of acetate present, it is assumed that the maximum number of acetate clusters will be formed. Acetate clusters were not produced below

2 μg mL^{-1} MQ (equivalent to 1.4 x 10^{-5} M), but $[2CQ^{+}+Ac^{-}]^{+}$ was formed with an abundance of 8% relative to base peak at 0.01 μg mL^{-1} (equivalent to 6.3 x 10^{-8} M) for CQ. At high concentrations of MQ and CQ the proportion of acetate cluster to free quat remained fairly constant (30 – 40%). The results indicate that the CQ acetate cluster, $[2CQ^{+}+Ac^{-}]^{+}$, is formed with greater relative ease than the corresponding MQ cluster, probably due to similar steric considerations to those proposed for chloride cluster formation. Clearly, the formation of clusters will influence the LOL and reproducibility of an analytical method.

4.2.1.5 Competition studies

Given that in the initial experiments (Section 4.2.1.2) the quats were introduced as the monochlorides, competition for the quat cation between chloride and acetate anions present within the analytical system is likely to have occurred. To ascertain which anion more readily associates with the quat cation, acetate was introduced into the system at the same concentration (6.6 mM) as chloride and the signals for the chloride and acetate cluster species were monitored. The spectra obtained for both MQ and CQ revealed that the chloride cluster had the more intense signal by about one order to magnitude, indicating greater thermodynamic stability, consistent with chloride having a higher charge density than acetate..

4.2.2 MS^n studies of the quats

4.2.2.1 Stability of trajectories of the quat precursor ions within the ion trap

The base peak ions observed in full scan MS studies of the quats can be subjected to MS/MS analysis. Solutions of the quats were infused and the signal response for these precursor ions was monitored over the full range of AQ values, whilst maintaining AA and AT at 0 V and 0.03 ms respectively. As observed previously for CQ (Section 3.2.3) the signal response for *m/z* 122 is zero for AQ values below ~0.1 and above 0.9 (Figure 3.9). The profile shows a steep sided plateau between 0.115 and 0.881, indicating the range of AQ values over which the precursor ion *m/z* 122 exhibits a stable trajectory and is trapped efficiently. Similar profiles, typically over

the AQ range 0.1 to 0.9, were observed for the MQ, DQ, PQ and DF precursor ions (Table 4.1).

The results in Table 4.1 reveal that ions with higher *m/z* ratios (DF, *m/z* 249) require lower AQ values to stabilise the ion trajectory. This observation is in good agreement with ion trap theory, which predicts that ions with higher *m/z* ratios have associated q_z (AQ) values near the origin (Section 3.1.1.4). Conversely, ions with lower *m/z* ratios (MQ, *m/z* 114) have higher associated q_z values and so the response plateau begins at a higher value of AQ. The table also shows that the upper AQ limit for the signal response plateau increases with increasing *m/z*. The AQ upper limit represents the q_z value at which the trajectory of the ion becomes unstable, resulting in exclusion from the trap without detection.

All five quat cations are shown to be trapped efficiently and to have stable trajectories within the trap over a broad range of AQ. Clearly, any loss in signal intensity during MS/MS analysis is not due to instability in the trajectory of the precursor in the trap, but is likely to be due to poor trapping of the product ions (Evans *et al.*, 2000a).

Quat	*m/z* of monocation	Signal Response Plateau	
		AQ lower limit	AQ upper limit
MQ	114	0.120	0.865
CQ	122	0.115	0.881
DQ	183	0.100	0.887
PQ	185	0.100	0.887
DF	249	0.085	0.890

Table 4.1: AQ limits observed for the signal response plateaux for the five quats.

4.2.2.2 CQ fragmentation pathways

As discussed previously in Chapter 3, the CQ^+ precursor at *m/z* 122 can be isolated efficiently (IW = 1.5 Th) and is amenable to MS^2 analysis if the operating conditions of the trap are optimised. In order to investigate the MS^2 fragmentation under conditions optimised for the maximum production of the ion at *m/z* 58, a solution of CQ (10 μg mL^{-1}) was infused into the mobile phase (50 mM ammonium acetate in 50/50 methanol/water) with the optimal trap settings of AA = 0.96 V, AQ = 0.354, AT = 35 ms. In addition to the main fragments at *m/z* 58 and 59 a range of product ions (*m/z* 106, 94, 86, 60) not reported previously for CQ was observed (Figure 4.7a).

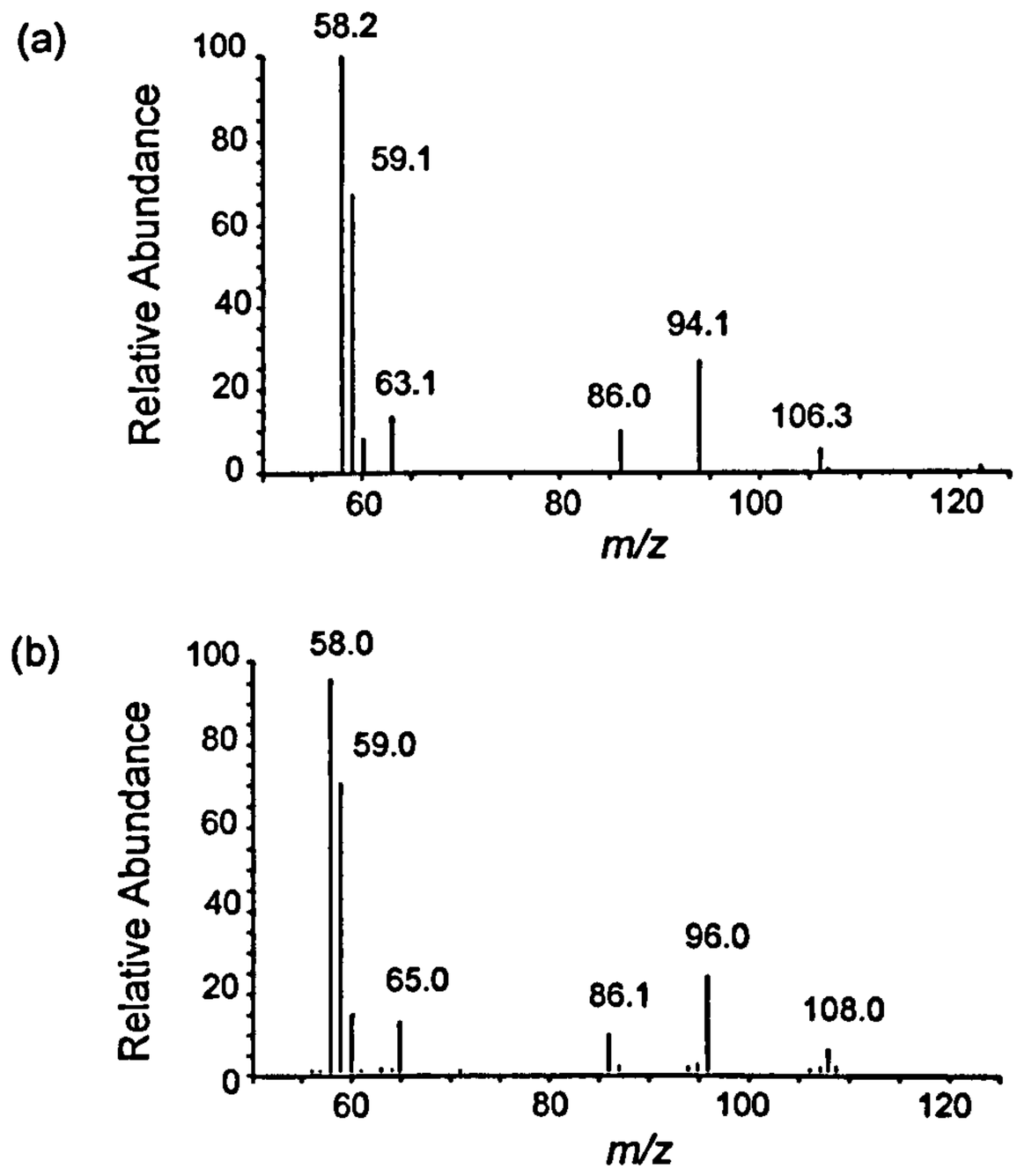


Figure 4.7: Full scan MS/MS spectra of CQ obtained under optimised conditions (AA = 0.96 V, AQ = 0.354, AT = 35 ms) (a) $^{35}Cl\ M_q^+$ at *m/z* 122 and (b) $^{37}Cl\ M_q^+$ at *m/z* 124.

Thus far, attention has focussed on fragmentation of the $^{35}Cl\ M_q^+$ ion at *m/z* 122. The fragmentation products from the $^{37}Cl\ M_q^+$ ion at *m/z* 124 have yet to be

considered. The ^{37}Cl M_q^+ ion at *m/z* 124 fragmented under the same conditions as *m/z* 122 to give the mass spectrum shown in Figure 4.7b.

The majority of product ions generated from *m/z* 124 have the same *m/z* values as those from *m/z* 122. Three of them, however, are 2 Da greater, indicating that they contain chlorine and aiding in their assignment (Figure 4.8). The ions at *m/z* 63/65 have also been reported by Hau *et al.* (2000), and have been assigned formally as $Cl\text{-}CH_2\text{-}CH_2^+$ (Hau *et al.*, 2000; Evans *et al.*, 2000a). The mechanism for production of *m/z* 94/96 probably involves an intramolecular elimination of ethene which results in the formation of the chlorotrimethylammonium ion (Figure 4.9), while formation of *m/z* 106/108 occurs by loss of methane (Figure 4.8).

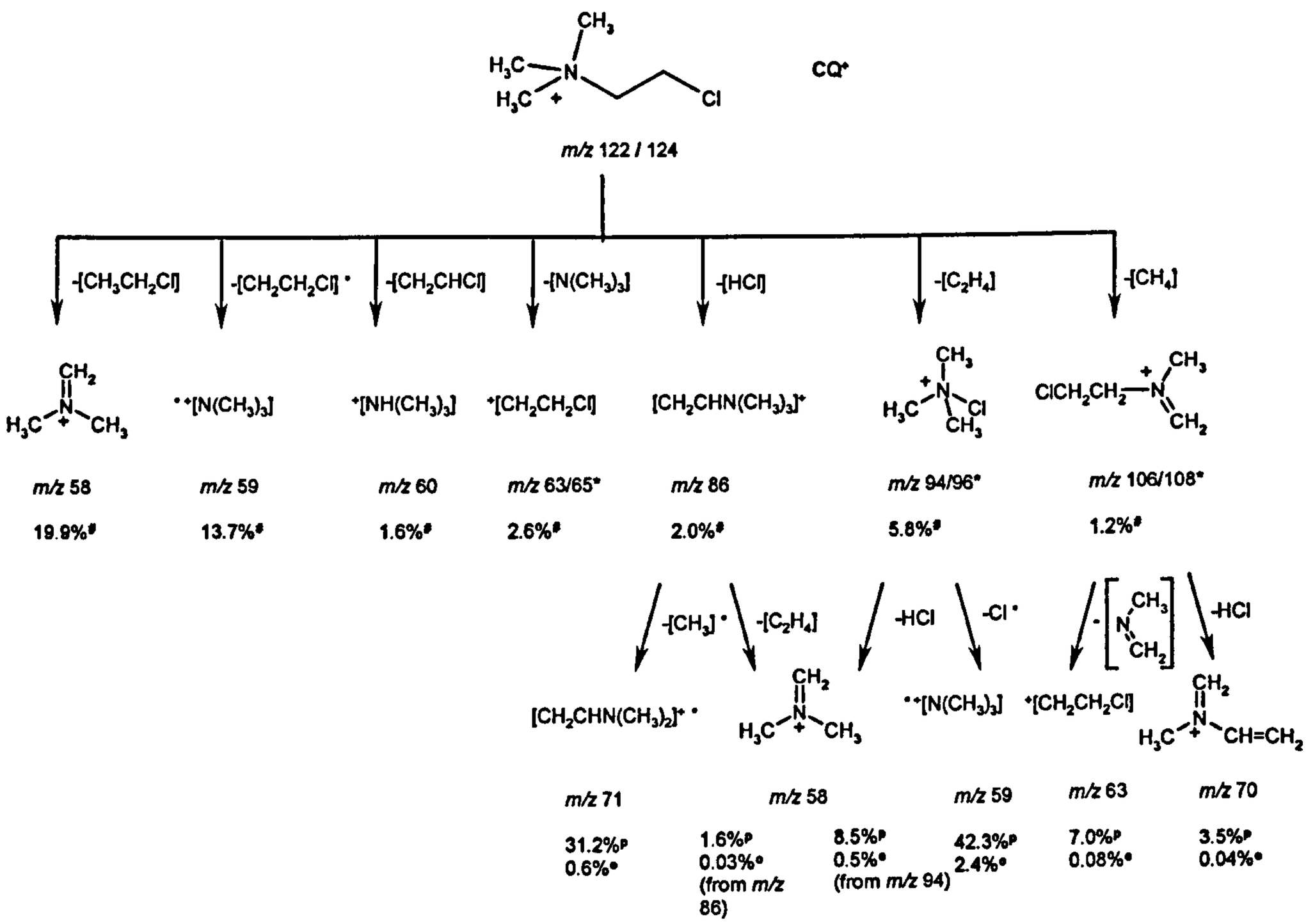


Figure 4.8: Fragmentation map for CQ
*** Ions formed only from *m/z* 124;**
 # Fragmentation efficiencies reported only for fragmentation of *m/z* 122;
p Fragmentation efficiencies from MS/MS precursor;
o Overall fragmentation efficiency from *m/z* 122.

Previously, Mol *et al.* (2000) assigned the product ions from *m/z* 122 to $[CH_2=CH-N(CH_3)H_2]^+$ (*m/z* 58) formed by exchange of 2 methyl groups for hydrogen combined with a loss of HCl, and attributed *m/z* 59 to the corresponding isotope peak. From our studies, however, it can be seen that *m/z* 59 is formed in high abundance (66% and 74%; Figure 4.7) from both ^{35}Cl and ^{37}Cl containing precursors. Thus *m/z* 59 is a true product ion, most likely a radical cation formed by loss of a chloroethyl radical, and is potentially of value for confirmatory detection. This assignment is consistent with the formation of the ion at *m/z* 58 through loss of chloroethane to form the dimethylimmonium ion $[CH_2=N(CH_3)_2]^+$ (Figure 4.8). The ions at *m/z* 86 and 60 arise from both *m/z* 122 and 124 and are attributed to the trimethylvinylammonium and trimethylammonium cations, respectively. These cations have also been reported as impurities in CQ standards analysed by CE-ES-MS (Wycherley *et al.*, 1996). Given the reports of these ions in the spectra of standards, their recognition alone is insufficient for the assignment of CQ. Hence, quantification of CQ based on detection of these ions (*m/z* 86 and 60) is only valid using MS/MS approaches.

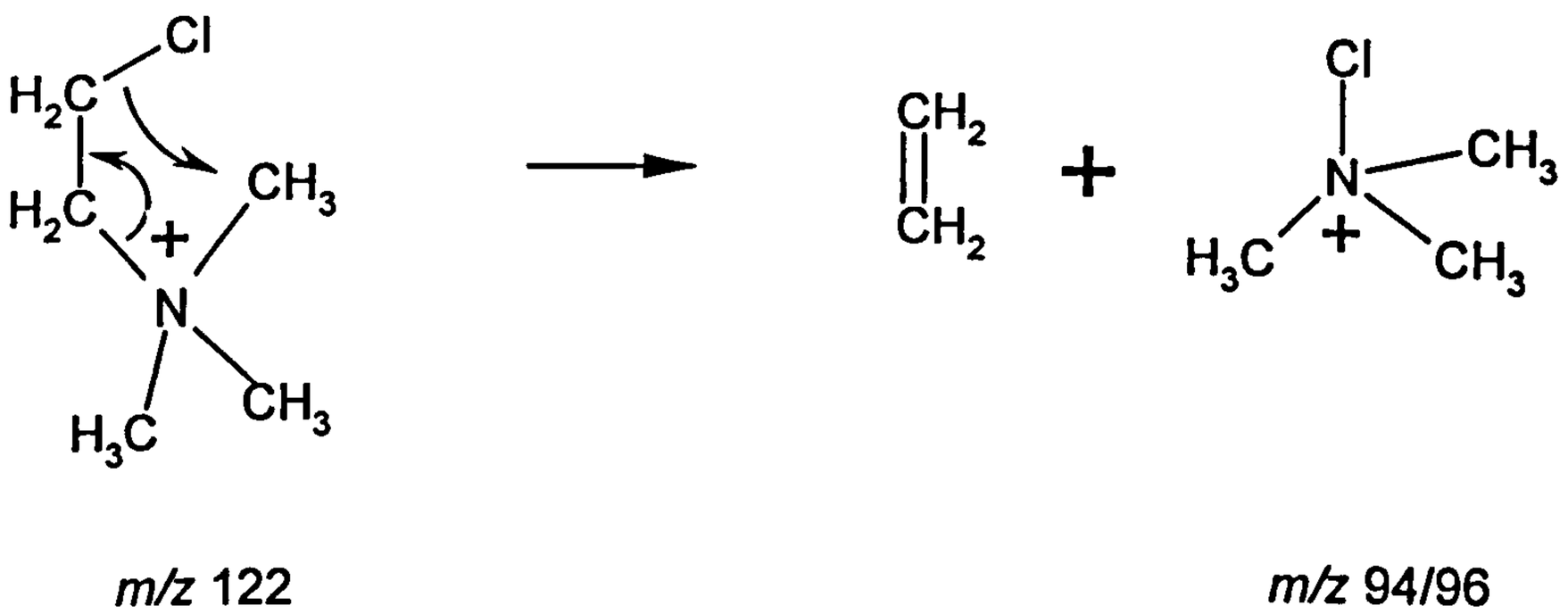


Figure 4.9: Proposed mechanism for fragmentation of CQ to *m/z* 94/96.

Further characterisation of the CQ fragmentation pathways can be achieved by performing MS^3 analyses. MS^2 product ions were subjected to MS^3 analysis with lower values of AA and with AT reduced to around 10 ms. Since the precursors are smaller, less energy is required to effect fragmentation. The MS^3 product ions were formed with low overall fragmentation efficiency (Figure 4.8), where the

fragmentation efficiencies were calculated for each of the MS^n product ions using Equation 4.1. It is noted that only MS^2 precursors with *m/z* values of 86 or above gave detectable products. This is attributed to the low *m/z* values of the product ions formed from *m/z* 63, 60, 59 and 58, leading to unstable trajectories within the trap under the conditions used here. It is noteworthy that only the MS^2 ions reported for the first time in this study gave detectable product ions in MS^3. Although the transitions for MS^2 to MS^3 were not optimised rigorously (*i.e.* using simplex) the overall efficiencies from *m/z* 122 (Figure 4.8) are sufficiently high to suggest that three of them (122 – 94 – 59, 122 – 94 - 58 and 122 – 86 – 71) would be suitable for highly specific quantification of CQ *via* a SRM strategy (Evans *et al.*, 2001a).

$$F = \frac{D}{P_o} * 100$$

Equation 4.1

where F = fragmentation efficiency; D = absolute product ion intensity observed in the MS^n spectrum and P = absolute precursor ion intensity observed under full scan MS conditions prior to fragmentation.

4.2.2.3 MQ fragmentation pathways

Previous MS/MS studies of MQ revealed an intense ion at *m/z* 98 (Moyano *et al.*, 1996; Castro *et al.*, 1999; Juhler and Vahl, 1999) and weaker ions at *m/z* 58 and 99 attributed to loss of $CH_3^{\bullet}$ as a radical (*m/z* 99), loss of methane (*m/z* 98) and loss of C_4H_8 (*m/z* 58; Moyano *et al.*, 1996; Castro *et al.*, 1999). A similar approach to that described above for CQ was used for a more complete MS/MS study of MQ. An aqueous solution of MQ (10 μg mL^{-1}) was infused into the mobile phase (50 mM ammonium acetate in 50/50 methanol/water) with instrument parameters on the LCQ operated under basic software control. An IW of 1.5 Th was found to trap the precursor ion *m/z* 114 efficiently. During MS/MS, however, no product ions were detected; this is attributed to the ions having unstable trajectories within the trap, as reported previously for CQ (Chapter 3; Evans *et al.*, 2000a).

Preliminary investigations varying the advanced trapping parameters revealed the presence of the ions *m/z* 99, 98 and 58. In addition, a weaker ion, not seen previously, was observed at *m/z* 69. This product ion is assigned formally as arising through loss of $HN(CH_3)_2$ (Figure 4.10). MS^n Fragmentation of *m/z* 99 and 98 produced a single product ion (*m/z* 70) as base peak, assigned formally as arising through loss of an ethyl radical from *m/z* 99 and ethene from *m/z* 98 (Figure 4.10).

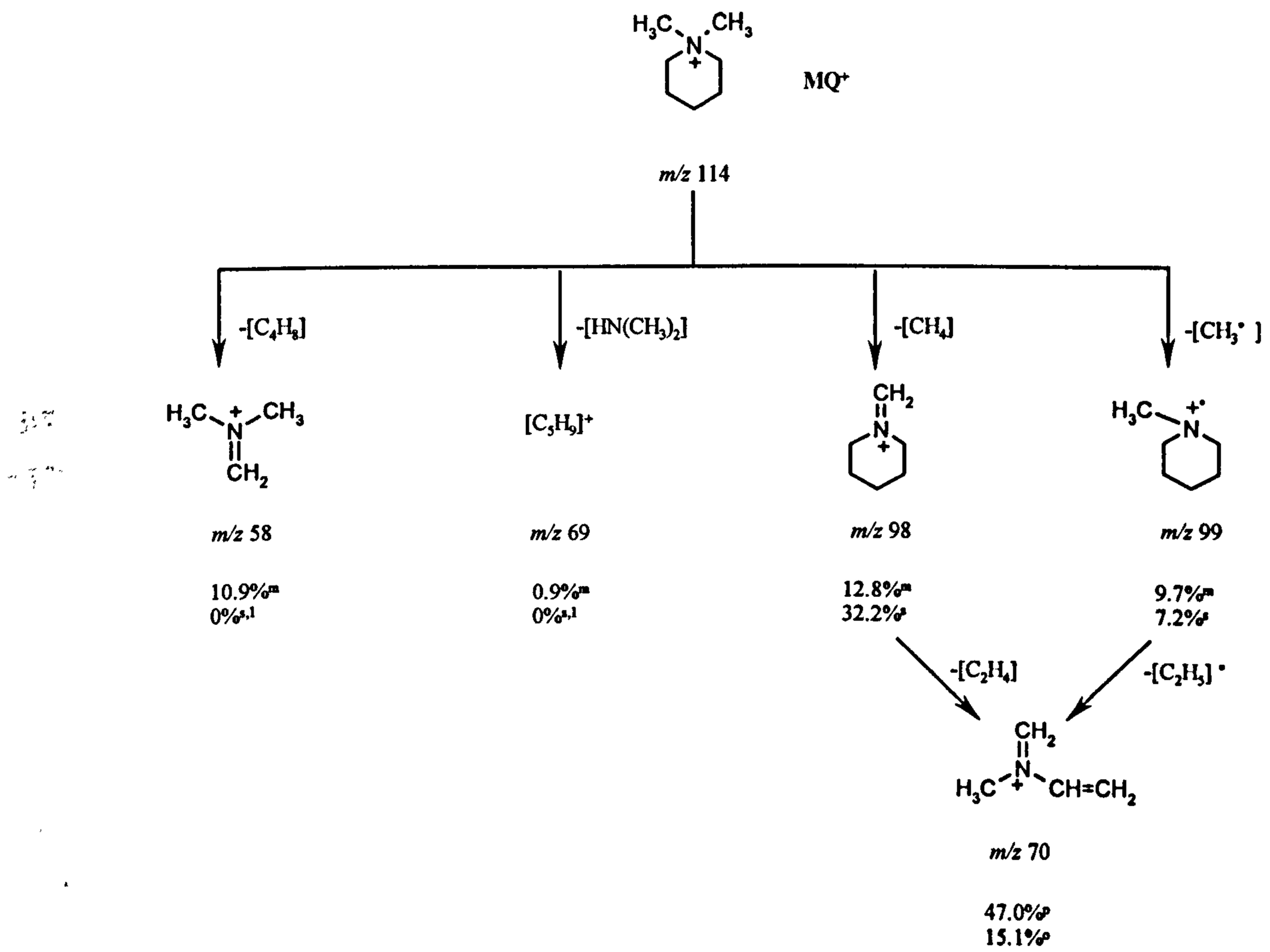


Figure 4.10: Fragmentation map for MQ ([m] Fragmentation efficiency under MIR conditions; [s] Fragmentation efficiency under conditions optimised specifically for the transition *m/z* 114 to 98; [o] Overall fragmentation efficiency from *m/z* 114; [1] *m/z* 69 and 58 are not observed due to the LMCO of the trap being set too high).

4.2.2.4 MQ optimisation of transitions

The MS/MS conditions employed in the analysis detailed above were not optimised. The benefit of optimising MS/MS conditions for product ion response has been established for CQ, and a similar strategy was employed for MQ. Variable size simplex optimisation of MS^2 trapping parameters was carried out in order to obtain the highest total ion count based on the four transitions (*m/z* 114 to 99, 98, 69 and 58), here termed maximum ion response (MIR; Evans *et al.*, 2001a). Since a product ion at *m/z* 58 was expected (Moyano *et al.*, 1996), the maximum value of AQ that could be employed was calculated using Equation 3.3 to be 0.461. The lower AQ limit (0.120) is determined by the stability of the trajectory of the precursor ion within the trap (Table 4.1). Under the MIR conditions (AA = 1.16 V, AQ = 0.364, AT = 36.58 ms) *m/z* 58, 98 and 99 were obtained in similar abundances (Figure 4.11), indicating similar efficiencies for their formation from *m/z* 114 (Figure 4.10). This contrasts with previous studies on quadrupole analysers where *m/z* 98 was found to be the most abundant product ion by a factor of 1.6 (Moyano *et al.*, 1996; Juhler and Vahl, 1999).

It is also possible to select trapping parameters that are optimal for a specific transition (Evans *et al.*, 2001a), and such conditions should give the best limit of detection (LOD) for SRM. The response for the transition *m/z* 114 to 98 was optimised at AA = 1.27 V, AQ = 0.593 and AT = 21 ms. Under these conditions *m/z* 98 was four times more abundant than *m/z* 99 (Figure 4.10). Owing to the high AQ value used, *m/z* 58 and 69 could not be detected (LMCO = *m/z* 74.5). Although the MS^2 to MS^3 transition *m/z* 98 to 70 was not optimised rigorously *i.e.* using simplex, it was found to be highly efficient at 47.0%, equating to an overall fragmentation efficiency of 15.1% from *m/z* 114. Thus, this appears to be the best candidate for selective detection and quantification of MQ using MS^3.

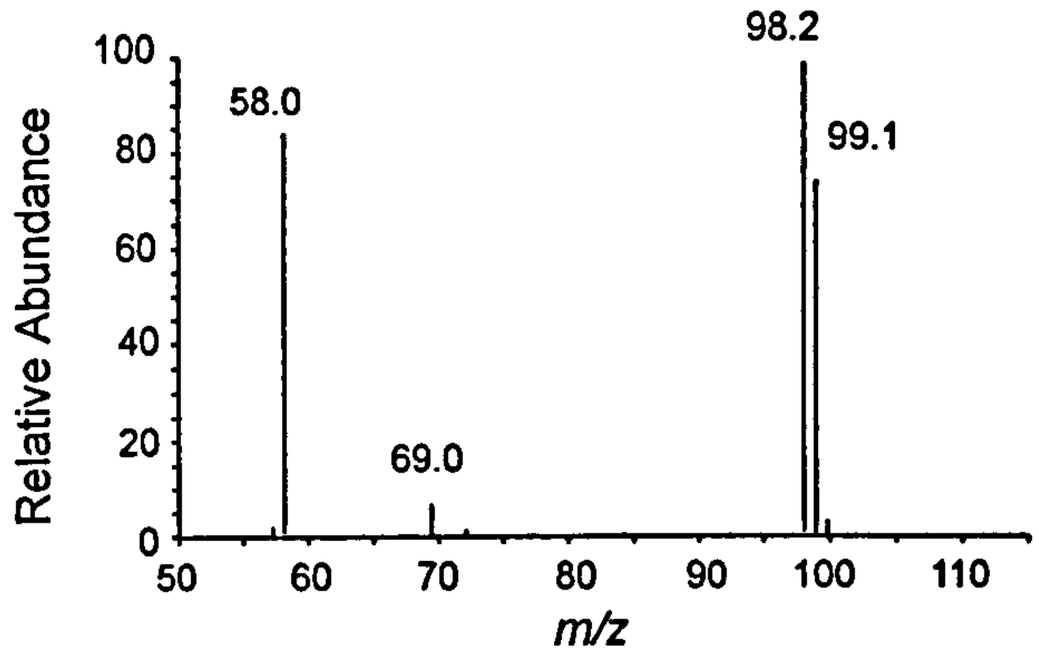


Figure 4.11: Full scan ES MS/MS maximum ion response (MIR) spectrum of MQ.

4.2.2.5 DF fragmentation pathways

Since the DF precursor ion (*m/z* 249) is of higher *m/z* than the MQ and CQ precursor ions it was amenable to MS^2 analysis using the basic software control with IW = 1.0 Th and a collision energy of 28% (based on tuning on the total ion current generated in MS^2). An isolation width of 1.0 Th was found to trap the $^{12}C\ M_q^+$ ion efficiently while excluding the $^{13}C\ M_q^+$ ion from the trap. The resultant MS^2 spectrum was characterised by base peak at *m/z* 208, corresponding to loss of 41 Da. Notably, this was not reported using either CE-ES-MS/MS (Moyano *et al.*, 1996) or LC-ES-MS with in-source CID (Castro *et al.*, 1999). However, it has been observed previously in low abundance using FAB-MS/MS where it was attributed to loss of either CH_3CN or of $CH_3C_2H_2$ (Tondeur *et al.*, 1987). Given that the precursor is an even electron ion, the former appears the more likely. The product ion at *m/z* 146, observed here for the first time, is attributed to the loss of C_6H_5CN (Figure 4.12). The MS^2 spectrum reveals the presence of a significant number of other ions and their assignment (Figure 4.12) is in good agreement with previous observations (Tondeur *et al.*, 1987; Moyano *et al.*, 1996).

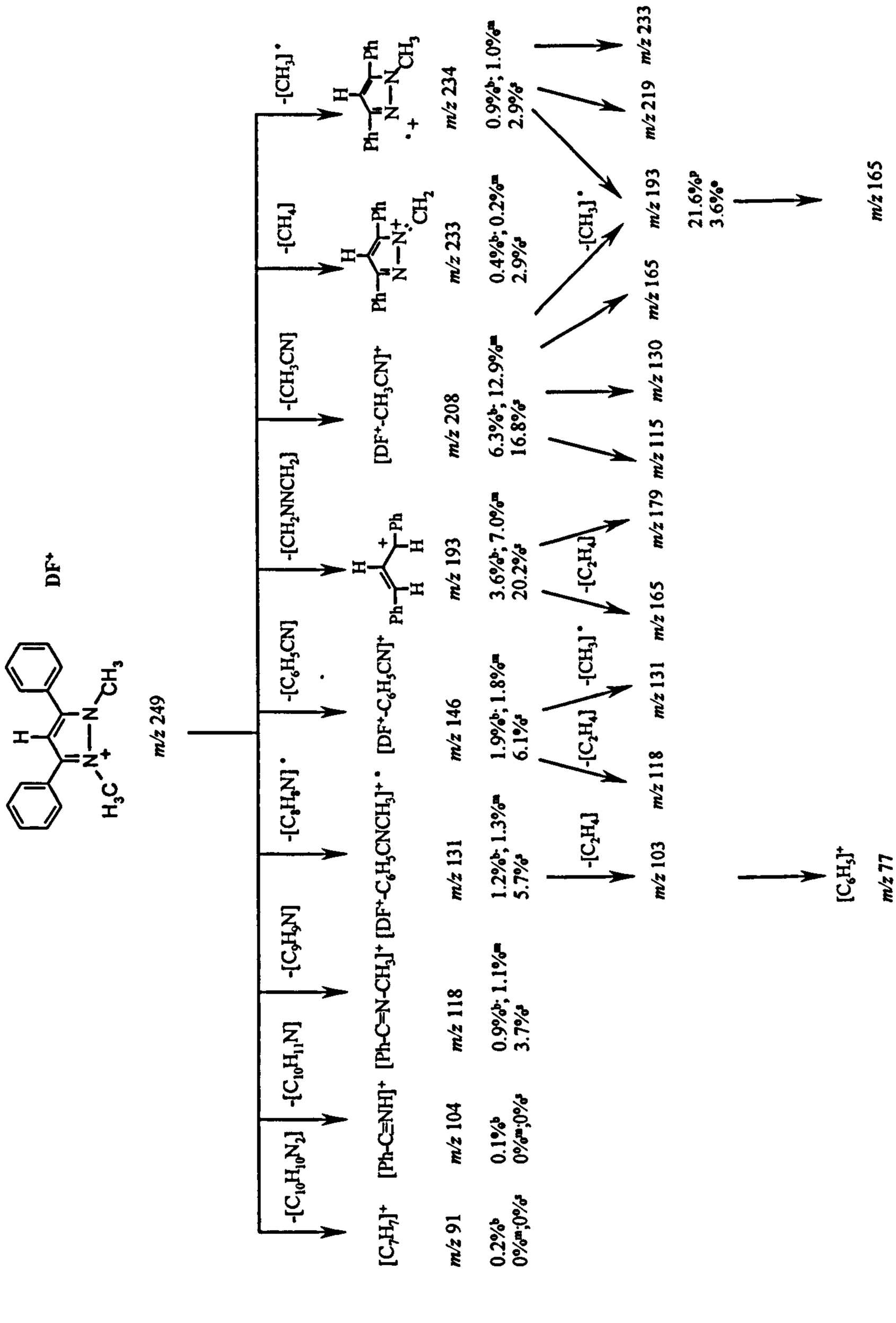


Figure 4.12: Fragmentation map for DF
[b] Fragmentation efficiency under basic conditions;
[m] Fragmentation efficiency under MIR conditions;
[s] Fragmentation efficiency under conditions optimised specifically for the transition *m/z* 249 to 193;
[p] Fragmentation efficiency from MS/MS precursor at *m/z* 208;
[o] Overall fragmentation efficiency from *m/z* 249.

MS^n analyses further characterise the fragmentation pathways for DF (Figure 4.12) and indicate a number of transitions with potential for highly specific detection. One such transition, *m/z* 249 – 208 – 193, appears particularly suitable for inclusion in an SRM strategy on an ion trap instrument.

4.2.2.6 DF optimisation of transitions

Using the basic settings some of the precursor ion (*m/z* 249) remained (12.4%), reducing the fragmentation efficiency. Thus, application of these conditions in an LC-MS^2 method would limit the overall LOD. To improve the fragmentation efficiency, the MS/MS conditions for DF were optimised using simplex. In order to detect the lowest mass ion reported previously, *m/z* 77 (Tondeur *et al.*, 1987; Moyano *et al.*, 1996), AQ has an upper limit of 0.266 (LMCO = *m/z* 73) and the lower limit is 0.085 (Table 4.1). The MIR conditions based on transitions from *m/z* 249 to the fragment ions *m/z* 234, 233, 208, 193, 146, 131 and 118 were found at AA = 1.43V, AQ = 0.259, AT = 22.53 ms (Evans *et al.*, 2001a). Under these conditions (Figure 4.13) the ions at *m/z* 208 and 193 increased in relative intensity but otherwise the spectrum remained essentially unchanged. Notably, however, the fragmentation efficiency for product ions in MS^2 increased to 25%, representing an improvement over basic conditions by a factor 1.6 (Figure 4.12). Thus, use of these advanced conditions within a MS^2 detection strategy would improve the sensitivity over that achievable using the basic conditions.

Optimisation of the MS^2 conditions to promote one precursor ion - product ion transition could further improve the sensitivity achievable in an MS^2 method. In order to determine which precursor ion - product ion transition would give the best LOD, the major transitions from *m/z* 249 were optimised individually using simplex. The greatest fragmentation efficiency, representing a threefold increase over the MIR conditions, was found for *m/z* 249 to 193 (AA = 1.68 V; AQ = 0.403, AT = 7.874 ms; Figure 4.12). Thus, this transition is optimal for specific detection of DF and further confirmation of identity is provided by the fragment ions at *m/z* 234, 233, 208, 146, 131 and 118 (Evans *et al.*, 2001a).

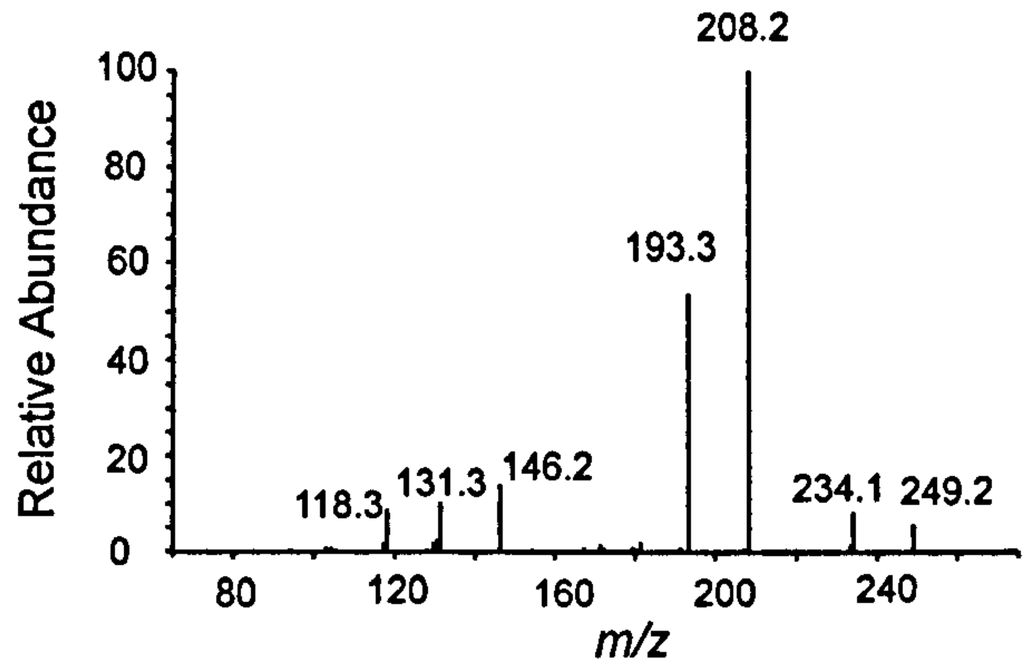


Figure 4.13: Full scan ES MS^2 maximum ion response (MIR) spectrum of DF.

4.2.2.7 PQ and DQ fragmentation pathways

PQ and DQ both gave radical monocations as base peak (*m/z* 185 and 183, respectively), as has been observed previously using a mobile phase containing ammonium acetate (Taguchi *et al.*, 1998) or the ion-pair agent nonafluoropentanoic acid (Takino *et al.*, 2000). In addition, the full scan MS spectra for DQ and PQ show the presence of *m/z* 92 and 184, and of *m/z* 93 and 186, respectively (Figure 4.1d,e). MS^n analysis confirms *m/z* 92 and 93 as the DQ and PQ dications by their fragmentation to give ions with greater *m/z* values, *e.g.* 157 and 171 (Figure 4.14 a,b). The absence in the MS^2 spectra of *m/z* 185 for PQ and *m/z* 183 for DQ suggests that these monocations do not form directly from the dications by way of loss of a proton.

In contrast, the MS^2 spectrum from *m/z* 186 for PQ revealed the presence of *m/z* 185, formed with a fragmentation efficiency of 4.6%. Given that *m/z* 186 ($PQ^{+\cdot}$) must be formed by one electron reduction of the dication (Moyano *et al.*, 1996; Song and Budde, 1996; Taguchi *et al.*, 1998), the results indicate that *m/z* 185 arises from fragmentation of the radical cation and not from proton loss from the dication as suggested previously. Thus, the PQ and DQ monocations at *m/z* 185 and 183 are assigned as $[M_q^{+\cdot}\text{-}H^{\cdot}]^+$ (Figures 4.15 and 4.16), consistent with the radical cation giving product ions derived mainly through losses of $R^{\cdot}$.

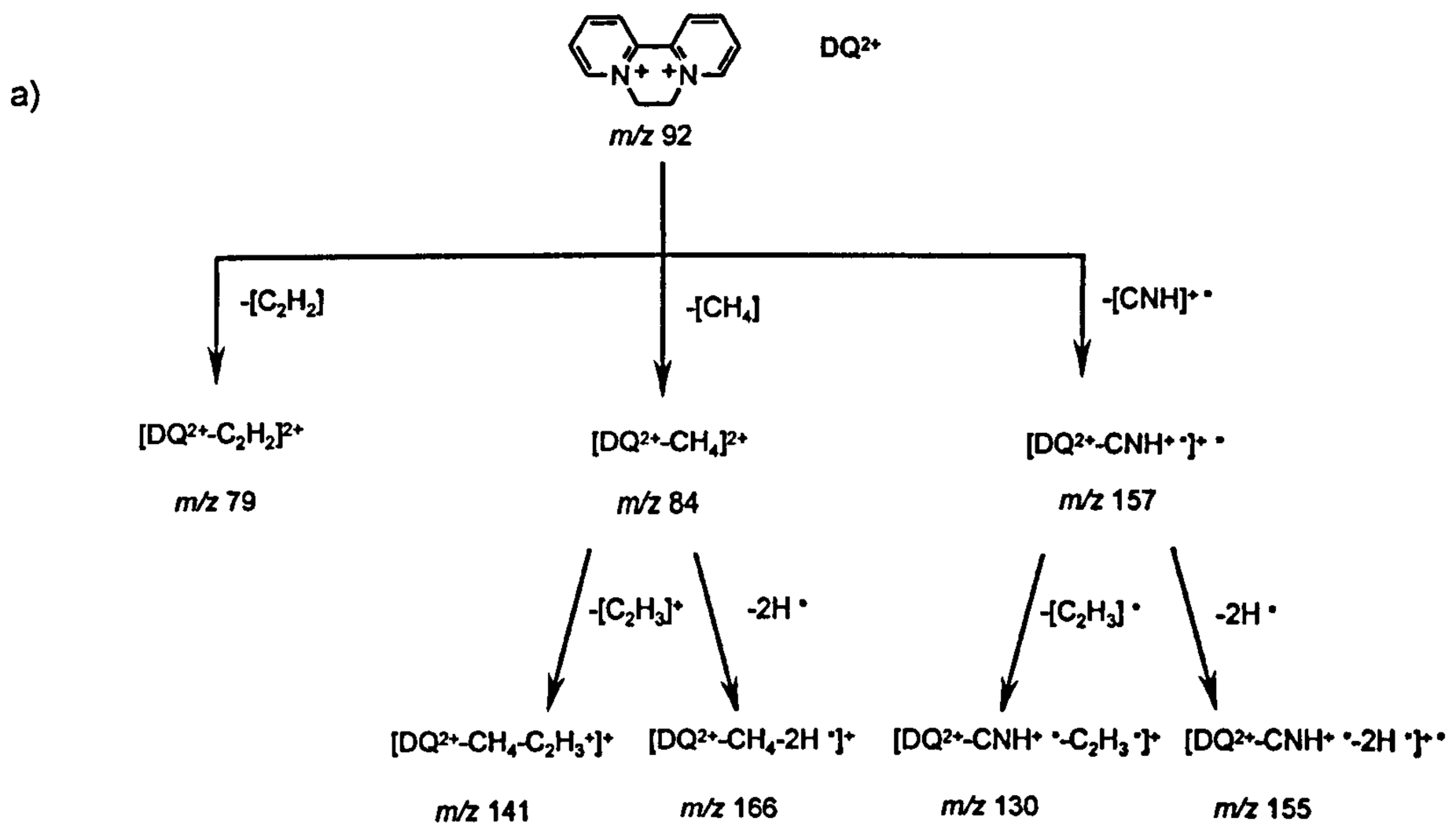


Figure 4.14: Fragmentation maps for (a) DQ^{2+} and (b) PQ^{2+}.

The tandem MS analysis of the monocations (*m/z* 183 and 185) was accomplished using the basic software controls; fragment ions were observed at *m/z* 157 and 168 for DQ using a collision energy of 22% (Figure 4.15), and at *m/z* 170, 158, 144 and 118 for PQ with a collision energy of 26% (Figure 4.16). These ions have been

reported previously using either an ionspray interface (all fragment ions for PQ and DQ; Marr and King, 1997), or FAB source (PQ: *m/z* 158, 144 and 118; Tondeur *et al.*, 1987). Fragmentation of the DQ product ion *m/z* 157, attributed to $[DQ^{+\cdot}\text{-}H^{\cdot}\text{-}C_2H_2]^+$, produced a single ion at *m/z* 130, assigned formally as arising through loss of HCN (Figure 4.15). Similarly, the PQ MS/MS product ion *m/z* 170, attributed to $[PQ^{+\cdot}\text{-}H^{\cdot}\text{-}CH_3^{\cdot}]^+$, fragmented under MS^3 conditions to give three product ions, *m/z* 169, 155 and 143 (Figure 4.16) assigned formally as arising from losses of $H^{\cdot}$, $CH_3^{\cdot}$ and HCN, respectively (Figure 4.16).

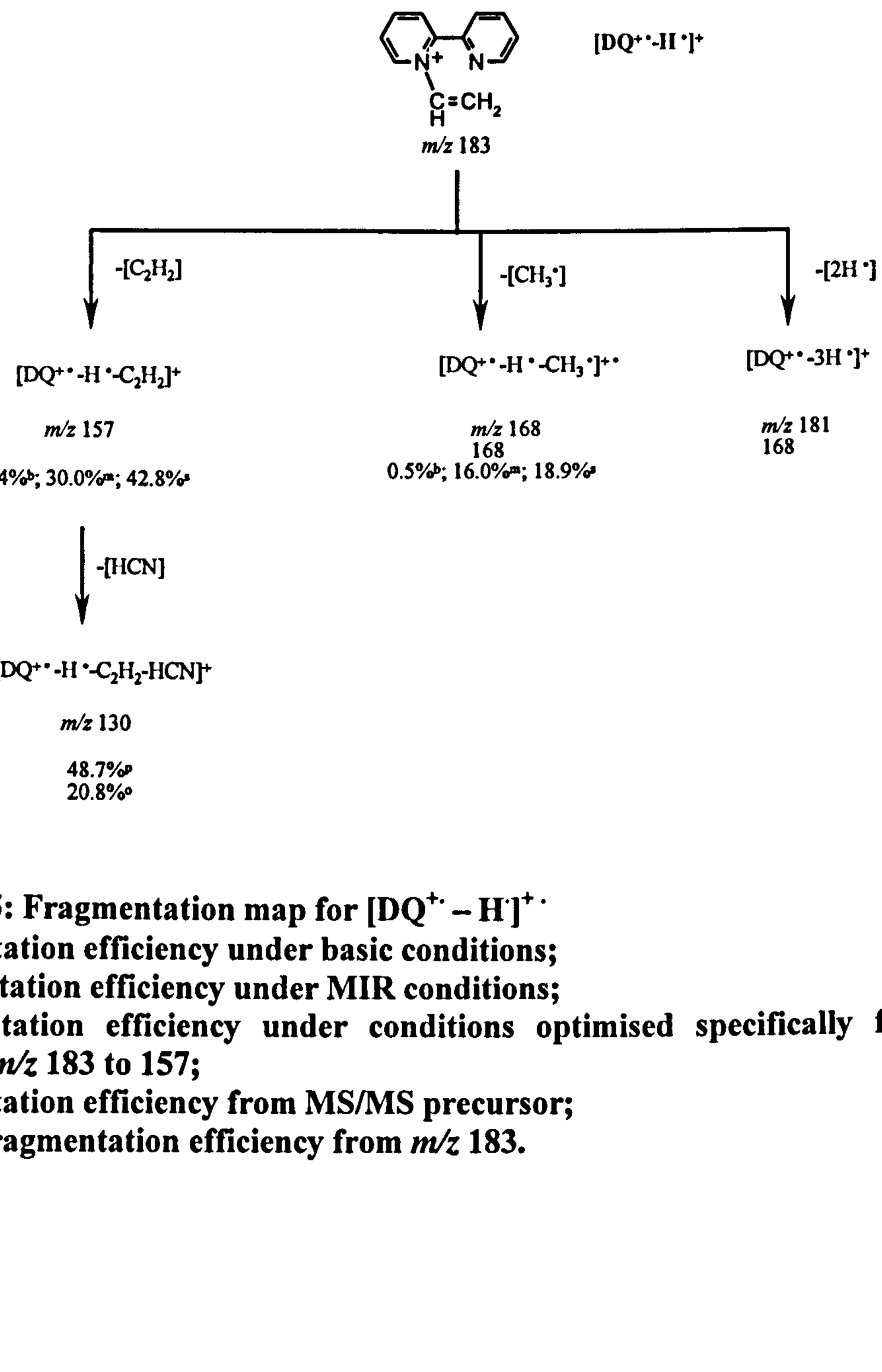


Figure 4.15: Fragmentation map for $[DQ^{+\cdot} - H^{\cdot}]^{+\cdot}$
[b] Fragmentation efficiency under basic conditions;
[m] Fragmentation efficiency under MIR conditions;
[s] Fragmentation efficiency under conditions optimised specifically for the transition *m/z* 183 to 157;
[p] Fragmentation efficiency from MS/MS precursor;
[o] Overall fragmentation efficiency from *m/z* 183.

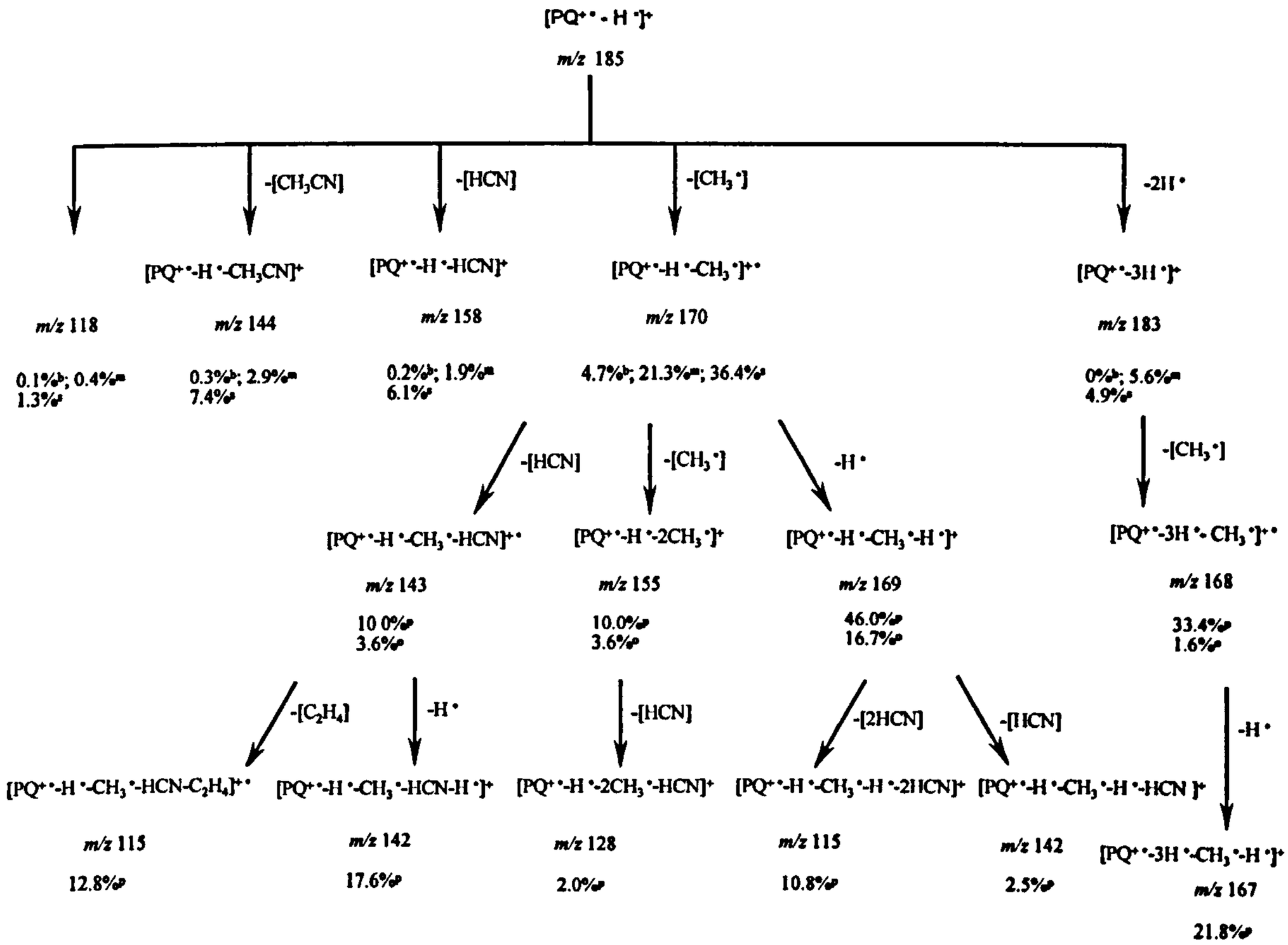


Figure 4.16: Fragmentation map for $[PQ^{+\cdot} - H^{\cdot}]^{+}$
[b] Fragmentation efficiency under basic conditions;
[m] Fragmentation efficiency under MIR conditions;
[s] Fragmentation efficiency under conditions optimised specifically for the transition *m/z* 185 to 170;
[p] Fragmentation efficiency from MS/MS precursor;
[o] Overall fragmentation efficiency from *m/z* 185

4.2.2.8 PQ and DQ optimisation of transition pathways

Using the basic settings, the efficiencies of fragmentation from the $[M_q^{+\cdot}\text{-}H^{\cdot}]^{+}$ precursor ions to the base peak ions at *m/z* 157 and *m/z* 170 were 10.4 % for DQ and 4.7 % for PQ (Figures 4.15 and 4.16). Fragmentation under MIR conditions, established using simplex based on *m/z* 168 and 157 for DQ (AA = 1.72 V; AQ = 0.371; AT = 8.6 ms) and on *m/z* 170 for PQ (AA = 1.28 V; AQ = 0.392; AT = 12.1 ms), improved the signal level for *m/z* 157 (DQ) and *m/z* 170 (PQ) by 3 fold and 4.5 fold, respectively. The MIR MS^2 spectra obtained from $[M_q^{+\cdot}\text{-}H^{\cdot}]^{+}$ (Figure 4.17) are

in good agreement with a previous report of the MS/MS spectra obtained on a triple quadrupole instrument (Marr and King, 1997) except for small differences in the relative abundances of the fragment ions. In the present study, the efficiency of fragmentation of *m/z* 185 (PQ) was greater (Figure 4.16), and one ion, *m/z* 170, dominates the MS^2 spectrum (Figure 4.17b).

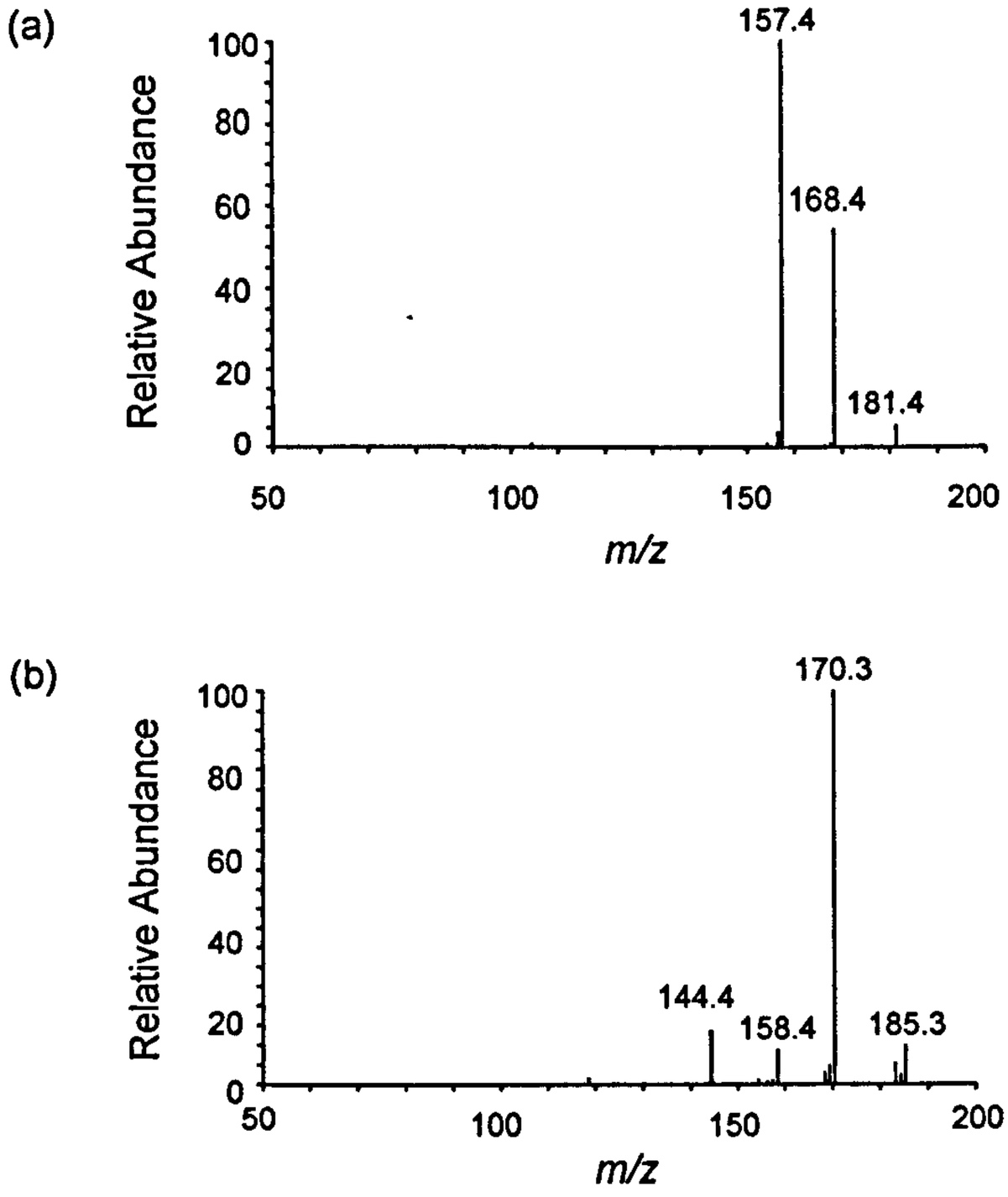


Figure 4.17: Full scan ES MS/MS MIR spectra (a) DQ and (b) PQ.

Optimisation of the DQ transition *m/z* 183 to *m/z* 157 (AA = 1.7 V, AQ = 0.500 and AT = 3 ms) resulted in an increase in fragmentation efficiency over that obtained under the MIR conditions (43% *vs.* 30%; Figure 4.15). MS^n analyses of the $[DQ^{+\cdot}\text{-}H^{\cdot}]^+$ ion indicate that the transition sequence *m/z* 183 - 157 - 130 has a high overall fragmentation efficiency (20.8%; Figure 4.15) and so would be suitable for specific monitoring of DQ. Similarly, optimisation of the transition (*m/z* 185 to *m/z* 170) for PQ (AA = 1.53 V; AQ = 0.494; AT = 9.3 ms) resulted in an increased fragmentation efficiency of 36.4% *cf.* 21.3% (Figure 4.16). Subsequent MS^n analysis

highlighted the transitions *m/z* 185 - 170 – 155 and 185 – 170 – 143 from $[PQ^{+\cdot}H^{\cdot}]^+$ as suitable for SRM as they have overall fragmentation efficiencies of 3.6% (Figure 4.16). Thus, optimisation of selected transitions is shown to give significant increases in the resultant MS^2 signal levels and will permit better LOD to be obtained for monitoring purposes using this analyser. In addition, MS^3 analyses can be incorporated into the methodology for increased specificity.

4.3 CONCLUSIONS

The ES mass spectra of the quaternary ammonium pesticides obtained over an extended mass range reveal that PQ forms cluster species with ammonia as well as an ion-pair complex with chloride. In addition, MQ and CQ form cluster species of the type $[2M_q^+\text{-}A^-]^+$ with chloride and acetate anions present in solution. By contrast, DQ and DF do not form detectable cluster species with any of the anions studied, probably due to steric constraints. MQ^+ and CQ^+ cations form clusters with chloride and acetate anions over a wide concentration range. Competition studies reveal that chloride anions have greater binding affinity for MQ and CQ than acetate anions, consistent with the higher charge density of chloride. Since these clusters can be formed over an analyte concentration range similar to that encountered routinely in pesticide analysis, their formation could affect the reproducibility and LOL of an LC-MS analytical method and has implications for quantification. Thus, formation of clusters should be considered when designing and validating analytical methods.

MS^n studies of the quaternary ammonium herbicides have been undertaken for the first time, and have allowed recognition of several fragment ions that have not been reported previously in the spectra of CQ and MQ and assignments to be proposed for them. In addition, a number of transitions that are highly specific to each quat have been identified. Optimal ion trap operating conditions, determined using simplex optimisation, can promote either detection of a particular fragmentation transition or a range of MS/MS product ions with a high overall signal response. The MS/MS conditions used have implications for the overall specificity, sensitivity and LOD of an LC-MS/MS method. It is clear that experiments can be designed either to obtain

the best LOD possible or to obtain the greatest specificity, e.g. incorporating MS^3 analyses. The approach described to optimise performance of the ion trap for selective and quantitative analysis is appropriate to a wider range of analytes than the pesticides considered here.

Chapter 5

Tandem MS Approach to the Quantitative Analysis of Quats

5.1 INTRODUCTION

5.1.1 Requirement for rapid analysis of pesticides

European and UK legislation stipulate the maximum residue levels (MRLs) of pesticides that are allowable in our food and drinking water (Statutory Instrument, 1997; Council directive, 1998). In order to monitor compliance with legislation, analytical methods must be developed and used in routine monitoring programmes. An increase in the number of pesticides applied (Chmil, 1996) and the requirement to monitor their levels in food and water has led to a significant increase in the number of analyses that need to be performed by regulatory laboratories. In order to meet these demands, rapid quantitative approaches are required.

Currently, the majority of quantitative analyses for the determination of pesticides are performed by gas chromatography (GC) with mass spectrometric (MS) detection or by liquid chromatography (LC) coupled with either UV or MS detection (Chmil, 1996; Hogendoorn and van Zoonen, 2000). Although the methods developed using these techniques are often specific and sensitive, many require long analysis times; for example, Takino's LC-MS method for paraquat and diquat has an analysis time of 30 min (Takino *et al.*, 2000). Development of methods with much reduced analysis time would give greater sample throughput, allowing analysis of greater numbers of samples per unit time.

Environmental and food regulatory laboratories are not alone in their need for rapid analytical techniques. Drug discovery laboratories synthesise hundreds to thousands of compounds each week (Whalen *et al.*, 2000), which need to be analysed. Due to the large numbers of compounds involved quick analysis methods must be utilised. Several approaches for rapid profiling by flow injection mass spectrometry (FI-MS) have been described. Rapid profiling includes assessment of the fidelity of synthetic pathways and determination of appropriate MS/MS parameters for the compound analysed (Wang *et al.*, 1998; Whalen *et al.*, 2000; Morand *et al.*, 2001). FI-MS involves injection of the analyte into a liquid carrier stream, which flows directly into the mass spectrometer, typically *via* an electrospray (ES) or atmospheric pressure

chemical ionisation (APCI) interface. The majority of FI-MS analyses carried out in drug discovery laboratories are automated. Sequential injection of samples from 96-well microtitre plates is performed by an autosampler, with automatic acquisition of the mass spectral data (Wang *et al.*, 1998; Whalen *et al.*, 2000). Using this sequential analysis protocol 96 samples contained on a microtitre plate have been analysed by FI-MS in under 48 minutes (Wang *et al.*, 1998).

Recently, FI with ES-MS detection in the selected ion monitoring (SIM) mode has been applied to the quantitative determination of analytes in complex matrices. Examples include analysis of the bis-indole alkaloids vincristine and vinblastine from plant matrices (Favretto *et al.*, 2000) and analysis of an antiepileptic drug, Topiramate, from human plasma (Chen and Carvey, 1999; 2001). Such techniques could be applied to the quantitative analysis of pesticide residues and increased specificity in detection could be achieved through incorporation of MS^n strategies. To date, there appears to be only one study reporting quantitative analysis by FI-MS^n (Prosek *et al.*, 2000): determination of caffeine in soft drinks. In studying plant matrices, Favretto *et al.* (2000) noted that due to the complex nature of the matrix analysed, suppression of the analyte MS signal was observed. No such suppression was noted by Chen and Carvey (1999, 2001) or by Prosek *et al.* (2000), though their analyses were performed following preliminary sample clean-up by liquid-liquid extraction and thin layer chromatography followed by solid phase extraction (SPE). Due to the nature of the matrices in which pesticide residues are analysed, FI-MS analysis is likely to be susceptible to matrix effects and so extraction of the analyte from the matrix (*i.e.* sample clean-up) might be required.

5.1.2 Removal of matrix interferences

Many approaches have been applied to the clean-up of "dirty" samples including liquid-liquid extraction, SPE, supercritical fluid extraction and gel permeation chromatography (Tekel and Hatrik, 1996). SPE is widely used in pesticide analysis (Tekel and Hatrik, 1996; Pico *et al.*, 2000b), due to the similarity of the stationary phases to those used in LC coupled with the possibilities of automation and

incorporation into on-line separation systems. SPE offers high extraction efficiency from the sample matrix and can also be used as a preconcentration technique (Pico *et al.*, 2000b). For analytes to be extracted using SPE they must have a greater affinity for the solid phase than for the sample matrix, thus ensuring that they are adsorbed onto the solid phase (Berreuta *et al.*, 1995). Compounds retained on the solid phase can be removed at a later stage by eluting with a solvent, for which the analyte has a high affinity (Berreuta *et al.*, 1995) or which competes with the analyte for the active sites on the solid phase.

Recently, the SPE methods employed in the determination of several quats were reviewed by Pico *et al.* (2000b). They grouped methods as being based either on ion exchange or on ion-pair associations. Ion exchange has been utilised both on dedicated cation exchange resins and also on silica sorbents (Pico *et al.*, 2000b). Ion-pair SPE methods have used apolar sorbents, typically C_{18} and C_8 chains bonded to silica, with ion pair formation achieved through addition of counter ions such as hexanesulfonate, heptanesulfonate and dihydrogen orthophosphate (Pico *et al.*, 2000b). The majority of SPE methodologies (*ca.* 40) have been developed for DQ and PQ (Pico *et al.*, 2000b and ref. therein), probably because these two herbicides are included in a priority list for monitoring established by the European Union. By contrast, there are few reports of the development and use of SPE methods for the analysis of CQ (Vahl *et al.*, 1998; Castro *et al.*, 1999, 2000; Juhler and Vahl, 1999; Hau *et al.*, 2000), MQ (Castro *et al.*, 1999, 2000; Juhler and Vahl, 1999) and DF (Ahmad, 1982 a,b; Ibanez *et al.*, 1996, 1997a,b; Tsukioka *et al.*, 1998; Castro *et al.*, 1999, 2000).

5.1.3 This Study

Previous reports have demonstrated the potential of FI-MS for rapid quantitative analysis (Section 5.1.1). The main aim of this work was to develop a similar approach using FI-MS^n for quantitative analysis of the quats, chlormequat (CQ), difenzoquat (DF), diquat (DQ), mepiquat (MQ) and paraquat (PQ). Highly specific detection will be achieved through selection of appropriate transition pathways for

the quats based on results of MS^n studies (Chapter 4). Previously, simplex optimisation allowed optimal resonance excitation conditions to be determined for maximum product ion response for MS to MS^2. The MS^2 to MS^3 conditions have yet to be optimised. Therefore, in order to achieve maximum sensitivity in the FI-MS^n method the MS^2 to MS^3 resonance excitation conditions will be optimised using simplex. The FI-MS^n method will be applied to the quantitative determination of CQ in pears. Due to the known susceptibility of FI-MS methods to matrix effects (Favretto *et al.*, 2001) an SPE method will be developed to remove matrix components.

5.2 RESULTS AND DISCUSSION

5.2.1 Potential for quantitative determination of the quats by FI-MS^n

In order to obtain the greatest sensitivity in a FI-MS^n method the MS^n resonance excitation conditions for the quats need to be optimised to provide maximum product ion response. Optimisation of MS to MS^2 transitions has been carried out previously (Chapter 4) and the conditions providing maximum ion response are given in Table 5.1, with the fragmentation efficiencies calculated using Equation 4.1. In order to determine the optimal MS^2 to MS^3 resonance excitation conditions for the quats simplex optimisation was performed using the protocol described previously (Chapters 3 and 4). Optimal conditions for MS^3 analysis are also given in Table 5.1; the results show that generally, high overall fragmentation efficiencies from the MS precursor ions are observed. It should be noted that in performing MS^3 analyses, initial fragmentation of the MS precursor ion is performed using the conditions for MS to MS^2 analysis, and then in the μscan window the resonance excitation conditions are changed to those for MS^2 to MS^3 analysis. For example, MQ^+ at *m/z* 114 is first fragmented using the MS to MS^2 conditions, yielding *m/z* 98, and the fragmentation conditions are then changed to those for MS^2 to MS^3 analysis, causing fragmentation of *m/z* 98 and resulting in the formation of *m/z* 70 (Table 5.1).

Quat	MS^2	Optimum conditions for transition MS to MS^2				Fragmentation efficiency from MS precursor / %	MS^3	Optimum conditions for transition MS^2 to MS^3				Overall fragmentation efficiency from MS precursor / %
		IW / Th	AA / V	AQ	AT / ms			IW / Th	AA / V	AQ	AT / ms	
MQ	114-98	1.5	1.27	0.593	21	32	114-98-70	1.5,	1.02	0.440	15	17
CQ	122-58	1.5	0.96	0.354	35	20	122-94-59	1.5,	0.80	0.362	7.0	2.5
DQ	183-157	1.5	1.70	0.500	3.0	43	183-157-130	1.5,	1.40	0.500	15	33
PQ	185-170	1.5	1.53	0.494	9.3	36	185-170-155	1.5,	1.80	0.522	5.0	6.4
DF	249-193	1.0	1.68	0.403	7.9	20	249-208-193	1.5,	1.30	0.380	21	5.3

Table 5.1: Optimal MS^n conditions and their fragmentation efficiencies for the five quats.

In order to investigate the suitability of FI-MS^n for the quantitative analysis of the quats, solutions of each of the quats in deionised water (concentration range 0.01 to 2000 ng mL^{-1}) were manually injected (5 μL; 12 injections) into a liquid carrier stream (50 mM ammonium acetate in 50/50 methanol/water). Detection was performed in full scan MS mode (first 3 injections); SIM mode for the quat precursor ion (next three injections); MS^2 mode (next 3 injections) and MS^3 mode (last three injections; Figure 5.1). The MS^n transitions previously identified as giving maximum signal response were employed at the optimal MS trapping conditions (Table 5.1).

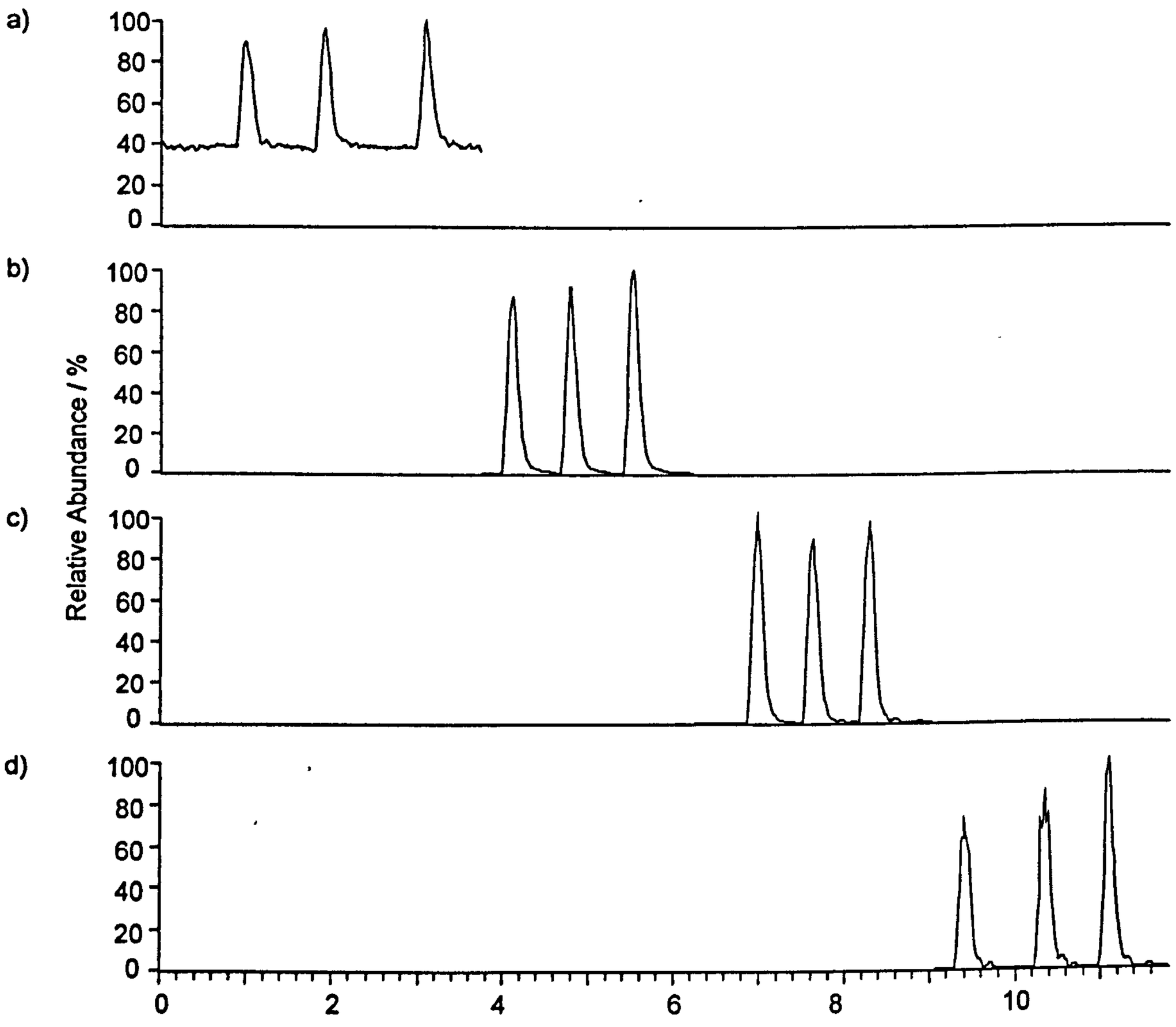
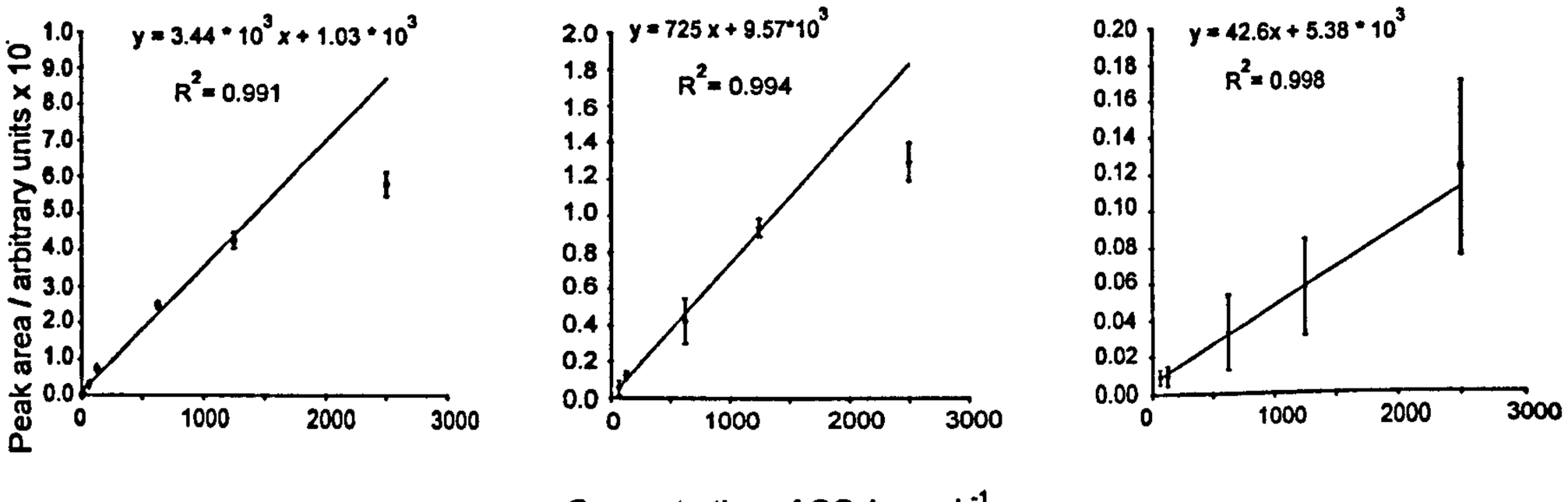


Figure 5.1: Fl-MS of DF at 500 ng mL^{-1}. Detection performed for a) Full scan MS b) SIM *m/z* 249 c) MS^2 *m/z* 249 to 193 and d) MS^3 *m/z* 249 to 208 to 193.

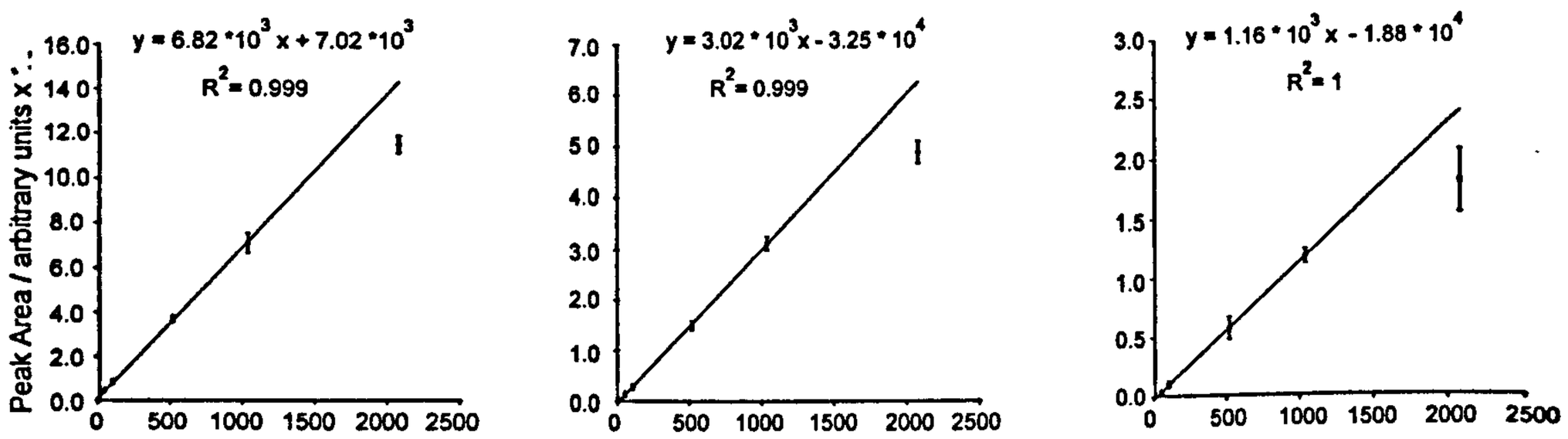
As the quat cations reached the mass analyser a sharp peak in signal intensity was observed and the analysis time per injection was 0.3 min (Figure 5.1). Good signal response is obtained for all MS modes. Since the resultant peaks are well defined, measurement of peak areas is straightforward. The repeatability of the peak areas obtained, and hence precision of the injections, was examined by performing ten replicate injections from a solution containing 500 ng mL^{-1} of CQ and monitoring in SIM mode. For the other detection modes, repeatability was evaluated for three injections of CQ. The peaks obtained for each injection of CQ were compared for each detection mode. Relative standard deviations (RSDs) for peak area measurement in the SIM, MS^2 and MS^3 modes were 6.6% (n = 10), 4.2 % (n = 5) and 18.9% (n=3), respectively. The low RSDs obtained for SIM and MS^2 indicate good repeatability in the peak areas obtained and hence good precision in the method. The higher RSD obtained for MS^3 analysis (*cf.* SIM and MS^2) is attributed to the low overall fragmentation efficiency associated with this analysis (see Table 5.1). Since good repeatability in peak area measurements was obtained, peak areas were used for quantification.

Peak area measurements were obtained for each sample injection in three detection modes (SIM, MS^2 and MS^3) and were used to construct calibration plots (Figure 5.2). Linear calibration plots up to 1000 ng mL^{-1} resulted for all five quats in the three detection modes (Figure 5.2). Slight curvature in the SIM plots is observed at higher concentrations between 1000 and 2000 ng mL^{-1} (Figure 5.2), and resulted in lower coefficients of determination (R^2) in the linear regression plots than those obtained for the calibration up to 1000 ng mL^{-1}. For example, SIM detection of CQ for the calibration over the range 0.01 to 2000 ng mL^{-1} an R^2 of 0.944 was obtained compared with a value of 0.991 for the range 0.01 to 1000 ng mL^{-1} (Figure 5.2). The flattening observed in the calibration curves at these higher analyte concentrations is attributed to the formation of clusters and ion-pair species as discussed in Section 4.2.1 and affects the limit of linearity of the calibration. Nevertheless, using this FI-MS^2 approach a wide linear dynamic range of two orders of magnitude was achieved for PQ and DQ and of three orders of magnitude for CQ, MQ and DF.

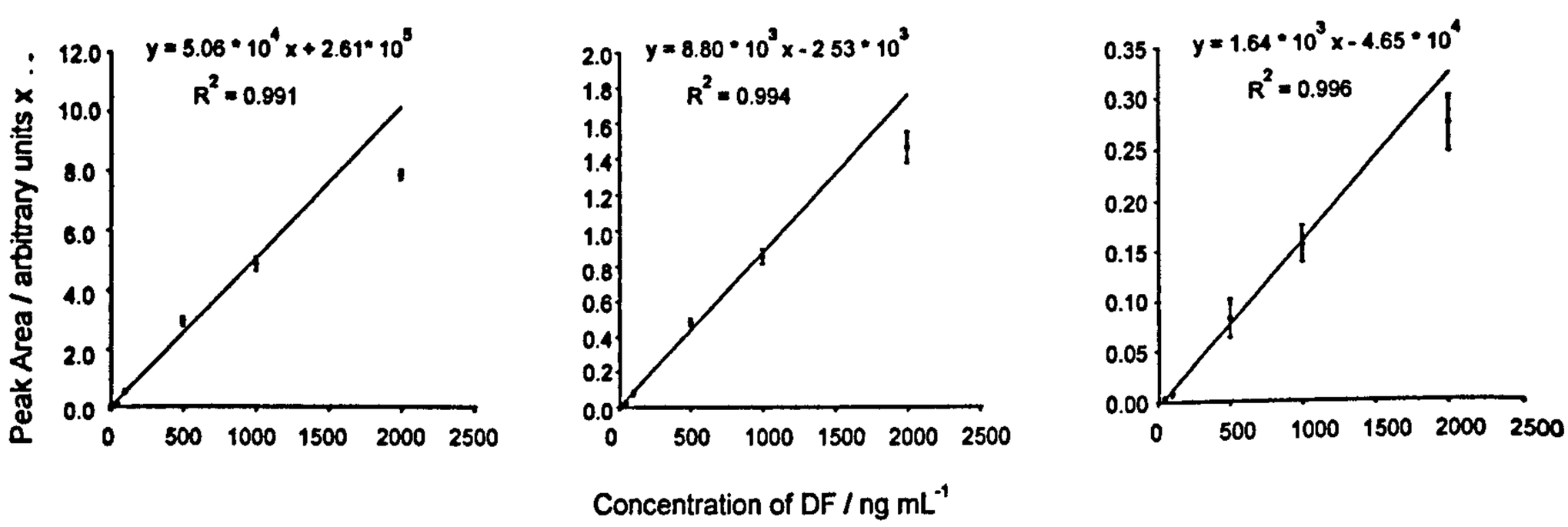
a)


y = 3.44 * 10^3 x + 1.03 * 10^3
R^2 = 0.991
y = 725 x + 9.57*10^3
R^2 = 0.994
y = 42.6x + 5.38 * 10^3
R^2 = 0.998
Peak area / arbitrary units x 10
Concentration of CQ / ng mL^-1

b)


y = 6.82 *10^3 x + 7.02 *10^3
R^2 = 0.999
y = 3.02 * 10^3 x - 3.25 * 10^4
R^2 = 0.999
y = 1.16 * 10^3 x - 1.88 * 10^4
R^2 = 1
Peak Area / arbitrary units x
Concentration of MQ / ng mL^-1

c)


y = 5.06 * 10^4 x + 2.61* 10^5
R^2 = 0.991
y = 8.80 * 10^3 x - 2 53 * 10^3
R^2 = 0.994
y = 1.64 * 10^3 x - 4.65 * 10^4
R^2 = 0.996
Peak Area / arbitrary units x
Concentration of DF / ng mL^-1

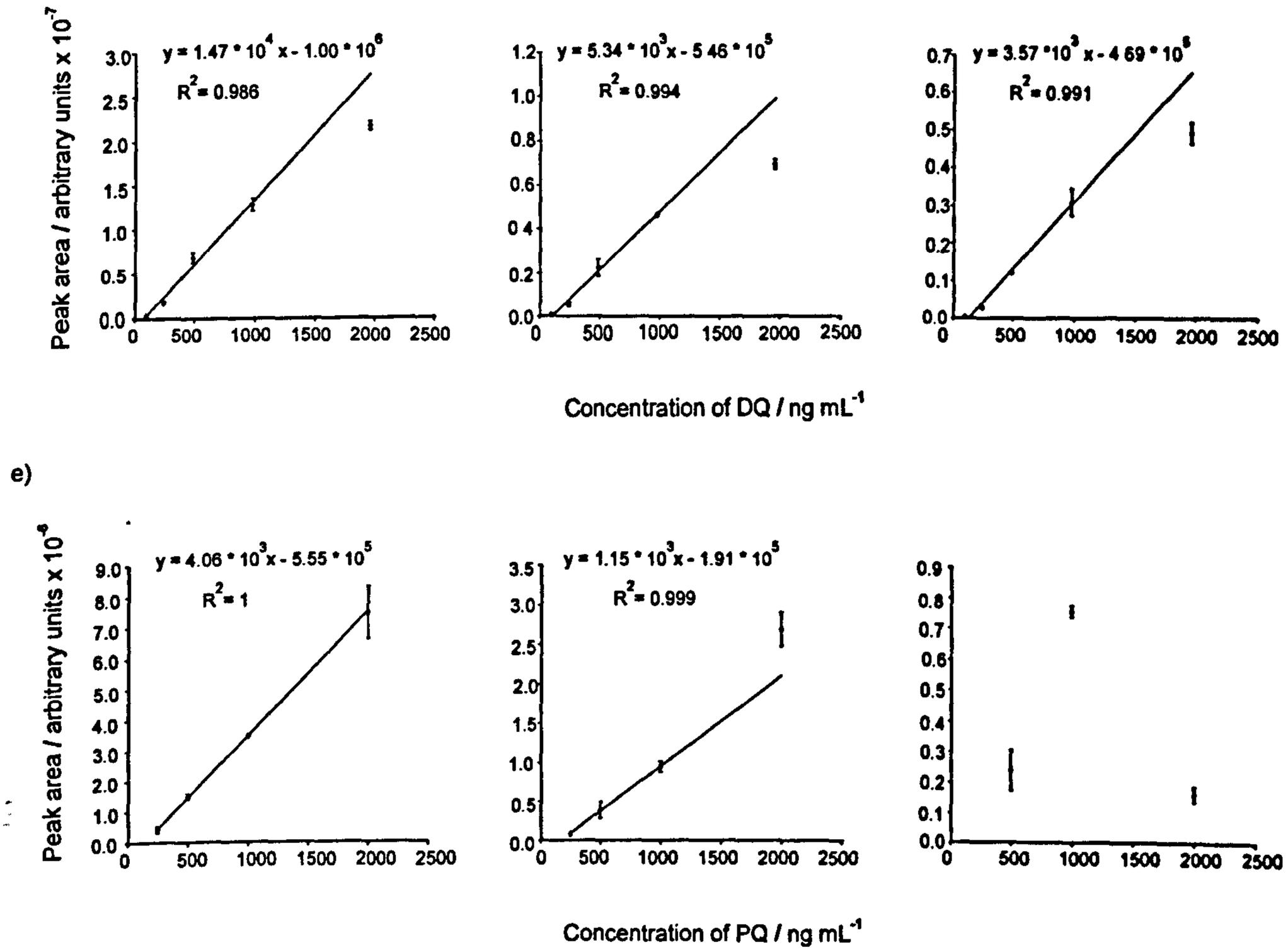


Figure 5.2: Calibration plots for FI-MS^n analysis of a) CQ b) MQ c) DF d) DQ and e) PQ and from left to right are for SIM, MS^2 and MS^3 detection modes. Regression lines are for the range of concentrations up to ~1000 ng mL^{-1} and are not forced through zero.

Limits of detection (LODs), based on three times signal to noise, were measured for each quat in the three MS modes (Table 5.2). It can be seen from the table that lower LODs were achieved for the monocations, MQ, CQ and DF, than for the dications, DQ and PQ. The FI-MS^n peak profiles obtained for PQ and DQ exhibited more tailing than those observed for the monocations, for example DF (Figure 5.1). The increased peak tailing observed for PQ and DQ probably resulted from interactions between the dications and the silanol groups on the fused-silica capillary, which transports the liquid carrier phase into the mass spectrometer *via* the ES interface. As the complexity of the MS detection mode increased, *i.e.* from MS to MS^2 to MS^3,

the LODs also increased. The LOD achieved for a particular MS^n detection step (Table 5.2) is related to its fragmentation efficiency (Table 5.1). For example, MS^3 analysis of MQ is highly efficient (17.2%) and results in a comparatively low LOD of 30 ng mL^{-1} (Table 5.2). Conversely, MS^3 analysis of CQ results in a higher LOD of 120 ng mL^{-1} due to the lower fragmentation efficiency (2.5 %). For MQ, the increased specificity of MS^3 detection is accompanied by a comparable LOD to that achieved with MS^2 detection (30 *vs.* 26 ng mL^{-1}), whereas detection of CQ in MS^2 mode resulted in a much lower LOD of 28 ng mL^{-1} than its detection in MS^3 mode (LOD = 120 ng mL^{-1}). Thus, for CQ, increased specificity is achieved at the expense of sensitivity. As a general observation, consideration must be given to both sensitivity and specificity when developing an analytical method. The LODs obtained for SIM detection (Table 5.2) are comparable to those achieved previously with an LC-SIM-MS method (Chapter 2). The linear dynamic ranges achieved in FI-SIM-MS are also comparable to those obtained for LC-SIM-MS, for example for CQ both methods are linear from the LOD to *ca.* 1250 ng mL^{-1} *i.e.* over two orders of magnitude.

Quat	LOD / ng mL^{-1}			
	LC-SIM-MS	FI-SIM	FI-MS^2	FI-MS^3
MQ	4.7	6.6	26	30
CQ	2.3	5.3	28	120
DQ	87	97	100	120
PQ	240	270	360	390
DF	0.02	5.5	12	30

Table 5.2: Comparison of LODs achieved for the five quats by FI-MS^n and LC-MS analysis.

5.2.2 Application to real samples – Analysis of CQ in pear matrix

5.2.2.1 Initial analysis of CQ in pear matrix

In order to assess the applicability of the method to the analysis of real samples, determination of CQ in pear matrix was undertaken. The LODs (Table 5.2) obtained for CQ in deionised water for three MS modes are all less than 120 ng mL^{-1}, and are below the MRL set for CQ in pear (3 mg kg^{-1} ≡ 600 ng mL^{-1}). This suggests that the FI-MS^n method could be applied in the regulatory monitoring of CQ residues. Initial experiments were carried out with flow injection analysis of solutions of CQ in pear matrix at concentrations covering the range 0.01 to 2000 ng mL^{-1}. These were unsuccessful; low signal response was observed for the precursor ion (*m/z* 122), even for samples containing high concentrations of CQ, and attributed to ion suppression effects due to the complexity of the matrix (Favretto *et al.*, 2001).

To investigate the extent of the matrix effect, solutions spiked with CQ at 250 ng mL^{-1} and containing different percentages of pear matrix in deionised water were analysed. Replicate injections of each solution were made and full scan MS detection for the CQ^+ ion at *m/z* 122 was performed. The average peak areas obtained for CQ from each solution were compared, and results are shown in Figure 5.3. With no pear matrix present in the sample solution (0%) a large peak area for CQ is obtained. As the percentage of pear matrix increased the peak area observed for CQ decreases. At 100% pear matrix the peak area obtained for CQ is less than 10% of that obtained in 100% deionised water. This proves that the flow injection method is susceptible to matrix effects, and that sample clean-up is required before this method can be applied to the analysis of real samples.

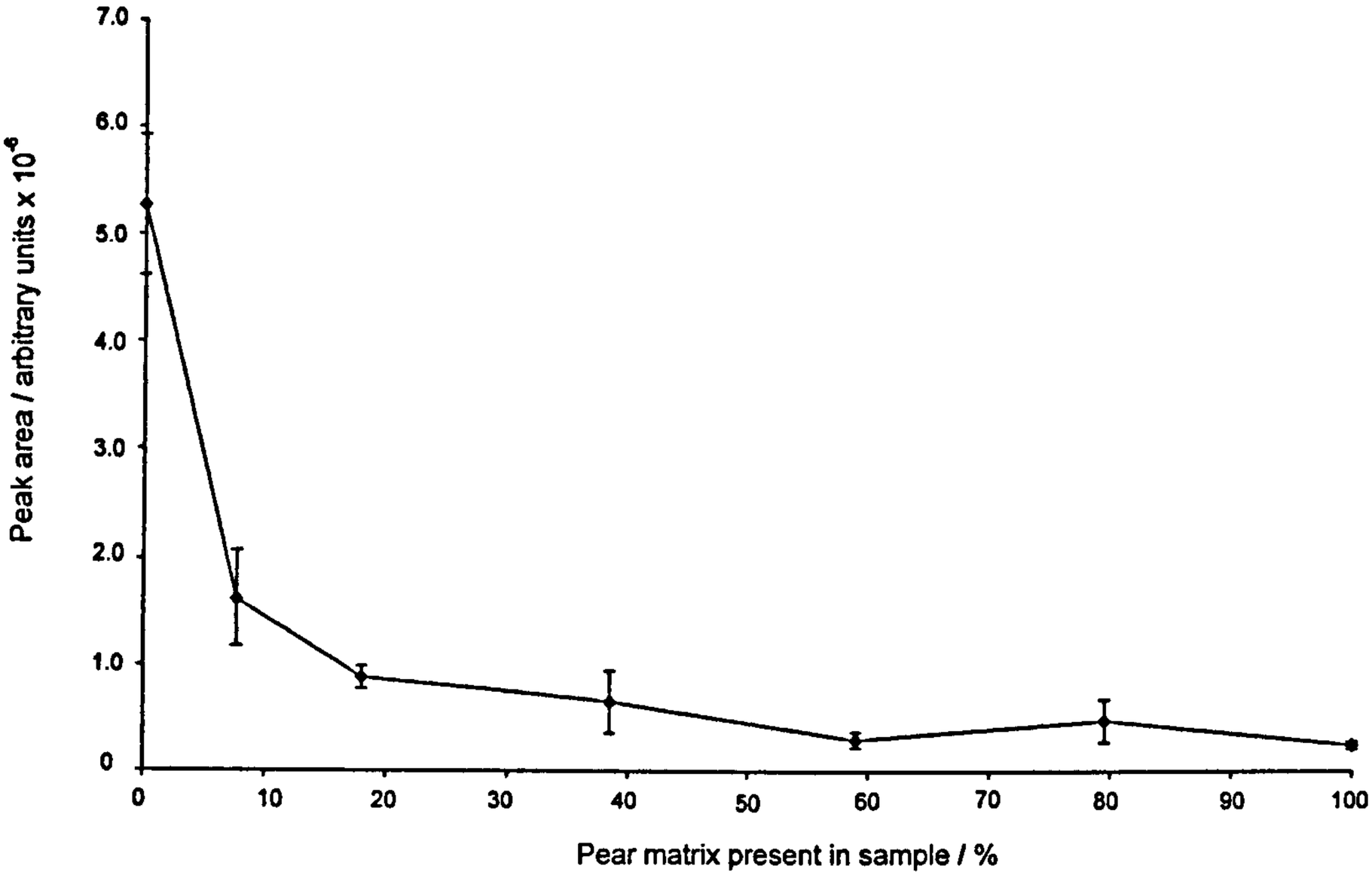


Figure 5.3: Effect of pear matrix on the peak area observed for FI-MS analysis of CQ at 250 ng mL^{-1}.

5.2.2.2 Solid phase extraction of CQ from pear matrix

In order to choose an appropriate solid phase for extracting CQ from pear matrix consideration was given to the stationary phases from which the quats eluted in LC. Analysis of the quats by LC-MS revealed that a non-endcapped cyano stationary phase was suitable for the retention and elution of all five quats (Chapter 2). Interaction between the quat cations and the cyano stationary phase was found to result mainly through ionic associations with residual silanol sites on the silica surface (Section 2.2.2.2). Consequently, elution of the majority of the quats was achieved through cation exchange. Due to the known similarities between LC and SPE (Majors, 1998) sample clean-up could be performed using an SPE protocol based on a cyano phase.

Extraction and clean-up of CQ from pear matrix was carried out using ISOLUTE SPE cartridges containing non-endcapped cyano phase. The protocol consisted of

five steps. In the first step, activation of the solid phase was achieved by preconditioning with 0.01% ammonia solution. This step caused deprotonation at the surface of the solid phase, thereby increasing the possible attraction of acidic species to the solid phase during the load step. The second step was the load step, in which a solution containing CQ was added to the cartridge. The solution passing through the cartridge was collected. MS analysis confirmed that no CQ was present within this solution, indicating that all CQ had been retained on the SPE cartridge. During the load step only species attracted ionically to the residual silanol sites, for example CQ, or through hydrophobically interactions with the cyanopropyl chains will be retained. In the third step, a wash with 100% water was used to remove any interfering compounds weakly retained during the load step. Again the washings were collected, and MS analysis confirmed that CQ was not present and so was still retained on the SPE cartridge. In the fourth step, CQ was eluted from the cartridge with 1% formic acid and collected. In the final step, the eluent was blown down at 50 °C using nitrogen gas; to avoid preconcentration, it was then reconstituted with deionised water to the initial volume added to the SPE cartridge. This reconstituted sample was then analysed by FI-MS^n.

To determine the recovery of CQ after SPE a volume of a solution containing a known concentration of CQ was taken through the procedure described above. The eluent was reconstituted to the original volume added to the SPE cartridge. Both the original solution and the reconstituted solution were then analysed by FI-MS. The peak area measured for the reconstituted solution compared with that obtained for the original solution determines the recovery of CQ after SPE. The recovery of CQ from deionised water was found to be 109 ± 9.9%, based on three replicate analyses (Table 5.3) and indicates that SPE on a cyano phase has potential as an extraction and clean-up method for CQ.

In order to validate the SPE method, a known volume and concentration of an internal standard needs to be added to all samples prior to SPE clean-up. An internal standard should have similar chemical and physical properties to those of the analyte. Tetraethylammonium (TEA) chloride was chosen as the internal standard because it

is likely to behave in a similar manner to CQ, due to its quaternary ammonium functionality, similar structure and size (Figure 5.4).

MS^2 analysis of TEA^+ (*m/z* 130) was performed using the same MS^2 conditions as those for CQ^+ *i.e.* IW = 1.5 Th, AA = 0.96 V, AQ = 0.354, AT = 35 ms. The MS^2 spectrum obtained (Figure 5.5) reveals that the base peak ion is at *m/z* 86, attributed to loss of C_3H_8 (Zhao *et al.*, 1997). This base peak ion was formed with a high efficiency (27.9%). Other product ions are observed at *m/z* 100 and 101 (Figure 5.5) and are formally assigned as arising through loss of ethane and an ethyl radical, respectively.

TEA chloride was added to all samples prior to SPE clean-up, and in subsequent FI-MS experiments. Detection of the internal standard (TEA) was also performed. MS analysis of the SPE washings confirmed that TEA was retained on the SPE stationary phase and was eluted with CQ by 1% formic acid.

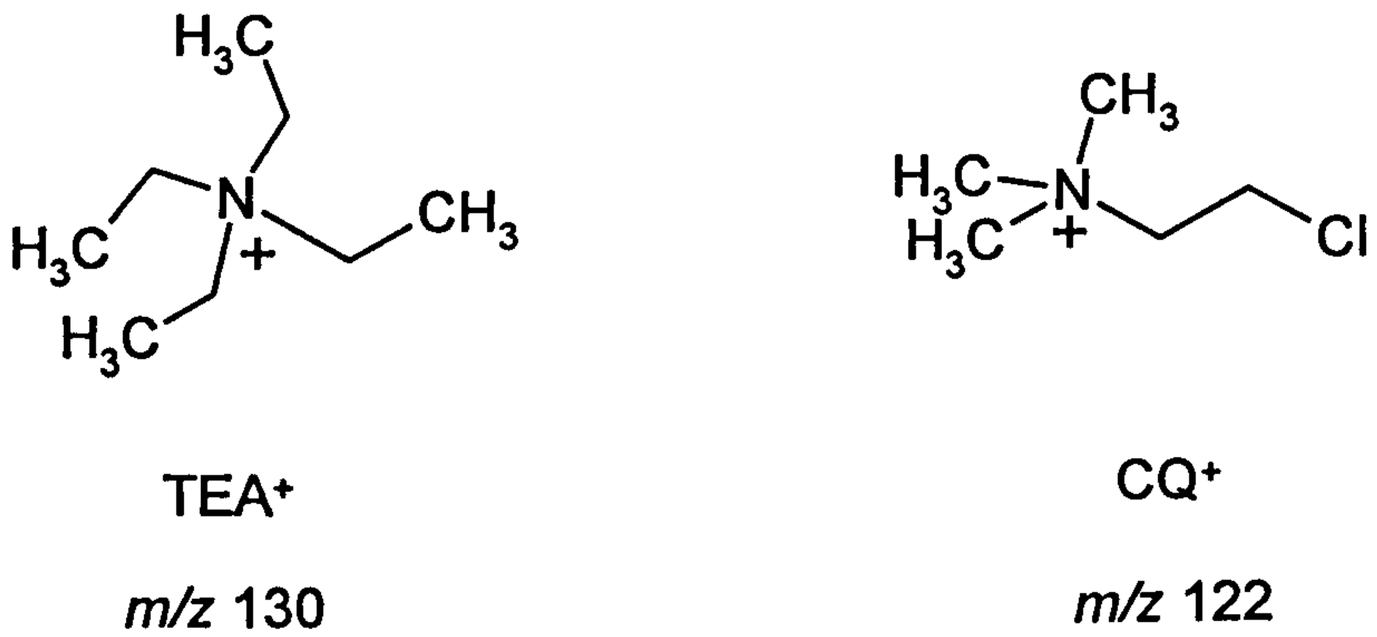


Figure 5.4: Structures of TEA^+ and CQ^+.

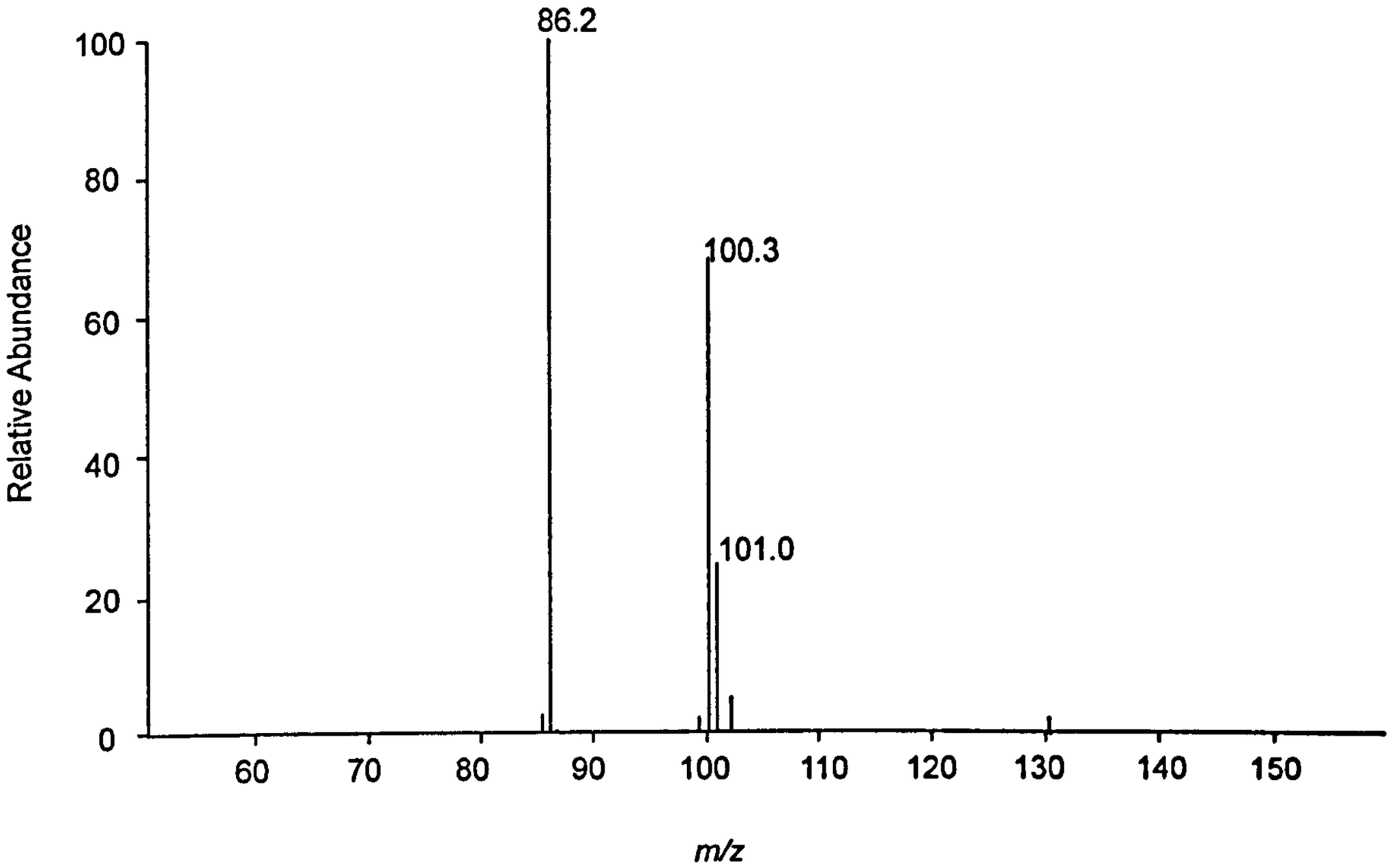


Figure 5.5: MS^2 spectrum of TEA^+.

In order to determine the absolute recoveries of CQ and TEA after SPE, the peak areas obtained for SIM detection of CQ and TEA in deionised water, tap water and pear matrix were compared with those obtained for the two analytes in deionised water (Table 5.3). The SPE method gave comparable absolute recoveries for CQ and TEA in the three matrices (Table 5.3), indicating that TEA is a good surrogate for CQ. Although TEA is a good internal standard for CQ analysis, use of an isotopically labelled chlormequat as standard, *e.g.* ^{13}C CQ^+, would be a better approach. The SPE method was susceptible to matrix effects as is demonstrated in the absolute recoveries obtained for TEA and CQ from deionised water, tap water and pear (Table 5.3), as the complexity of the matrix increased the recoveries decreased. The influence of the matrix on the extraction is understandable, since both CQ and TEA interact with the solid phase through ionic associations, similar to those utilised in LC-MS (Chapter 2) and both analytes subsequently elute *via* a cation exchange mechanism. Ion exchange is affected by ionic strength and ionic composition of the sample (Pico *et al.*, 2000b). The order of conductivity is deionised water < tap water < pear matrix, and since ionic strength and conductivity

are normally well correlated this could account for the order in recoveries observed (Table 5.3). However, the recovery of CQ from pear (Table 5.3) is low compared with previous studies (~94%) using SPE cartridges containing a strong cation exchange resin (Hau *et al.*, 2000) or a C_{18} phase (Vahl *et al.*, 1999). As the recoveries of CQ and TEA in the three matrices are similar and change systematically with the matrix, quantification based on the relative response of CQ to that of the internal standard TEA should compensate for the effect of matrix.

Matrix	Recovery / %	
	TEA	CQ
Deionised water	92 ± 4.5	109 ± 9.9
Tap water	64 ± 5.1	66 ± 5.8
Pear matrix	39 ± 13.0	40 ± 13.0

Table 5.3: Recoveries for TEA and CQ after extraction from a variety of matrices by SPE ($n = 3$).

To investigate the repeatability of the SPE method, ten extractions of samples containing the internal standard TEA at 250 ng mL^{-1} in pear matrix were performed and the reconstituted eluent analysed by FI-MS. Recoveries were determined for each sample and an RSD of 19% (n=10) was observed. The variation observed probably reflects differences in the composition of the solid phase between SPE cartridges.

5.2.2.3 Off-line SPE – FI-MS^n of CQ in pear matrix

To investigate the applicability of the overall method for analysis of CQ in pear samples the SPE method was used in association with analysis by FI-MS^n. Standard solutions of CQ covering the range of concentrations 0.5 to 2000 ng mL^{-1} were prepared in pear matrix, spiked with internal standard (TEA), extracted using SPE and then analysed by FI-MS^n. Peak areas were determined for both TEA and CQ

peaks and used to construct calibration plots for the three MS detection modes (Figure 5.6). Calibrations were linear over at least two orders of magnitude for MS^2 and MS^3 analyses and over three orders of magnitude for SIM analysis. LODs similar to those obtained for deionised water were achieved (Table 5.4). For the analysis of CQ in pear, all are lower than the MRL of 3 mg kg^{-1} (equivalent to 600 ng mL^{-1}) required for regulatory detection. Thus, the low LOD coupled with the wide linear dynamic range indicate that this method could be used for regulatory purposes for rapid, sensitive, highly specific detection and quantification of CQ in food matrices.

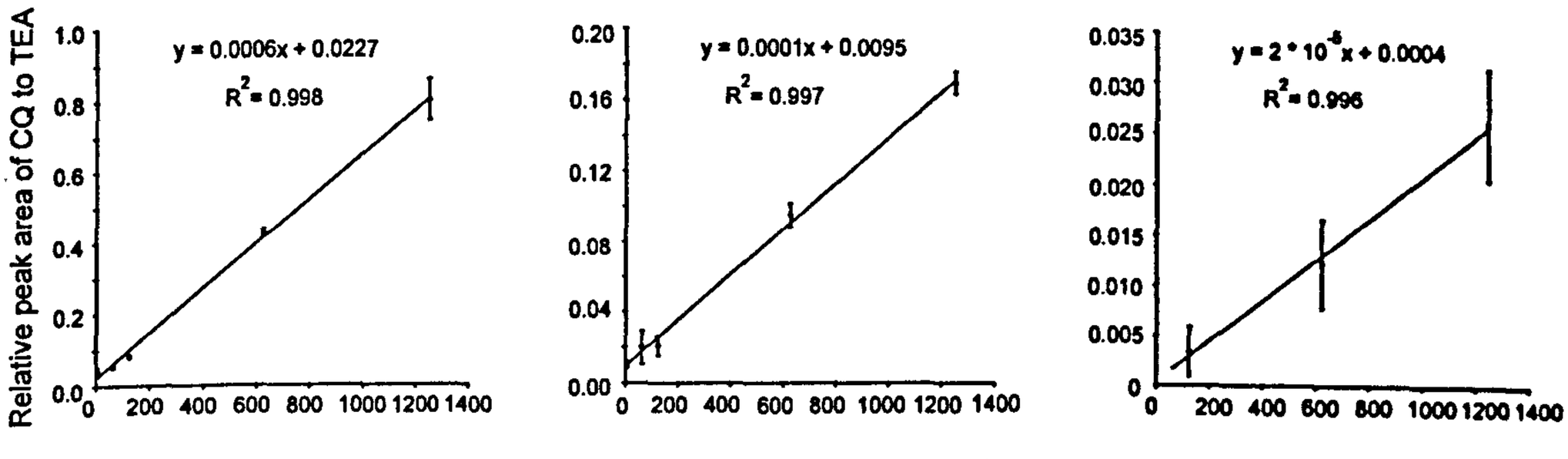


Figure 5.6: Calibration plots obtained for off-line SPE-FI-MS^n analysis of CQ in pear, from left to right for SIM, MS^2 and MS^3 detection.

Matrix	LOD / ng mL^{-1}		
	SIM	MS^2	MS^3
Deionised water	5.3	28	120
Pear (SPE)	13	36	125

Table 5.4: LODs obtained for CQ from solutions prepared in deionised water and from pear matrix following SPE.

5.3 CONCLUSIONS

An FI-MS^n method has been developed for the quantitative analysis of the quats, and LODs and linear dynamic ranges similar to those obtained using an LC-MS method were achieved. The FI–MS^n method has significant advantages over the LC-MS method in the greater specificity and speed of analysis (0.3 min *vs* 20 min for LC-MS). The FI-MS^n method allowed the rapid, sensitive and specific determination of the five quats in deionised water. In this study, manual injection of the samples into the liquid carrier stream was performed. Use of an automated injection system would reduce the dead time and increase the sample throughput (*cf.* Wang *et al.*, 1998). Recently, the use of multiprobe autosamplers, so called multiplexed systems, has been reported and shown to further increase the sample throughput in FI-MS (Wang *et al.*, 1998; Whalen *et al.*, 2000; Morand *et al.*, 2001). In regulatory monitoring detection need only be performed in one MS^n mode therefore, triplicate analysis of a sample would be possible in less than 1 minute.

Application of the FI-MS^n method in the analysis of real samples was undertaken for the determination of CQ in pear matrix. The FI-MS^n method was found to be susceptible to matrix effects and so sample clean-up was required. Off-line SPE using a non-endcapped cyano phase enabled extraction of CQ from pear and tetraethylammonium chloride was used as an internal standard to compensate for recovery losses. Off-line SPE-FI-MS^n analysis of CQ in pear resulted in linear calibrations spanning over two orders of magnitude. LODs in all three MS modes (SIM, MS^2, MS^3) were below that required for regulatory monitoring of CQ in pear, and so all three could be employed in the determination of CQ. The increased specificity offered by MS^2 and MS^3 detection is necessary in the analysis of CQ from complex matrices, which could contain isobaric interferences. If SPE was used as a preconcentration technique, this off-line SPE-FI-MS^n method could be applied to the analysis of CQ in drinking water.

This SPE-FI-MS^n method has the potential for full automation for the quantitative analysis of the quats in a range of matrices. Through the incorporation of automated SPE instruments and multiprobe autosamplers, analyst intervention would be

minimised and sample throughput increased. This approach could be applied to the rapid quantitative determination of analytes other than those considered here.

Chapter 6

Conclusions and Future Work

Chapter 6: Conclusions and Future Work

6.1 OVERALL SUMMARY AND CONCLUSIONS

The work presented describes the analytical determination of quaternary ammonium pesticides (quats). The previous chapters have outlined (i) the development of separation methods for the analysis of quats (ii) ion trap mass spectrometric analysis of the quats including investigation of the MS^n fragmentation pathways and optimisation of quat MS^n response, (iii) a rapid quantitative flow injection mass spectrometric method for the analysis of quats.

Comparison of two previously published methods for the regulatory analysis of chlormequat (CQ) in foodstuffs was performed. The methods employed either a strong cation exchange (SCX) column (Brewin and Hill, 1996) or a non-endcapped C_{18} (Spherisorb ODS1) column (Vahl *et al.*, 1998). The basis of separation of CQ on the ODS1 stationary phase was examined and was found to result from ion exchange processes involving residual silanol sites present on the stationary phase. Separation of CQ on the ODS1 phase resulted in a shorter analysis time, narrower peak width, better peak symmetry, lower limit of detection (LOD) and wider linear dynamic range than obtained on the SCX column. The better chromatographic separation on ODS1 combined with MS detection on an ion trap instrument allowed direct quantification of CQ in drinking water at levels well below those required for regulatory monitoring.

Capillary electrophoretic and liquid chromatographic methods were developed for the separation of the five quats, CQ, difenzoquat (DF), diquat (DF), mepiquat (MQ) and paraquat (PQ). Knowledge of the importance of ion exchange processes on a non-endcapped stationary phase aided the development of a generic LC method for the quats. Separation of the five quats from a non-endcapped cyano phase was achieved using salt/solvent gradient elution. Four of the quats, CQ, DQ, MQ and PQ, were retained through ionic interactions with residual silanol groups and they were eluted *via* cation exchange with ammonium ions. The fifth quat, DF, was retained on the stationary phase predominantly through hydrophobic interactions with the cyano chain, and was eluted though increasing the percentage of the organic modifier in the mobile phase. The generic LC method was found to be unaffected by

the nature of the matrix containing the quats. In contrast to the CE-MS method, which had limited sensitivity and dynamic range, the generic LC-MS method achieved low LODs and linear calibrations over a wide dynamic range in deionised water, tap water and pear matrix. The generic LC-MS method could potentially be applied as a multi-residue approach to the simultaneous analysis of the five quats in one sample, or to the consecutive analysis of each quat in different samples.

The LC-MS method was applied to the analysis of CQ in pear and achieved the necessary levels of sensitivity for its inclusion in regulatory monitoring of foodstuffs. The LOD achieved was, however, 40 fold greater than obtained using the ODS1 column, even though similar peak widths and peak symmetries were observed for both. The difference in sensitivity obtained for the two methods is attributed to a mass spectrometric dependence on the LC eluent composition present at the electrospray needle during elution of CQ: the lower LOD was achieved with a higher organic content present within the electrospray. Although it might be possible to enhance the sensitivity of the generic LC-MS method through incorporation of a make-up flow, consideration would have to be given to a possible dilution effect..

Increased specificity in detection for the quats can be achieved through incorporation of MS^n strategies into the analytical methodology. The MS^2 analysis of CQ was investigated using an ion trap mass spectrometer. Since CQ^+ is of low mass the conditions employed for trapping and fragmentation were critical to the performance of the ion trap in MS^2 mode. Use of advanced software controls on the LCQ ion trap allowed greater operator control of the MS^n fragmentation process than is possible using the basic software settings. The most critical parameters for MS^n in the ion trap were found to be the activation amplitude and the activation Q (AQ). It was found that low mass ions, like CQ, required a higher AQ value to promote fragmentation than that set automatically by the software. Use of the advanced parameters allowed a significant improvement in the sensitivity of MS^2 detection of CQ. Maximum sensitivity in MS^2 detection was achieved by optimising the parameters controlling fragmentation. The use of simplex optimisation, particularly variable size simplex, allowed rapid ascent toward optimum conditions. MS^2

analysis of CQ under optimum fragmentation conditions resulted in a 1000 fold increase in the response observed for the base peak product ion over that achieved with the basic software controls. Incorporation of the optimal fragmentation conditions for CQ into an LC-ion trap-MS/MS method increased the specificity in detection, and comparable analytical performance to that of a LC-triple quadrupole-MS/MS method was achieved.

Since optimisation of the mass spectrometric response had a beneficial effect on the analysis of CQ, comprehensive mass spectrometric studies of the five quats were performed. The full scan electrospray mass spectra of the quats revealed that three of them formed detectable ion-pair or cluster species with other molecules or anions present in solution. PQ was observed to form an ion-pair complex with chloride anions as well as cluster species with ammonia. MQ and CQ readily formed cluster species of the type $[2M_q^+ + A^-]^+$ with chloride and acetate anions. Since the clusters formed over a wide quat concentration range, encompassing the range encountered for pesticide residue monitoring, their formation could affect the reproducibility and limit of linearity of an analytical method employing electrospray mass spectrometric detection.

The MS^n fragmentation pathways of the quats were explored and several fragment ions not reported previously were observed, for which assignments have been proposed. Furthermore, highly specific MS^n transitions were identified for each quat and the resonance excitation conditions promoting these transitions were optimised using simplex. These MS^n transitions could potentially be used for detection of the quats with high specificity.

Incorporation of MS^n strategies into existing analytical methodology, for example the generic LC-MS method using the cyano column, would be one approach to the highly specific determination of the quats. However, the majority of the existing analytical methods for the analysis of the quats have long analysis times and so sample throughput is limited. A rapid method for the quantitative analysis of the quats was developed using flow injection – mass spectrometry (FI-MS) and

incorporated MS^n detection. Similar linear dynamic ranges and LODs to those obtained by LC-MS for the analysis of the quats in deionised water were achieved, but with a much reduced analysis time per injection (0.3 min *vs.* 20 min). For the analysis of real samples solid phase extraction (SPE) was performed on a non-endcapped cyano phase in order to remove matrix components, which had a detrimental effect on the mass spectrometric response. Off-line SPE–FI-MS^n analysis of CQ in pear matrix gave linear calibrations over two orders of magnitude, and LODs below those required for the regulatory monitoring of CQ in pear were obtained. The method could be fully automated and triplicate sample analysis in one MS^n mode could be performed in under 1 minute.

In conclusion, consideration of their chromatographic behaviour on a variety of stationary phases together with detailed examination of their mass spectrometric fragmentation pathways and subsequent identification of highly specific MS^n transitions has enabled the development of an SPE-FI-MS^n method for the rapid, specific, sensitive and quantitative analysis of the quats.

6.2 FUTURE WORK

The benefits and implications of the work presented here are not restricted to the analysis of quaternary ammonium pesticides.

Examination of the retention mechanism of cationic species on various stationary phases revealed that stationary phases based on Spherisorb silica contain a high proportion of silanol groups. Interaction of cationic species with the silanol groups causes the stationary phase to behave like a weak cation exchange medium. This could be exploited in the development of LC methods, though due to the presence of different types of silanol sites, peak tailing can be problematic. This could be overcome by combining the selectivity of the stationary phase with the efficiency of an electrophoretic separation through the development of a capillary electrochromatography (CEC) method. For the analysis of the quats CEC using a non-endcapped cyano phase could be explored. The residual silanol sites present in

the non-endcapped cyano phase would be advantageous in a CEC method. When the silanol groups exist in their anionic form (SiO^-), cations are attracted to them, forming an electrical double layer and creating a potential difference, known as the zeta potential (Heiger, 1997). When a voltage is applied across the capillary, the cations forming the double layer migrate toward the cathode. Because these cations are saturated, their movement drags the bulk solution in the capillary toward the cathode and an electroosmotic flow (EOF) results (Heiger, 1997). The zeta potential and hence the magnitude of the EOF is essentially determined by the surface charge on the base silica and therefore, the number of silanol sites present in their anionic form. Thus, in a CEC method, the residual silanol sites are needed to drive the liquid flow within the capillary and so the non-endcapped cyano phase is likely to be an ideal stationary phase for the analysis of the quats by CEC.

The statistical method, *i.e.* simplex optimisation, used to achieve rapid ascent to optimum MS^n fragmentation conditions could be applied to the optimisation of other analytical techniques. For example, optimising the separation of the quats in a CEC method, where parameters likely to influence the separation are the separation pH, the separation potential, the length of capillary and concentration of salts in the mobile phase. .

The parameters controlling MS^n fragmentation in an ion trap have been shown to exert a large influence on the signal response observed, particularly in the analysis of low mass ions. Optimisation of MS^n parameters has resulted in significant gains in the sensitivity levels achieved in subsequent analytical methods. Thus, optimisation of MS^n conditions would be beneficial for other low-mass analytes requiring specific, trace-level determination, for example, in the trace level analysis of explosive residues, such as nitroglycerine.

The SPE-FI-MS^n method could be used in two ways for the analysis of quats. Firstly, it could be employed as a qualitative technique for initial screening of quats within food, or environmental matrices. Alternatively, it could be included in regulatory monitoring programmes for the rapid quantification and highly specific

determination of the quats. In either case, further work would need to be carried out to determine the suitability of the SPE-FI-MS^n method to the analysis of the quats in a range of matrices. In particular, the SPE protocol developed for CQ using a non-endcapped cyano phase would need to be investigated to determine its applicability to the analysis of the other quats, and to the analysis of CQ in matrices other than pear and water. The SPE-FI-MS^n method could be extended further to incorporate a preconcentration factor into the SPE methodology so that rapid determination of pesticide residues in drinking water could be performed. In addition, the development of a fully automated online SPE-FI-MS^n method would be beneficial for the employment of the technique in regulatory laboratories.

The FI-MS^n method could be applied to the rapid analysis of other analytes requiring either initial qualitative screening or highly specific quantification. For example, potential uses include regulatory monitoring of other pesticides, testing for drug abuse in sports including equine testing, and trace level determination of pollutants in environmental matrices.

Chapter 7

Experimental and Analytical Procedures

7.1 GENERAL PROCEDURES

7.1.1 General considerations

Prior to all experiments, the glassware was washed with Decon 90 to remove any residual organic compounds and then rinsed with deionised water. All solvents used were HPLC grade and all mobile phase additives were analytical reagent grade.

The doubly charged cationic species diquat (DQ) and paraquat (PQ) are prone to sorptive interactions (Taguchi *et al.*, 1998). In order to prevent losses of PQ and DQ through adsorption onto glassware surfaces, all glassware (vials, volumetric flasks, syringe barrel) that would come into contact with PQ and DQ was deactivated by silanisation (Section 7.1.2). Silanisation chemically bonds a thin water-repellent film to glass giving neutral, hydrophobic and non-oily surfaces that are not affected by solvents and are not easily hydrolysed (Doughty, 2000). Where possible polypropylene labware was used for the quat dications.

7.1.2 Silanisation protocol

Silanisation was achieved using Sylon-CT, a solution of 5% dimethyldichlorosilane in toluene (Supelco, Poole, UK) as follows:

1. The glass surface was coated with Sylon-CT by rinsing with the reagent for 10 to 15 s. The reagent was then discarded.
2. The glass surface was rinsed twice with toluene
3. The glass surface was rinsed three times with methanol and the final washings were checked to ensure they were pH neutral.
4. The surface was dried under a stream of nitrogen gas.

7.1.3 Standards

Standard solutions of 0.01 mg mL^{-1} chlormequat (CQ) chloride, mepiquat (MQ) chloride, difenzoquat (DF) methyl sulfate, PQ dichloride and DQ dibromide (Qmx, Thaxted, UK) were prepared by diluting stock solutions of concentration 1 mg mL^{-1}

prepared in 50/50 (v/v) water/methanol. Calibration solutions were prepared by diluting these standard solutions with deionised water, tap water or pear matrix, as appropriate.

7.1.4 Preparation of pear matrix

Pear matrix (methanolic pear extract) was supplied by Central Science Laboratory, York, and was prepared according to the method described in Startin *et al.* (1999).

7.2 INSTRUMENTATION

7.2.1 Mass spectrometric analyses

7.2.1.1 Ion trap mass spectrometric analyses

The majority of mass spectrometric analyses were performed using an ion trap LC-MS system, which comprised a Thermo Separations liquid chromatograph (P4000) and autosampler (AS3000) and a Finnigan LCQ equipped with an ES interface and operated in positive ion mode using Finnigan Navigator software version 1.2 (Finnigan, Hemel Hempstead, UK). Finnigan developmental software was used to access the advanced controls on the ion trap, which allow adjustment of the activation amplitude (AA), the activation time (AT) and the value to which Q is set during fragmentation (AQ). All solvents were degassed continuously using helium.

7.2.1.2 MS analyses on a triple quadrupole mass spectrometer

MS/MS analyses were performed using a Micromass Quattro tandem quadrupole mass spectrometer (Manchester, UK) operated in positive ES mode and equipped with a Gilson 231 autosampler (Villiers le Bel, France) and a Waters 600MS HPLC pump (Milford, MA, USA).

7.2.2 Capillary electrophoretic analyses

Capillary electrophoresis was performed using a ThermoQuest CE Ultra instrument (ThermoQuest, Stroke-on-Trent, UK) coupled *via* a Finnigan CE-MS interface kit to a Finnigan LCQ mass spectrometer fitted with an ES interface. MS detection was performed in positive ion mode.

7.3 LC-MS ANALYSES

7.3.1 Ion exchange LC-MS for the analysis of CQ

7.3.1.1 Ion exchange chromatographic conditions

A Partisil 10 SCX column (150 mm x 2.1 mm; Capital HPLC, Broxburn, UK) was used at ambient temperature with a mobile phase comprising 50 mM ammonium acetate in 50/50 (v/v) methanol/water flowing at 0.2 mL min^{-1}. The injection volume was 20 μL (5 x loop overfilled). The column, which had been used almost exclusively for the analysis of chlormequat, was equilibrated with fresh mobile phase for an hour prior to LC experiments. The sample syringe was flushed with 50/50 methanol/water (1 mL) before each injection to prevent carry-over. The column dead volume was measured using β-carotene as unretained species.

Calibration solutions of chlormequat cation in pear matrix covered the range 1.6 to 1620 ng mL^{-1}. To convert ng mL^{-1} to the usual units for reporting pesticide residues, mg kg^{-1}, multiply by a factor of 0.005. These solutions were also used to determine limits of detection (LODs).

7.3.1.2 Ion trap mass spectrometric conditions for ion exchange LC-MS analyses

LCQ MS conditions were: sheath and auxiliary gas flows 55 and 30 arbitrary units respectively, capillary temperature 200 °C, and spray voltage 4.0 kV. Selected ion monitoring (SIM) of both *m/z* 122 and *m/z* 124 was performed during the same analysis. Since MS/MS experiments required the LC and autosampler trigger signals to be disconnected from the LCQ, these analyses were performed during a separate

acquisition. Optimised MS^2 ion trap parameters for CQ were as follows: isolation window 1.5 Th, AA = 0.96 V, AQ = 0.354 and AT = 35 ms. SIM and MS/MS experiments used the divert valve for the first 7.5 min to avoid blocking the heated capillary by the build up of sugars around the orifice.

7.3.1.3 Triple quadrupole mass spectrometric conditions for ion exchange LC-MS analyses

The triple quadrupole MS (Quattro Tandem quadrupole mass spectrometer) conditions were: source temperature 120°C; nebuliser and bath gas flows 15 L h^{-1} and 350 L h^{-1}, respectively, cone voltage 25 V, capillary and HV lens voltages 3.6 kV and 0.15 kV respectively. For LC-MS/MS, CID was performed using argon as collision gas at 5 $\times 10^{-3}$ mbar and a collision energy of 35 eV. MS and MS/MS were employed concurrently, using SIM on Q1 and selected reaction monitoring (SRM) for the CID transitions from the ^{35}Cl and ^{37}Cl isotopes of the chlormequat precursor ion (*m/z* 122.07 and 124.07) to the common product ion at *m/z* 58. The dwell time for each of the four channels was 0.2 s, the interchannel delay was 0.02 s and the mass span was 0.2.

7.3.2 Reversed-phase LC-MS analysis of CQ

7.3.2.1 Reversed-phase chromatographic conditions

A Spherisorb S5 ODS1 column (250 x 4.6 mm; Phase Separations Ltd, Deeside, UK) was used at ambient temperature with a mobile phase, comprising acetonitrile/methanol/water/acetic acid (53/21/25/1) and being 50 mM in ammonium acetate, flowing at 1 mL min^{-1}. A 20 µL injection loop was fitted and overfilled 5 times. With the exception of the manufacturer's tests, the column had been used solely for the analysis of CQ. The column was equilibrated with fresh mobile phase for an hour prior to LC experiments. The sample syringe was flushed with 50/50 methanol/water (1 mL) before each injection to prevent carry-over.

Experiments to examine the retention mechanism of CQ on C_{18} phases were carried out using either a Hypersil ODS 5 μm fully endcapped column 250 x 4.6 mm (HPLC Technology, Macclesfield, Cheshire, UK) or a Hypersil BDS C_{18} 5 μm column 250 x 4.6 mm (Shandon, supplied by Alltech, Carnforth, UK), in addition to the Spherisorb ODS1 column. The ammonium acetate and acetic acid contents of the mobile phase was varied, as detailed where appropriate, to effect changes in the ionic strength and pH. Column dead volumes were measured using sodium nitrite as unretained species.

Solutions for calibration were prepared in deionised water, tap water and pear matrix and covered the range 1.6 to 1620 ng mL^{-1}. For determination of LODs the calibration solution of lowest concentration was diluted further with the appropriate matrix.

7.3.2.2 Mass spectrometric conditions for reversed phase analysis

The LCQ MS conditions used were: sheath and auxiliary gas flows 65 and 20 arbitrary units respectively, capillary temperature 230 °C and spray voltage 3.5 kV. SIM experiments used the divert valve for the first 4.5 min.

7.3.3 Development of a generic LC-MS method for analysis of the quats

7.3.3.1 Chromatographic conditions

The following 5μm stationary phases were evaluated: Spherisorb ODS1, silica, cyano, all in 250 x 4.6 mm columns (PhaseSeparations, Deeside, UK) using an isocratic solvent system flowing at 1 mL min^{-1} and comprising acetonitrile/methanol/water/acetic acid (53/21/25/1) containing a final concentration of 50 mM ammonium acetate. After initial evaluation, elution of the quats from various cyano phase columns: 3 and 5 μm Spherisorb cyano 4.6 x 150 or 250 mm (PhaseSeparations); ACE 3μm CN, 150 x 2.1 mm, (HiChrom, Reading, UK) was carried out using a mobile phase gradient (Method A; Table 2.5) comprising

acetonitrile, water and 200 mM ammonium formate in water containing 5 % formic acid (pH ~ 3.5) flowing at 1 mL min^{-1} (except as noted in Chapter 2). Slight modification of the solvent programme was required (Method B; Table 2.5) for the S3 cyano column (150 x 4.6 mm, Phase Separations).

Solutions for LC-MS calibration and LOD determination were prepared in deionised water, tap water or pear matrix and covered the range 0.01 to 2000 ng mL^{-1}. LODs and calibration plots were determined using Method B (Table 2.5) on a S3 cyano column (150 x 4.6 mm, Phase Separations).

7.3.3.2 Mass spectrometric conditions for the generic LC-MS method

LCQ MS conditions used were: sheath and auxiliary gas flows 65 and 30 arbitrary units, respectively, capillary temperature 230 °C and spray voltage 4.5 kV. SIM was performed for *m/z* 122 (CQ); *m/z* 114 (MQ); *m/z* 92 and 183 – 184 (DQ); *m/z* 93 and 185 – 186 (PQ) and *m/z* 249 (DF). For experiments carried out in pear matrix the flow was diverted for the first 3.4 min.

7.4 CE-MS ANALYSES

Separations were carried out using an uncoated fused-silica capillary (86 cm x 75 μm i.d., Composite Metal Services, Hallow, UK) tapered 1 cm at the interface end. Tapering was performed by drawing out the capillary in a Bunsen burner flame (McClean *et al.*, 2000). The end was then trimmed to remove any potential blockages, which might have occurred due to heat sealing of the tip. The capillary was positioned protruding 0.5 mm from the ES needle using a micrometer. The capillary was rinsed or conditioned by pressure-driven flow (100 psi) from the inlet vial. Prior to analysis of the quats, the capillary was treated with a rinse of 1 M sodium hydroxide solution at 50 °C for 0.4 min followed by conditioning, by leaving the solution in the capillary at 50 °C for a further 5 min. The capillary temperature was adjusted to 30 °C and conditioning continued by flushing in turn with 0.1 M sodium hydroxide, deionised water and with run buffer, each for 0.4 min. The run buffer comprised 50 mM ammonium acetate in water at pH 4.7 and was prepared by

adjusting the pH of a solution containing 1.42 mL of glacial acetic acid in 475 mL of water to pH 4.7 with 10% ammonia solution.

Hydrodynamic injections of 5 s duration at 1.6 psi were used to introduce the sample into the capillary, which was thermostatted at 25 °C for the CE runs. A separation potential of 30 kV was applied. The ES spray voltage was set to 0 V for the first 30 s of the analytical run and subsequently to 3.6 kV, resulting in an overall separation potential of 26.4 kV. Each vial of run buffer was only used for three analytical separations before switching to another run buffer vial, to ensure repeatability. In between analytical runs the capillary was flushed (100 psi) for 0.4 min at 25 °C with run buffer. The sheath liquid flow, comprising 1% acetic acid in methanol, was introduced into the ES interface at 2.5 μL min^{-1} using a Harvard Apparatus syringe pump, Model '22' (Harvard Apparatus Ltd., Edenbridge, Kent, UK). The LCQ sheath gas flow was set to 5 arbitrary units, no auxiliary gas was used, and the heated capillary temperature was set to 150 °C.

Calibration solutions of the five quats were prepared in deionised water and covered the range 0.01 to 100 μg mL^{-1} of quat cation. All solutions used in CE-MS analyses were filtered through 0.45 μm filters and were degassed daily.

7.5 MASS SPECTROMETRIC INVESTIGATIONS

7.5.1 Response for precursor and product ions at varying AQ values

Standard solutions of the quats (0.01 mg mL^{-1}) were introduced individually into the LCQ mass spectrometer by infusion (10 μL min^{-1}), and the signal response for the precursor ion was monitored as a function of AQ while AA and AT were held constant at 0 V and 0.03 ms (the lowest setting achievable). Similarly, MS^2 product ion intensity was monitored as a function of AQ, while AA and AT were held constant.

7.5.2 Optimisation of product ion intensity

7.5.2.1 Preliminary optimisation of product ion intensity

Preliminary optimisation of CQ product ion intensity was conducted with direct introduction of mobile phase, comprising 50/50 methanol/water containing 50 mM ammonium acetate (0.2 mL min^{-1}), into the LCQ. CQ solution (0.01 mg mL^{-1}) was introduced into the mobile phase *via* loop injections (5 μL) using the rheodyne injector port on the LCQ. For each set of MS^2 resonance excitation conditions the signal response at *m/z* 58 was monitored and averaged over three replicate injections. Improvements in ion intensity were achieved by varying one parameter (AA, AQ, AT) at a time. Other LCQ conditions used were: sheath and auxiliary gas flows 55 and 35 arbitrary units, respectively, capillary temperature 200 °C and spray voltage 4.0 kV.

7.5.2.2 Simplex optimisation of product ion intensity

Individual solutions of the quats (0.01 mg mL^{-1}) were used for the simplex optimisation of MS^n product ion intensities. The experimental conditions and procedure for simplex optimisation was identical to that described above (Section 7.5.2.1), except that improvements in MS^n ion intensity were achieved by varying the three parameters AA, AQ and AT simultaneously according to the protocol outlined in Chapter 3 (Section 3.2.4).

7.5.3 Cluster studies

The LCQ MS conditions used were those described above (Section 7.5.2.1), except that the auxiliary gas flow was 30 arbitrary units. Individual solutions of the quat cations (10 μg mL^{-1}) were introduced by loop injection (20 μL loop) into a mobile phase comprising 50/50 methanol/water containing 50 mM ammonium acetate. ES-MS spectra were acquired over various mass ranges (*m/z* 50 – 500; 50-1000; 50-1250; 50-2000). The stability of clusters over a range of AQ values and their MS^n fragmentation pathways were determined with MQCl and CQCl (10 μg mL^{-1}) infused at 10 μL min^{-1} into the LC stream (flowing at 0.2 mL min^{-1}) using the

syringe pump provided with the LCQ. Similarly, MQ and CQ solutions were infused for in-source CID experiments; the collision energy was increased incrementally from 0% to 100% in 10% steps. Experiments investigating the effect of ammonium acetate concentration upon MQ and CQ cluster formation were carried out using mobile phases containing a range of ammonium acetate concentrations (between 0 and 50 mM), while the concentration of the quat solution injected was kept constant (10 μg mL^{-1}). Experiments investigating acetate cluster formation over a range of MQ and CQ concentrations (0.01 to 10 μg mL^{-1}) were performed by loop injection of the quat solution into a mobile phase containing 50 mM ammonium acetate in 50/50 methanol/water. Competitive binding experiments for MQ and CQ with chloride and acetate were performed by loop injection of solutions of MQCl and CQCl (6.6 mM) into a mobile phase with total ammonium acetate concentration at 6.6 mM.

In between experiments involving different mobile phase compositions, the LCQ was flushed with fresh mobile phase for at least 30 minutes. All experiments were repeated three times.

7.5.4 Examination of the fragmentation pathways by MS^n

Solutions of the quats (0.01 mg mL^{-1}) were introduced by infusion (10 μL min^{-1}) *via* a T-piece into the mobile phase comprising 50/50 methanol/water containing ammonium acetate at a final concentration of 50 mM. MS^n analyses were performed using the advanced controls on the mass spectrometer according to the conditions found previously using simplex optimisation (Section 4.2.2). The LCQ MS conditions used were: sheath and auxiliary gas flows 55 and 35 arbitrary units, respectively, capillary temperature 200 °C and spray voltage 4 kV.

7.6 FI-MS^n ANALYSIS OF THE QUATS

7.6.1 FI-MS^n procedure

FI-MS^n experiments were performed using a mobile phase comprising 50/50 (v/v) water/methanol containing ammonium acetate at a final concentration of 50 mM and flowing at 0.2 mL min^{-1} into the LCQ mass spectrometer. Samples were introduced into the mobile phase *via* loop injections (5 μL loop, 5 x overfilled) using the rheodyne injector port on the LCQ. LCQ conditions used were: sheath and auxiliary gas flows 65 and 30 arbitrary units, respectively, spray voltage 4.5 kV and capillary temperature 230 °C. MS detection was performed in full scan, SIM, MS^2 and MS^3. MS^2 and MS^3 analyses were performed using the resonance excitation conditions found previously by simplex optimisation (Table 5.1). All solutions were analysed by 12 injections; three replicates for each of the MS detection modes. Individual calibration solutions of the quats (0.01 ng mL^{-1} to 2000 ng mL^{-1}) in tap water and deionised water were analysed. In order to assess the application of the method to the analysis of real samples, analysis of CQ in pear matrix was undertaken. Solutions for calibration in pear matrix were prepared and covered the range of concentrations outlined above. These were analysed by FI-MS^n after SPE extraction (Section 7.6.3).

7.6.2 Effect of matrix on FI-MS^n

Solutions spiked with CQ at 250 ng mL^{-1} were prepared with different ratios of pear matrix to deionised water. The solutions were analysed by FI-MS with full scan MS detection. Three replicate injections were performed per solution.

7.6.3 SPE protocol

SPE was achieved using non-endcapped cyano phase extraction cartridges (Isolute CN; 100 mg sorbent mass and 3 mL reservoir volume; Jones Chromotography, Hengoed, UK) and the extraction protocol outlined in Table 7.1. Extraction cartridges were held in an SPE vacuum manifold. The vacuum was maintained using a water pump regulated to achieve a flow rate of 1 mL min^{-1}. The SPE cartridges

were not allowed to run dry. Tetraethylammonium chloride hydrate was added as an internal standard to all samples subjected to SPE analysis, so that the final TEA concentration present in the sample was 250 ng mL^{-1}.

Step	Action
Precondition column	2 mL of 0.01% ammonia solution
Load	1 mL of sample
Wash	1 mL of water
Elution	1 mL of 0.1% formic acid
Reduction	Blow down sample at 50 °C under nitrogen stream
Reconstitution	1 mL of deionised water

Table 7.1: SPE protocol for the extraction of CQ.

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