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The *in vitro* bioavailability of
components of some dental restorative resins

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ABSTRACT OF THE THESIS

Resin-based materials are important restorative materials in clinical dentistry with increasingly diverse applications being suggested. These materials, although possessing properties necessary for their successful use in the restoration of teeth, have also been associated with untoward clinical effects. The loss of components from the material in service has been suggested as one such cause for these effects. An investigation was conducted to examine component loss from the material into water, over a time course of up to 100 days, by high performance liquid chromatography (HPLC) and mass spectroscopy (MS). It has also been suggested that dentine possesses a protective function, perhaps by preventing the passage of released components through to the pulp space. It was then important to examine the effect of dentine on the passage of released components. For this investigation, an *in vitro* tooth model, based on that developed by Hume (1985), was used. This model enabled restorative procedures to be carried out, in a simulation of clinical procedures, whilst allowing retrieval of the sample from the "pulp space" cavity at intervals. The "pulp space" samples were then subjected to chemical analysis by HPLC and MS.

The monomer component, TEGDMA, was found to be released from composite resin into water throughout the time course. The highest rate of release was observed in the earliest time interval, that is, under 4.32 minutes. The highest cumulative release was seen when the composite resin was uncured or when air-inhibition of the surface during curing had occurred. A range of composite resins showed similar patterns of TEGDMA release.

TEGDMA was also observed to diffuse from composite resin through dentine, *in vitro*. Dentine, however, delayed the time of attainment of highest rate of diffusion. It is proposed that this delay and its effect on local concentrations within the pulp is the basis of the protective effect of dentine against the cytotoxicity of resin components. When a dentine bonding system was used, the resin reinforced layer did not prevent TEGDMA diffusion from the composite resin and the monomer, HEMA, a component of a dentine bonding system, was

also observed to diffuse through dentine in a similar pattern to that of TEGDMA. Physiological positive (outward) hydrostatic pressure from the pulp space did not affect the TEGDMA or HEMA diffusion observed, but high hydrostatic pressure muted TEGDMA diffusion. Amounts of TEGDMA and HEMA diffusing through dentine were found to be highest when dentine had been pre-treated with phosphoric acid etch, and were lowest when dentine was untreated or treated with other common etchants, namely, EDTA and maleic acid.

These findings provide insight into the mechanism of adverse responses noted in pulpal cytotoxicity studies (for example, Qvist *et al.*, 1989) and into the nature of the interdiffusion zone. The data also have significance for potential untoward reactions, such as allergy, following the clinical use of resin-based restorative materials.

PUBLICATIONS AND PRESENTATIONS OF THE WORK DESCRIBED IN THE THESIS

Papers:

Gerzina TM and Hume WR (1994)
Effect of dentine on release of TEGDMA from resin composite
in vitro.
J Oral Rehabilitation 21:463-468.

Gerzina TM and Hume WR (1995)
Effect of hydrostatic pressure on the release of monomers
through dentine *in vitro*.
J Dental Research 74(1):369-373.

Gerzina TM and Hume WR
Diffusion of monomers from bonding resin-resin
composite combinations through dentine *in vitro*.
J Dentistry (in press)

Hume WR and Gerzina TM
The influence of some physical variables on TEGDMA release
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J Dental Research (submitted)

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Cytotoxic evaluation of leachable components from orthodontic adhesives.
J Dental Research 73: 436

STATEMENT OF AUTHORSHIP

The work presented in this thesis is wholly the work of Tania M Gerzina, except where stated, undertaken at the Institute of Dental Research between March 1991 and December 1992 and within the Department of Restorative Dentistry and Dental Public Health and Hygiene in the Faculty of Dentistry, University of California, San Francisco USA between January 1993 and December 1993, towards the degree of Doctorate of Philosophy at the University of Sydney.

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1.1 Introduction

The aim of the present work was to determine the patterns of release of components of resin restorative materials into the adjacent aqueous phase.

If they occur, local toxicity (for example, to pulpal or periodontal cells), systemic toxicity and atopy (allergy) must all be mediated by released components, because such responses occur at a distance from the restorative material. Knowledge about which chemicals move out of resin-based materials, in what amounts and for how long, is very likely to aid in both the safe use of the present generation of resin bonding agents and tooth restoratives, as well as in the design of future resin-based materials. Increasing our general understanding of component release from restorative materials and the effects of dentine on such release may also aid in the safe use and future design of other classes of restorative materials.

Before addressing specific issues associated with the release of components from dental restorative resins, it is relevant to consider the general nature and the sequence of development of the synthetic, polymeric resin materials which preceded the development of dental resins.

1.2 General polymer theory

Polymers are large molecules consisting of smaller, repetitive units called mers or monomers. Generally, to be considered a polymer a molecule must have a molecular weight of at least 1000 and a large number of mer-derived units. Smaller polymers are called oligomers. Polymeric molecules may be *linear*, with monomer-derived units joined end to end; *branched*, where multiple interconnections between units occur, or *networked* where even more complex arrangements occur. The primary bonds joining monomeric components in most polymers are covalent. Various intramolecular forces such as dipole moments and hydrogen bonds act to fix the individual chains into solid spatial arrangements. Many natural organic polymers, such as polysaccharides and proteins, occur in plants and animals. The majority of synthetic polymers are also organic. There are some synthetic inorganic polymers, such as silicones, which are used in dentistry.

Polymers were first described as such by Staudinger in 1920, who coined the term "macromolecule". Definitive establishment of the molecular

hypothesis of polymer structure is attributed to Carothers of the Du Pont Company, who in 1929 demonstrated the relationship between properties and structure of polymers. The company applied his concept to the development of synthetic rubber and nylon, then to many other polymers.

Carothers suggested a polymer classification based on the nature of the polymerization reaction, namely *condensation* or *addition*. Condensation and addition reactions have also been termed *step* and *chain* reactions respectively.

Condensation reactions involve the loss of a molecule, usually water or an alcohol, as two monomer units join together. Examples of condensation reaction polymers are the phenol-formaldehyde resin, Bakelite and polyester, which is poly(ethylene terephthalate). In step reactions, there is a gradual build up of high molar mass material. The complexity of the final structure will ultimately depend on the number and location of functional groups on the parent monomers and the reactions which take place between them. Multiple functional groups will yield polymers with an extensive and highly branched or networked character.

In contrast, addition polymers are the product of the reaction of unsaturated monomers which cleave to allow polymerization with no reaction product. Polymerization of most restorative composite resins occurs this way. Monomer units of addition polymers must therefore contain an accessible, unsaturated bond. Addition of molecules occurs at activated sites within the reactive molecule. A relatively rapid growth of macromolecules can occur, but the process generally gives rise to only linear or simply branched structures. Because of their relatively weak intermolecular bonding, such polymers are generally more sensitive to penetration by solvents and have a higher tendency to degrade over time.

General aspects of polymer chemistry which are most important to dental composite resin will be briefly considered in the following sections. In light of this background, specific characteristics of dental composite resin will then be presented. The importance of the bioavailability of components from dental materials will then provide the rationale for the experimental objectives of the current work. As initiation of polymerization is a crucial aspect of polymer kinetics, this will be discussed first.

1.2.1 Initiators and accelerators

Addition polymerization takes place in three stages. The first of these is initiation (activation) where a chemical initiator breaks down liberating free radicals which open double bonds in monomers nearby to start polymerization. A common example in dentistry is benzoyl peroxide, an initiator for poly-methyl methacrylate (PMMA) resins. Benzoyl peroxide degrades when heated to release two radicals each of which opens a double bond in a nearby methacrylate monomer. Initiators in composite resins have been of three types, chemical, photo and a combination of these two types.

Chemically-initiated polymerization uses a tertiary amine accelerator. When added to a thermochemical initiator (such as benzoyl peroxide), an electron exchange between these compounds, at room temperature, liberates a free radical which then initiates polymerization. Application of this particular reaction allowed the development of the first direct filling (restorative) materials (Bowen, 1962). Initiators are particularly sensitive to decomposition by heat, light and some chemicals, resulting in spontaneous initiation and, in dental composite resins, this decomposition reduces colour stability. Examples of accelerators include N,N-dimethyl-para-toluidine and N,N-dihydroxyethyl-para-toluidine (Craig, 1981).

In addition to chemical-initiation, another form of initiation is photostimulation (or photoinitiation) which is commonly used in dental composite resin polymers. In photoinitiation, generation of free radicals is accomplished by using normal light at various wavelengths, argon laser (Blankenau *et al.*, 1991), γ -rays (Curtis Jr. *et al.*, 1991), X-rays or through electrochemical means. In photoinitiated polymerization, functional oligomers and monomers are polymerized and cross-linked by photogenerated initiators. Light-initiation of polymerization offers several advantages over chemical-initiation (Pappas and Woods, 1991). Amongst these advantages are the rapid formation of a polymer network, and the control of both organic emissions and heat generation. Several disadvantages also exist such as the dependence of cure depth on photoinitiator concentration. The inclusion of filler particles also effects polymerization of the resin material by interfering with light transmittance due to the opacity of the particles. Commonest photoinitiated systems in dental composite resin have used ultraviolet (UV) and visible-light sources.

In the UV-initiated systems, on exposure to UV-light (365 nm) a photoinitiator, for example, benzoin methyl ether, undergoes fragmentation or photolytic dissociation with the formation of polymerization-initiating radicals (Craig, 1981). These systems also require the presence of catalysts capable of diffusing through into unexposed regions (Pappas and Woods, 1991). Other examples of UV-photoinitiators include 4,4'-dimethylbenzophenone and 4-methacryloxybenzophenone. The requirement of these materials for reactive UV-sensitive diluents also reduces inherent viscosity, thereby reducing the need for solvents.

Many dental composite resins commonly used, employ visible-light initiated polymerization. Visible-light initiated composite resin systems typically contain a two-component initiator system of an α -diketone photo-sensitizer and a tertiary amine reducing agent. A commonly used ketone is di-2,3-diketo-1,7,7-trimethylnorcamphane or camphorquinone (CQ) which absorbs radiation energy with a wavelength maximum of 468 nm and enters an excited state. At the triplet state, CQ can then combine with a reducing agent (for example, N,N-dimethylaminoethyl methacrylate) to form an excited state complex which breaks down to liberate free reactive radicals (Ruyter, 1985). The content of CQ varies in different resin materials and has been determined by gas-liquid chromatography (GLC) to be 0.03-0.09% w/w was found in resin pastes, and 0.05-0.08% in bonding agents (Shintani *et al.*, 1985). Higher concentrations, such as 0.17-1.03% w/w, are found in composite resins (Taira, *et al.*, 1988). More CQ is included in more highly filled composite resins to compensate for the reduced light intensity received by these composite resins as a result of increased light scatter (Shintani *et al.*, 1985; Taira *et al.*, 1988). CQ is an intensely yellow crystal solid with a broad absorption band, and may impart this colour to the resin when used in larger amounts.

Several reducing agents are used in resins such as the aliphatic tertiary amine dimethylaminoethyl methacrylate (DMAEMA) (Craig, 1981), the aromatic tertiary amine di-methyl-p-toluidine (DMPTI) which is also an accelerator for self-cure resins, and ethyl 4-dimethylaminobenzoate (EDMAB) (Stansbury and Antonucci, 1992). The diketone absorbs radiant energy in the blue zone range of the visible spectrum (440-480 nm) (Ruyter, 1985; Taira *et al.*, 1988). In the excited state, the diketone extracts electrons from the amine to produce at least two free radicals, one from the

diketone and one from the amine. These radicals then participate in the polymerization process in a similar way to other methods of initiation.

Other photoinitiators have been suggested, for example, 2-hydroxy-3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-N,N,N-trimethyl-1-propanaminium chloride (QTX) with an absorption maximum of 402 nm (Hayakawa and Horie, 1992). The addition of QTX in a dentine bonding resin primer solution, is intended to facilitate polymerization initiation in monomers which have diffused into dentine. Visible-light initiation offers several advantages over UV-light initiation in the use of a safer wavelength, the achievement of a greater depth of cure, greater reliability of the curing sources available and more reliable cure through enamel (Shintani *et al.*, 1985).

In some clinical circumstances, it is desirable for polymerization to be both chemical- and photoinitiated simultaneously, such as in light-compromised areas. Combination-initiated (dual-initiated) materials provide this facility. In the dual-initiated systems both chemical and photoinitiated methods affect polymerization in initiation and chain growth respectively. The chemical polymerization in these materials has been referred to as "dark cure" (Pappas and Woods, 1991). In resin-modified glass ionomers and polyacid-modified composite resins a similar dual facility is incorporated together with the acid-base reaction and setting of a variable quantity of the ionomer component.

1.2.2 Conversion

A critical index of the efficiency of polymerization is the *degree of conversion*. Conversion, or the extent of polymerization, describes the breaking of double bonds within the developing macromolecule to enable chain growth and networking. Degree of conversion may be considered to be the extent to which the properties of a polymer approach those of the functionally cured state and implies cross-linking between monomers and oligomers with an inherent degree of unsaturation (Davies, 1991). In general, any physical or chemical property of a polymer which changes as polymerization takes place can be used to monitor degree of conversion. Commonest measurements of degree of conversion can be classified into two main types based on the timing of measurements (Davies, 1991): *real time* and *intermittent time*. In real time monitoring, the measurement of a property is made continuously whilst curing takes place. This is possible

with infra-red (IR) spectroscopy, calorimetry or dilatometry. In intermittent measurement of degree of conversion, the material is given a certain radiation dose after which measurements are made, measurements are again taken after another dose of radiation. IR spectroscopy is a sensitive quantitative technique which uses real time and gives a direct measure of the concentration of aliphatic carbon-carbon double bonds which disappear on conversion. This form of analysis also allows assessment of how much of the polymerization reaction has taken place and provides the facility to change parameters to determine the rate of polymerization (Davidson *et al.*, 1991).

In both chemical and photoinitiated composite resins a proportion of the methacrylate groups (25 - 55%) remain unreacted and the degree of successful conversion in an "optimally" cured composite resin may only be up to 75% (Ruyter, 1985). Conversion in dimethacrylate monomer systems can be determined by measuring the quantities of these unreacted methacrylate groups. This is accomplished by measuring the decrease in the carbon-carbon double bond (C=C) absorbance of light with wavelength in the 1635-1640 cm^{-1} range using, for example, multiple internal reflection (Ruyter, 1985). This is calculated as a percentage of the total amount of methacrylate groups in the unpolymerized material. Unconverted double bonds remaining at the end of early initiation tend to decay over the first 24 h after initiation and the half life of remaining reactive radicals is only an additional 30 h (Eliades *et al.*, 1987, Burtscher, 1993). This residual unsaturation exists mainly in the form of unconverted pendant methacrylate groups.

In dental resins, the amount of converted methacrylate groups per unit volume largely determines polymerization shrinkage (Venhoven *et al.*, 1993). During polymerization, double and Van Der Waals bonds are replaced by single covalent bonds in the polymer. This replacement is particularly sensitive to the amount of diluent monomer since methacrylate monomers are able to diffuse through the polymeric matrix to the reactive ends of the polymerizing chain.

A common oligomeric component of the polymeric matrix of composite resins is 2,2-bis[4-(2-hydroxy-3-methacryloxypropoxy)phenyl]propane (bisphenol-A-glycidyl dimethacrylate or Bis-GMA) (see section 1.4.1). The viscosity of this oligomer is generally reduced by the addition of diluent

monomers but these monomers also influence conversion. Conversion in resins containing dimethacrylates, for example, triethylene glycol dimethacrylate (TEGDMA), as diluent monomers is low compared with those containing monomethacrylates because heavy cross-linking in the monomethacrylates hastens "vitrification" of the polymer (Venhoven *et al.*, 1993). Conversion in dimethacrylates is also limited by the presence of pendant methacrylate groups which are unable to diffuse through the polymeric matrix (Venhoven *et al.*, 1993).

Of particular interest to the current experimental work is the role that resin composition has in conversion. Composition influences the quantity of remaining double bonds in chemically-initiated composite resins. The TEGDMA/Bis-GMA ratio in these composite resins, for example, determines conversion such that conversion decreases with decreasing TEGDMA and increasing Bis-GMA (Ruyter, 1985). The quantity of double bonds in these materials, however, is inversely related to amine, peroxide and inhibitor contents. However, where the concentration of these components is held constant, the quantity of remaining double bonds is found to vary inversely with concentration of diluting monomer concentration; this relationship is also applicable to light-initiated composite resins (Asmussen, 1982; Ruyter, 1987).

Other factors are associated with conversion in light-initiated composite resins. Common to both UV-initiated and visible-light-initiated resins is the identification of depth-dependence, beyond which conversion rapidly decreases. Better conversion is sometimes demonstrated in UV-initiated resins which is due to a higher time dependent quantum yield for radical formation in these materials (Ruyter, 1985). The depth of optimal conversion varies according to resin composition, efficiency of the activating light source and exposure time (Ruyter and Øysæd, 1982) and also with the nature and distribution of filler. Visible-light-initiated composite resins are especially sensitive to ambient operatory lighting which can provide an additional light intensity of 20 klx supplementing the 200 klx provided by an activating source. Prolonged exposure times are accompanied by elevated rates of free radical formation. Microfilled resins exhibit smaller depths of cure and higher amounts of unconverted bonds (Ruyter and Øysæd, 1982). This can be monitored by following concurrent hardness values as an indicator of degree of cure. Below the surface, adequate curing is achieved up to a certain depth; below this depth both

hardness and quantity of remaining C=C bonds change rapidly due to attenuation of initiating light as a function of distance. These variables are formally united in Lambert-Beer's equation which states that the rate of free radical generation (that is, rate of cure) is proportional to both the intensity of radiation at the surface of the resin and the concentration of photoinitiator.

Another important factor which determines conversion in resins is the nature of monomer and oligomer content, which differs between composite resin formulations. Oligomers, like Bis-GMA and monomers like TEGDMA and ethylene glycol dimethacrylate (EGDMA) are commonly used. However, other combinations are also used, including oligomers based on urethane methacrylates and products synthesized from Bis-GMA and hexamethylene diisocyanate, namely 2,2-Bis[4-(2-hydroxypropoxyphenyl)]propane and 2-hydroxypropylmethacrylate (Ruyter, 1987). The influence of monomer blends on conversion has been found to vary widely for different composite resins (Ruyter and Györösi, 1976; Stansbury, 1990; Rueggeberg *et al.*, 1990; Chung, 1990; Spencer *et al.*, 1992; Van Meerbeek *et al.*, 1992a; Yoshida *et al.*, 1992). Generally, increased concentrations of aromatic oligomers (such as Bis-GMA and Bis-MA) result in increased remaining methacrylate groups. Smaller quantities of remaining methacrylate groups are seen in resins containing higher amounts of the nonfunctional monomers (such as 2-hydroxyethyl methacrylate or HEMA) and oligoethylene glycol dimethacrylates (Spencer *et al.*, 1992). Relatively rigid monomers such as urethane dimethacrylate (UDMA), containing di(urethanophenyl)methane groups exhibit less conversion. Similar conversion has been demonstrated in visible-light-initiated and chemically-initiated composite resins containing the same monomer blend (Ruyter, 1987).

Filler content (see below) is also significant in conversion, with better conversion found in conventionally-filled resin (coarser ground particles) compared to "microfills" (Ruyter and Øysæd, 1982) where depth is comparable. The colloidal silica particles in microfilled composite resins scatter incoming light (so reducing radiation intensity) which reduces conversion in these composite resins. Light scattering is considered to be due to the reflection, refraction and diffraction of light from the particle surface. Larger particles exhibit mostly reflective scattering which is the smaller contributor to overall scatter and maximum scattering of light occurs as particle diameters approach half the wavelength of the activating

light. Composite resins containing larger, coarser particles are more likely to admit deeper penetration of activating light. Mismatch in the refractive indices of filler and resin matrix components also affects light transmittance (Eliades *et al.*, 1987; Patel *et al.*, 1992).

Heat absorption of the filler can also influence conversion. In heavily filled materials, heat absorption capacities of the filler particles become significant enough to influence setting reactions causing reductions in chain length and therefore disturbance of networking (Eliades *et al.*, 1987).

The degree of residual unsaturation of composite resin materials can determine optimal mechanical properties such as hardness, strength, flexural modulus and wear (Young *et al.*, 1978; Salako and Cruickshanks-Boyd, 1979; Asmussen, 1982; Ruyter and Øysæd, 1982). In an examination of double bond conversion in light-initiated composite resins by micro-attenuated infrared spectroscopy, for example, increased inorganic loading and decreased filler particle size increased the degree of unsaturation (Van Meerbeek *et al.*, 1992b). Following polymerization in TEGDMA-containing resins a network, in which about 30% of the double bonds remain unreacted, is formed (Kalachandra, 1989). Polymerization ultimately reaches a point beyond which no further networking occurs and this point is known as *termination*.

1.2.3 Termination

Polymerization does not generally continue until all monomer supply is exhausted because this would demand limitless activity of the radicals with time. Termination, therefore, marks the end of polymer growth and is effected by the reaction of two active radicals by either combination or disproportionation. Combination involves the reaction of two radical species (for example, the reactive ends of two polymer species) to form a single bond and one reaction product (Nicholson, 1991c). This product is a large, stable molecule. In disproportionation, a hydrogen atom is transferred from one radical to another leading to the formation of two reaction products, one saturated and the other unsaturated. A form of disproportionation is *chain transfer* where a growing polymer chain may terminate by acquiring a hydrogen atom from another molecule resulting in the formation of a new radical which itself initiates a new molecular chain. The donor molecule of the hydrogen atom is usually a neutral saturated agent (for example, a solvent) and may be purposefully added to

control polymer size. Usually, termination occurs by any of these methods and occurs before all of the monomer molecules have been initiated; as a result, residual monomer remains in the solid polymer.

The growing polymer is a very actively evolving molecule which by virtue of this activity is prone to spontaneous polymerization if unchecked. Because photoinitiated resin is particularly susceptible to spontaneous polymerization, a vital component which prevents these reactions is the inhibitor.

1.2.4 Inhibitors

Inhibitors or retarders are molecules which form free radicals of such high stability that they effectively stop chain growth. These components, therefore, function to prevent spontaneous polymerization of a monomer. Benzoyl peroxide is particularly sensitive to decomposition by heat, light and some chemicals resulting in spontaneous initiation. This necessitates the inclusion of inhibitors into the formulation and recommendations for light-safe, cool storage of the material.

Inhibitors commonly used with diacrylates include 4-methoxyphenol (Craig, 1981). Other inhibitors include members of the quinone family, such as hydroquinone (for example, monoethyl ether) and 2,4,6-tritertiarybutyl phenol (butylated hydroxy toluene or BHT), which are used in very low concentrations (for example, 0.005%). Inhibitors can be distinguished from retarders in that they halt the polymerization reaction completely. Chemically-initiated composite resins also contain colour stabilizers such as 2-hydroxy-4-methoxybenzophenone, 2(2'-hydroxy-5'-methylphenyl) benzotriazole and phenylsalicylate. Another aspect of polymers pertinent to composite resin is the susceptibility of polymerization initiation to inhibition by oxygen.

1.2.5 Oxygen inhibition of cure

The early stage of polymerization in all types of initiation systems is inhibited, for a time, by oxygen (Finger and Jørgensen, 1976; Reinhardt and Vahl, 1978). This period of inhibition is directly related to the initial oxygen concentration and to the inhibitor content (Asmussen, 1981). Oxygen diffusing into the polymerizing material successfully competes with monomer for reaction with radicals and causes the formation of a poorly initiated or *air-inhibited* resin layer on the surface. This layer is similar in

composition to uncured resin but now lacks sufficient initiator-activator content to polymerize. Rueggeberg and Margeson (1990) found the degree of conversion in unfilled resins polymerized in an argon atmosphere, which is effectively air-excluded, was four times that found in unfilled resins polymerized under air-inhibited conditions. The thickness of the inhibited surface layer in the two cases differed by a factor of 12. This air-inhibited layer contrasts with the anaerobic cure achieved clinically, when a polyester matrix is applied before photoinitiation.

The thickness of this layer varies for different resin compositions and initiation systems and can be measured by surface profilometry (Davies, 1991). UV-initiated composite resins exhibit a thicker oxygen-inhibited layer possibly as a result of the greater tendency of UV-light to be scattered compared with visible light (Ruyter and Øysæd, 1982). Resins exhibiting high viscosity (with lower diluent monomer contents) also exhibit thicker unpolymerized layers than resins of low viscosity (Rueggeberg and Margeson, 1990). The inhibited layer on unfilled resins, used as bonding agents, provides a source of radicals capable of diffusing into the associated composite resin layer to facilitate polymerization. Subsequently, as the bulk of composite resin contracts towards the centre of the mass and takes with it the well-bonded adhesive layer, there is the potential for the development of a contraction gap at the resin/substrate interface.

Measurement of the air-inhibited layer, accomplished by surface profilometry (Davies, 1991), can enable the relating of thickness to photoinitiator type and concentration, prepolymer resin and monomer, inhibitor concentration, light intensity and exposure time as shown in the following equation:

$$\Delta t_{o_2} = \frac{A}{I[PI]} + \frac{B}{E[PI]} \quad (1.1)$$

where:

- t = time,
- A, B = constants,
- I = intensity,
- PI = photoinitiator concentration,
- $E = (It) =$ total exposure energy.

1.2.6 Cross-linking

Cross-links are defined as the covalent interatomic bonds that occur between macromolecules (Nicholson, 1991d). The presence and frequency of these cross-links can determine some properties of the polymer. Resistance to dissolution in solvents, for example, is imparted by heavy cross-linking and this is dependent on the density of the cross-linking. Weakly cross-linked polymers more readily admit solvent, probably becoming softer and swelling with the effect, and under certain circumstances this effect is reversible. Mechanical properties are also dependent on cross-linking density. Heavily cross-linked materials typically contain rigid three-dimensional networks which make the material brittle. This brittleness is constant at a range of temperatures.

Cross-linking is introduced into the polymer at either polymerization or after the initial macromolecule has formed. This second category requires the formation of a prepolymer followed by cross-linking. This has the effect of reducing the tendency of the material to flow at low temperatures and is referred to as *curing*. Davies (1991) suggested that curing implies the "cross-linking between multifunctional monomers and oligomers".

1.2.7 Polymerization kinetics

In materials which harden by polymerization, such as polymers, the phenomenon of molecular movement during polymerization is particularly important in a consideration of stability of the material. The rate of polymerization over time in methacrylate resins for example, is non-linear resulting from increasing viscosity of the developing polymer which causes radicals to lose mobility at about 20% conversion (Ruyter, 1985).

Of particular interest to the current work are the functions of composite resin components in polymerization kinetics. In polymerization, decomposition of the initiator molecule occurs during the rate determining step of initiation, liberating free radicals. These free radicals then interact with reactive sites on the monomer and a rate of initiation can be determined. A series of propagation steps then occur culminating in termination which takes place by either combination or disproportionation. A steady state of free radical concentration occurs early in polymerization when rates of initiation and termination become equal and maximum monomer consumption takes place during propagation. Nicholson (1991b) has described polymerization kinetics in an equation relating several

variables which demonstrates that several components of the polymer are critical participants, at different times, in polymerization (equation 1.2).

$$R_p = k_p \left(\frac{fk_d [I]}{k_t} \right)^{1/2} [M] \quad (1.2)$$

This equation indicates that, in the early stages of polymerization, rate of reaction (R_p) is directly proportional to square root of the initiator concentration (I) when the fraction of initiator fragments causing polymerization (f) is independent of monomer concentration (M). Other variables in the equation are rate constants. When poorly efficient initiators are used, this fraction may be proportional to the monomer concentration. This indicates that the monomer content of resin may have a profound effect on the rate of polymerization.

With respect to visible-light-initiated composite resins, a consideration of polymerization kinetics can enable a better understanding of polymerization shrinkage (see below) which is a serious disadvantage in the clinical use of these materials. Contraction during polymerization is time-dependent, occurring both before and after the gel point has been reached (Bausch *et al.*, 1982). The gel point represents a critical stage in step polymerization; when the polymer abruptly loses fluidity, viscosity rises sharply and further diffusion through the gel area is limited. The gel area increases in size as polymerization continues through the gel point (Nicholson, 1991c) and the growing polymer progressively gains rigidity.

In dentistry, conversion in composite resins has been determined by forms of IR spectroscopy such as transmission (Asmussen, 1982), IR multiple internal reflection (Ruyter and Øysæd, 1982; Ruyter, 1987), micro-attenuated total reflection IR (Eliades *et al.*, 1987), and by Fourier Transform IR Spectroscopy (FTIR) (Ferracane and Greener, 1984; Rueggeberg and Craig, 1988; Rueggeberg *et al.*, 1990; Caughman *et al.*, 1991). Estimation of degree of conversion from volumetric contraction has also been used (Venhoven *et al.*, 1993).

Calorimetric measurement of conversion exploits the exothermic nature of double bond breaking during polymerization. The heat of the photoinitiated chemical reaction can be determined by detecting the temperature difference between the sample and a reference cell (Davies, 1991).

Dilatometry monitors photopolymerization in solution since a volume change accompanies the growth of the polymer. The quantity of polymer formed following each radiation dose is determined by pouring the mixture into a quantity of solvent in which the polymer will precipitate. The polymer is then filtered off, dried and weighed. Characterization can then be completed by gas-chromatography.

The polymerization of the oligomer in composite resin is accompanied by a degree of shrinkage, as networking contracts intermolecular distance, which has been measured (Dennison and Craig, 1972; Goldman, 1983). Watts (1991) predicted an exponential relationship between shrinkage and time if there is a steady concentration of radicals formed and consumed and if shrinkage follows cross-linking polymerization. The direction and magnitude of the shrinkage also varies, according to the method of polymerization of the composite resin, so that photoinitiated resins contract least and toward the source of initiation whereas chemically-initiated materials shrink toward their centres (Jensen and Chan, 1985). The addition of 50% by volume of inorganic phase and the use of Bis-GMA reduces the polymerization shrinkage in dental restorative materials, compared with unfilled resin, which has been estimated at between 1 and 3% (Bandyopadhyay, 1982; Feilzer *et al.*, 1988; Feilzer *et al.*, 1989; Rees and Jacobsen, 1989; Iga *et al.*, 1991; Watts and Cash, 1991; Sakaguchi *et al.*, 1992; de Gee *et al.*, 1993). This shrinkage is responsible for a tensile stress at the resin/tooth interface reported in the order of 130 kg/cm² (Craig, 1985). Hygroscopic expansion, which follows polymerization shrinkage, is generally of the order of 0.07 to 0.80% and is unlikely to compensate for volume changes associated with polymerization shrinkage (Jensen and Chan, 1985).

The clinical effects of shrinkage include contribution to breakdown of marginal seal, loss of adaptation to cavity walls and possible accompanying post-operative sensitivity, and the development of internal stresses resulting in microcracks (Bowen, 1967; Asmussen, 1975b; Bausch *et al.*, 1982; Lambrechts *et al.*, 1982; Davidson and de Gee, 1984). These effects are not removed by either enamel etching or the use of dentine bonding agents and probably the most potentially damaging clinical manifestation is microleakage occurring at the cervical margin of proximal restorations.

1.3 Physical and chemical properties of polymers

Several physical properties of polymers can be directly related to component loss from the material including solubility, hydrophobicity, water sorption and viscosity. An understanding of these factors is of relevance to the current work.

1.3.1 Solubility

The extent to which a compound dissolves into solution may be discussed in terms of solubility and molecular movement, and is determined by the laws of thermodynamics where "like dissolves like" (Nicholson, 1991a). Solubility of a material overall is based on the solubility of its constituents or components in a solvent.

A factor derived for both solvent and polymer, the *solubility parameter*, can assist in determining whether a polymer is susceptible to dissolution. Where there is no particular interaction between polymer and solvent, the polymer will dissolve in the solvent when the difference in solubility parameters is less than $4 \text{ MJ}^{1/2}/\text{m}^{3/2}$ (Nicholson, 1991b). The interaction of composite resin materials with hydrated dentine structure may involve some degree of degradation due to solubility. Generally, solubility parameters of common composite resin monomers fall within the range of $18.4 \text{ MJ}^{1/2}/\text{m}^{3/2}$ for TEGDMA to $23.3 \text{ MJ}^{1/2}/\text{m}^{3/2}$ for HEMA. Values for Bis-GMA and for UDMA are $21.5 \text{ MJ}^{1/2}/\text{m}^{3/2}$ and $21.9 \text{ MJ}^{1/2}/\text{m}^{3/2}$ respectively (Asmussen and Uno, 1993).

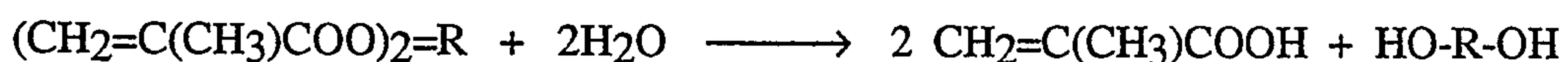
The solubility parameter, together with the polarity of a compound, also determines the *wettability* of the compound (Asmussen and Uno, 1993). This property is of interest in bonding to dentine where, ideally, the wetting characteristics of an adhesive should match that of dentine. When a dentine conditioning procedure modifies the dentine so as to raise or lower the solubility parameter of the dentine to match that of the adhesive resin, softening followed by penetration of the adhesive into the dentine occurs.

1.3.2 Hydrophobicity and hydrolysis

Another factor related to solubility of polymers is *hydrophobicity* which can be defined as the quality of a surface which forms a contact angle greater than 90° with a hydrophilic liquid such as water. Most polymers consist of a non-polar backbone supporting polar functional groups. Most composite resins exhibit a contact angle with water on their surface of

about 65° and so are classed as hydrophilic (Craig, 1981). This parameter is of particular significance in resin bonding agents which, clinically, are intimately applied to dentinal structure.

Related to hydrophobicity is the susceptibility of a polymer to *hydrolysis* since the collection of water molecules on an interface directly promotes the propensity for water interactions. Hydrolytic interactions between resin materials and water are enhanced in the clinical situation by the action of bacterial enzymes, such as bacterial hydrolases, dissolved in saliva (Munksgaard and Freund, 1990). Most susceptible to these enzymes are the diluent monomers, such as TEGDMA, which undergo hydrolysis resulting in regression to EGDMA and methacrylic acid fractions:



Equally susceptible to this hydrolysis is diethylene glycol dimethacrylate (DEGDMA) and UDMA whereas Bis-GMA is least susceptible. A significant aspect of this breakdown relates to component release and its contribution to wear of the material and to changes in microhardness (Freund and Munksgaard, 1990) and fracture toughness. The degree of conversion after polymerization also affects the resistance of the composite resin to chemical degradation (Ruyter, 1985). Residual double bonds in the formed polymer make the materials less resistant to degradation reactions. Glass fillers are susceptible to hydrolytic degradation, which results in an almost linear loss of filler over time (Söderholm, 1985). This effect is a direct result of weakening of the siloxane bonds between coupling agent and the filler surface, which in a wet environment, requires significantly less initiation energy to initiate hydrolysis (Söderholm, 1985). Barium glasses, in addition, have an alkaline reaction with water. This reaction results in a movement of hydrogen ions into the silica network which, after attaining a critical pH, can cause breakdown of the siloxane bonds.

1.3.3 Water sorption.

This property has been defined as weight gain in a specimen over time following immersion in water (Misra and Bowen, 1977). All composite resins suffer water absorption to differing degrees and therefore undergo hygroscopic expansion (Cook *et al.*, 1985). For the linear polymer, PMMA, water sorption plots, relating weight gain to time, are characterized by definite physical events which approximately conform to Fick's laws of

diffusion (Kalachandra, 1989) although the intrinsic heterogeneity of filled composite resins influences this significantly. Generally, water sorption starts immediately on immersion, probably as a result of simple void replacement in the resin. Much smaller weight gains (and sometimes weight losses) are then seen for about 4 days, during which unpolymerized resin dissolves. Kalachandra (1989) suggested that the slow and small continual weight gain after 4 days reflected interactions at the critical interfacial region between filler particles and resin matrix and possibly facile diffusion. However, this has been refuted by Ferracane and Condon (1990) who found elution of leachable species from composite resin was completed by 24 h. Composite resins containing hydrophilic components such as HEMA are known to have higher values for water sorption, in the range of 0.64 ± 0.09 to 1.24 ± 0.04 mg/cm² (Dulik *et al.*, 1981), whereas the presence of 4-methacryloxy ethyl trimellitic anhydride (4-META) acting as an effective coupling agent reduces water sorption (Kalachandra, 1989). Composite resins containing dimethacrylate diluents suffer less water uptake due to partial cross-linking of the diluent. Incomplete polymerization is also associated with increased solubility and water sorption (Pearson and Longman, 1989).

1.3.4 Viscosity

This property in composite resins is dependent on structure and molecular weight but is inversely related to temperature and concentration of diluent monomer (Dulik *et al.*, 1981). Fewer hydroxyl groups in the monomer side chain also reduces viscosity (Chung, 1990). An important characteristic influencing viscosity is high inorganic loading, especially with fumed silica (pyrolitic silica) which causes a marked increase in viscosity (Roulet, 1987). Very high viscosity of the composite resin is associated with more difficult clinical manipulation of the material.

1.4 Dental composite resins

Two important predecessors of dental composite resin in restorative dentistry were silicate cements and epoxy resins. Silicate cement, consisting of a powder (containing silica and alumina acid-soluble glasses, sodium fluoride and calcium fluoride) which was mixed with phosphoric acid to form a gel, was an early restorative material introduced in the late

19th century (Paffenbarger *et al.*, 1938). The material had good aesthetic qualities and thermal dimensional stability and the advantage of fluoride release (Kent *et al.*, 1970; Wilson *et al.*, 1970; Phillips, 1973) but showed relatively rapid solubility (Wilson and Batchelor, 1967; de Freitas, 1968; Kent *et al.*, 1971).

Epoxy resin materials are prepared from polymers containing a minimum of two terminal 1,2-epoxy-groups per molecule. The epoxy group is relatively reactive and so many product molecules are known; among the most common are those based on bisphenol A and epichlorohydrin (Nicholson, 1991a). Epoxy resins are prepared in two stages; an initial reaction yielding a linear prepolymer (for example, diglycidyl ether) followed by a reaction which introduces cross-linking between molecules during a curing stage. Cross-linking of diglycidyl ether resins may be carried out in two ways, either by the use of catalytic curing agents or stoichiometric cross-linkers. These resins exhibit little shrinkage as they harden at room temperature and are relatively insoluble. Nicholson (1991a) noted that epoxy resins were originally developed by Castan in the late 1930s whilst working for the dental company De Trey in Switzerland. These resins were considered for dental use but had little success as they showed slow hardening. These materials contained quartz or porcelain particles distributed within the epoxy resin and could therefore be considered experimental composite resins.

1.4.1 Bowen's resin.

In 1956, Bowen synthesized a product of methacrylic acid and an epoxy resin, DER332, but found it to have an undesirable effect with water (Bowen, 1956). Although the material exhibited low viscosity, use of MMA imparted the product with high polymerization shrinkage. Bowen then combined bisphenol A with glycidyl methacrylate to produce the condensation product, 2,2-bis[4(2-hydroxy-3-methacryloyloxy-propyloxy)-phenyl]propane (bisphenol-A-glycidyl dimethacrylate) or Bis-GMA (Fig. 1.1).

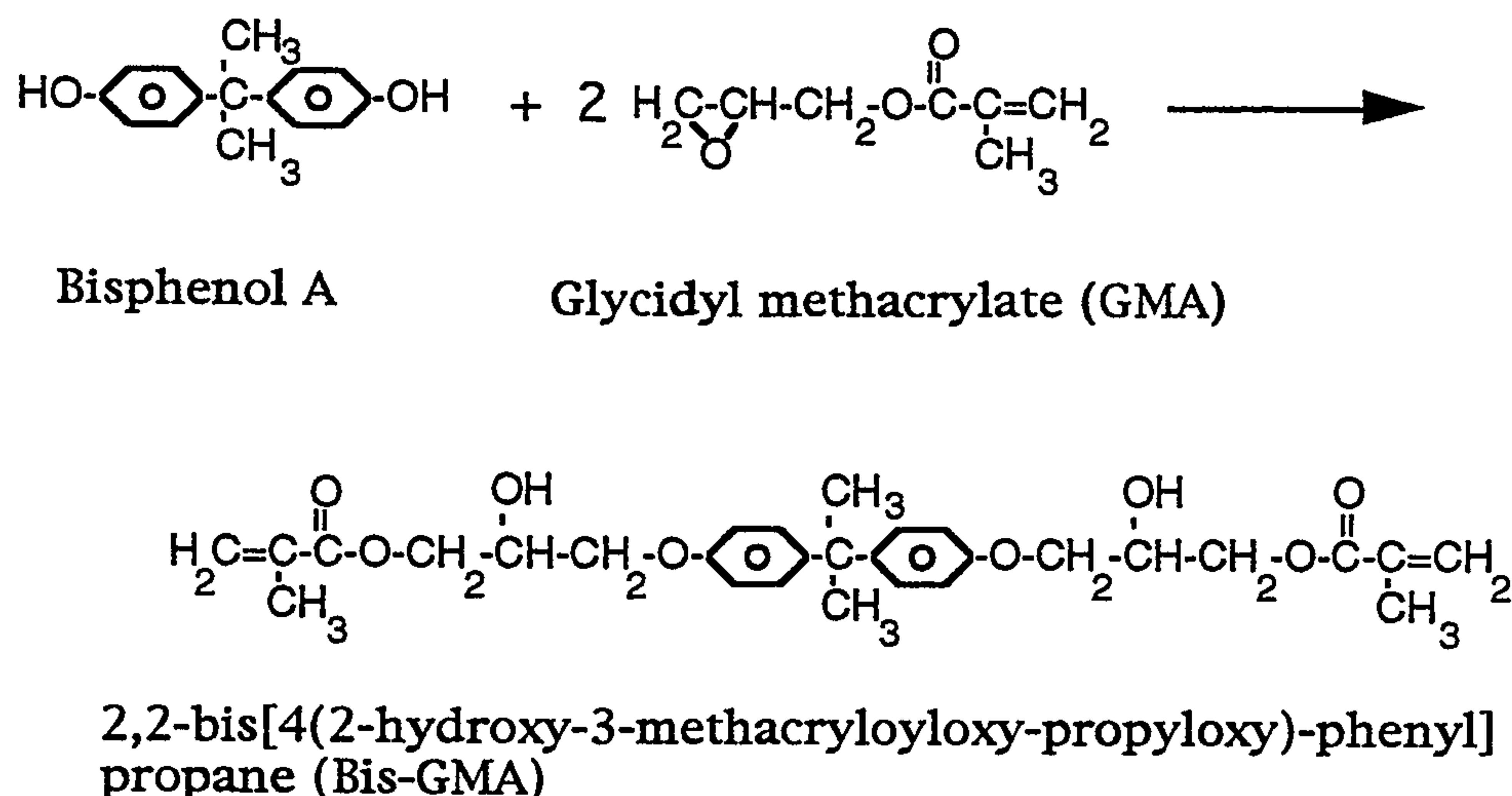


Figure 1.1 Reaction producing Bis-GMA.

By then adding vinyl silane-treated fused silica particles to the Bis-GMA, Bowen went on to patent a new “dental filling material” in 1962 and continued its development (Bowen and Rodriguez, 1962; Bowen, 1963, Bowen, 1964; Bowen *et al.*, 1968; Bowen and Reed, 1976a; Bowen and Reed, 1976b).

The difunctional Bis-GMA molecule exhibits many advantages including an inherent stiff structure (due to the central linked aromatic rings) and a high viscosity (due to hydrogen bonding via the hydroxyl group on the propoxy group). Difunctionality imparts a high capability for cross-linking forming a strong homopolymer which binds well and appreciably resists hydrolysis (Glenn, 1982). When thinned with TEGDMA and combined with an activation catalyst, the resin “cured” within 3 minutes. The addition of fillers to the material reduced both the coefficient of thermal expansion and the polymerization shrinkage and produced a satisfactory restorative material (Paffenbarger *et al.*, 1953; Bowen, 1958). Commonly used oligomers and monomers in commercial composite resins include isomers and derivatives of Bis-GMA such as 2,2-bis(4-methacryloyloxy-phenyl)propane or Bis-MA (Craig, 1981), 2,2-bis[4-(3-methacryloyloxypropoxy)phenyl]propane or Bis-PMA and 2,2-bis[4-(2-methacryloyloxyethoxy)-phenyl]propane or Bis-EMA (Ruyter, 1985). In common composite resins, linear and branched isomers of Bis-GMA are found in the ratio of 3:1 and a double branched isomer of Bis-GMA can also be found up to 2% of the content (Ruyter, 1987).

Disadvantages of Bis-GMA, however, include high viscosity requiring inclusion of a diluent, and questionable purity producing batch-to-batch variation. Clinically, polymerization is also strongly air-inhibited and occurs with marked volumetric shrinkage. These problems have encouraged the consideration of alternative oligomers including 1,6-bis(methacryloxy-2-ethoxycarbonylamino)-2,4,4-trimethylhexane (urethane dimethacrylate or UDMA). Urethanes offered several real advantage in higher molecular weight, lower viscosity and superior toughness (Glenn, 1982). Together with UDMA, Bis-GMA remains the most commonly used base oligomers in dental restorative resins although other large monomers such as bis(acryloyloxymethyl)tricyclo(5.2.1.0^{2,6})decane, bis(methacryloyloxymethyl)tricyclo(5.2.1.0^{2,6})decane (Ruyter, 1985), and 2,2-bis(4-methacryloxyphenyl)propane or Bis-MPEEP (Chung, 1990) and novel monomers such as fluorinated dimethacrylates, unsaturated spiro-ortho carbonates (Cook *et al.*, 1985) and cyclophosphazenes (Anzai *et al.*, 1992) have also been examined. Yet other formulations use Bis-GMA/TEGDMA/UDMA combinations. Different *diluent* monomers considered include EGDMA, diethyleneglycol dimethacrylate and tetraethyleneglycol dimethacrylate (Te-EGDMA) (Ruyter, 1985). Monomers used in other fields have also been examined for potential application to dental materials. One of these, α -methylene- γ -butyrolactone (MBL), a cyclic analog of MMA, shows promise as an alternative diluent monomer and, in fact, significant improvements in conversion have been observed when small amounts of MBL (up to 5 weight % to Bis-GMA) are added to unfilled TEGDMA/Bis-GMA resins (Stansbury, 1990; Stansbury and Antonucci, 1992). MBL is a highly polar low viscosity monomer and so it acts as a good solvent for oligomers participating in cyclopolymerization by incorporating a cyclic ether structure into the polymer backbone. This kind of polymer is non-cross-linked featuring pendant ester groups and exhibits a high glass transition temperature. Such cyclopolymerizable comonomers are also associated with lower polymerization shrinkage which makes them of continued interest.

Analysis of monomer content in composite resins has employed many analytical techniques: nuclear magnetic resonance (NMR) spectrometry (Asmussen, 1975a), micro high performance liquid chromatography (Miyazaki *et al.*, 1981), IR spectroscopy (Pashley, 1979; Miyazaki *et al.*, 1981; Ferracane and Condon, 1990), liquid chromatography/mass spectroscopy (Miyazaki *et al.*, 1981) and high performance liquid chromatography (Munteanu *et al.*, 1987b).

1.4.2 Filler systems and resin/filler interactions

Another important component of composite resins is the filler, included to improve overall toughness and strength (Craig, 1981; Söderholm, 1985; Roulet, 1987). Many early filler particles, such as glass beads and fused silica, were too hard to allow adequate polishing or were insufficiently radiopaque. The greatest disadvantage in the use of the quartz was the higher thermal coefficient of expansion of this filler than that of surrounding tooth (Söderholm, 1985). Present filler particles include glasses, silicates, ytterbium trifluoride, polystyrene latex, calcium metaphosphates and $\text{TiO}_2\text{-SiO}_2$ (Craig, 1981; Roulet, 1987; Suzuki *et al.*, 1991; Willems *et al.*, 1992). Classification of composite resins has usually used filler particle size or inorganic filler loading as a defining index (Lutz and Phillips, 1983; Albers, 1985; Leinfelder, 1985; Marshall *et al.*, 1988; Khan *et al.*, 1992; Lang *et al.*, 1992) but many of these classifications offer limited sensitivity to the range of commercial composite resins now emerging. A more comprehensive classification has been suggested by Willems and his co-workers (1992) and this system will be used in the current work (see Table 1.1).

Of interest to the current work is the influence of filler morphology, volume and distribution on the patterns of component release from the composite resin. Viscosity of the composite resin, for example, is reduced markedly with the inclusion of particles around 50 nm in diameter (Söderholm, 1985). This reduced viscosity improves clinical handling of the composite resin but may increase susceptibility to solvent breakdown. A high filler loading can also reduce polymerization shrinkage by reducing the overall volume occupied by shrinkage-prone matrix resin. Internal stresses accompany the inclusion of filler particles in the resin since the filler-resin interface will be differentially reactive to polymerization forces. This may cause filler particle loss from the material and contribute to overall wear (Bayne *et al.*, 1992). A clinical goal in composite resin formulations has been the simultaneous incorporation of optimal filler loading and particle size. Roulet (1987) considered that an 'optimal', homogeneous, microfilled composite resin would contain a combination of an organic matrix and directly admixed microfillers with an average particle size of 0.04 - 0.2 μm .

Fibre inclusion in composite resins is an alternative to inclusion of traditional fillers. Commonest types of fibres used in composite resins

include glass, carbon, kevlar, poly(ethylene) and polyester and can be unidirectional or braided tubular and may be uncoated (Ekstrand *et al.*, 1987; Krause *et al.*, 1989; Goldberg and Burstone, 1992) which are sized to prevent fibre-fibre contact and to enhance interaction with the resin matrix. Fibres provide advantages over conventional fillers in reducing peak temperature of polymerization and reducing rate of polymerization thus improving mechanical properties of the composite resin. Physically, fibres act as a diluent in the formulation, probably by disturbing particle ordering; this also reduces the exothermic reaction during polymerization (Krause *et al.*, 1989; Willems *et al.*, 1992). Practical difficulties, however, associated with fibre incorporation, such as imperfect fibre/matrix adherence, sensitivity to water sorption and cytotoxicity associated with dry composites, have been reported in fibre-filled composite resins (Suzuki *et al.*, 1991; Krause *et al.*, 1989). These factors may contribute to potential component release.

Amongst the more commonly used glass fillers are strontium, barium or lanthanum which impart radiopacity to the composite resin and are softer than quartz (Draughn *et al.*, 1985). Particle size ranges are 5-30 μm in macrofilled ("traditional") composite resins and 0.5-8 μm in hybrid ("fine" and "ultrafine") composite resins.

Amorphous (or colloidal) silica particles are manufactured by hydrolysis and precipitation averaging 0.04 μm in size (microfilled composite resins). Colloidal silica is also used as a filler in composite resins. A *colloid* refers to a continuous phase in which very small dispersed particles are suspended (Laubengayer, 1957). Such suspensions lie between homogeneous solutions and mixtures and *gels* (for example, silica gel) describe colloids where the dispersed and continuous phase are interlaced. The dispersed phase in colloids is under continual motion and presents a very large surface area which is highly reactive due to unsatisfied valence and intermolecular forces. There is tremendous potential, therefore, for the surfaces within these suspensions to strongly attract and interact with other substances in adsorption. Silicon dioxide is an example of a colloid used as a composite resin filler (Söderholm, 1985). Such colloidal particles have a strong attraction for resins but suffer water adsorption which results in hydrolytic degradation. These fillers typically include particles of very small diameter exhibiting a low thermal expansion coefficient but lack adequate radiopacity (Söderholm, 1985).

To improve the quality of the interface between filler particles and resin matrix, a silane coating, having the basic structure $Q_3Si(CH_2)_nR$ where $n = 0$ to 3 and Q is a hydrolyzable silicon group (Roulet, 1987), is used. Typically, filler particles used in composite resins are treated with a silane coupling agent, such as γ -methacryloxypropyltrimethoxysilane or vinyl triethoxysilane, to facilitate a bonded interface between filler particles and resin matrix. Intermediate silanol (Si-OH) groups are generated from the hydrolyzable groups in aqueous solution which then are available to bond to the oxides of the glass surface in the presence of water. Any free silanol groups are available to reform or construct new bonds with the surface so that replenishment may occur. The silanated layer acts as a flexible layer able to deform easily and absorb stress although "slipping" occurs with silanated fibres leading to early failure under load. Silanation thus improves the mechanical properties of the composite resin by reducing the occurrence of debonding of particles from the resin matrix, such as that due to hydrolytic degradation, and by providing stress absorption at the interface between filler particle and resin.

1.4.3 Classification of composite resins

A classification of current composite resin materials has been presented by Willems and his co-workers (1992). This classification will be used in the current work and is shown in Table 1.1.

Table 1.1 Classification of composite resin materials
(after Willems *et al.*, 1992)

Class	MPS ¹ (μm)	Y-Mod ² (GPa)	Filler (Vol %)	CS ³ (MPa)	HV ⁴ (kg/mm ²)
Ultrafine Midway-filled (U M-F) ⁵	0.7-3.6	13.2-17.5	49.9-70.0	328-483	65-97
Ultrafine Compact-filled (U C-F)	1.0-3.6	19.7-26.2	66.5-77.0	340-448	106-159
Fine Midway filled (F M-F)	4.2-9.3	13.3-18.4	49.0-59.9	343-450	79-144
Fine Compact filled (F C-F)	3.6-9.6	19.9-27.3	68.4-74.0	277-450	10-186
Microfine with Splintered particles (MFspf)	0.04	5.4-9.5	17.1-45.3	262-474	43-59
Microfine with Agglomerated particles (MFapf)	0.04	9.6-10.6	39.7-49.1	300-500	55-63
Fibre-reinforced (f)	11.2	23.9	-	-	125
Traditional (t)	5.1-15.4	17.9-22.5	53.0-66.2	234-350	93-160

1 Mean Particle Size

2 Young's Modulus of elasticity

3 Compressive Strength

4 Vickers Hardness

5 Codes to be used in the current work

"Splintered pre-polymerized" particles are made from microfilled composite resin which has been heat cured and then ground into particles ranging in size from 1-200 μm (Roulet, 1987). These particles are then added to the resin as a "filler" to reduce polymerization shrinkage. "Agglomerated particles" are made from microfiller particles (1-100 nm size range) which are heat-treated (causing agglomeration) to form larger particles (0.5-50 μm). The inclusion of these agglomerated particles improves filler loading of the composite resin (Roulet, 1987).

Willems and his co-workers used this classification in making clinical recommendations. Posterior teeth support higher occlusal loads and so the

composite resin of choice will ideally have high compressive strength, high filler content, good hardness and a modulus which matches that of dentine. A composite resin from the U C-F group was suggested in this circumstance. Anterior teeth support less direct loading but are highly aesthetic areas and so require a composite resin exhibiting both low surface roughness and resistance to chipping. MF materials have sufficient strength and small mean particle size and can be used in small interproximal cavities in anterior teeth but larger anterior cavities may require U M-F materials (Willems *et al.*, 1992).

1.4.4 Components of the composite resin "Foundation"

The principal composite resin used in the current study was Foundation (Nulite Systems International, Hornsby, NSW, Australia). This material was chosen as it was Bis-GMA-based and the resin was a relatively common formulation. Specific components, according to the manufacturer, are described below together with component function and some physical details. According to manufacturer data, 70.56% of the volume of Foundation composite resin was filler, 29.5% of the volume of material was resin. Proportions for each component stated below are from manufacturer data; volume and weight percentages refer to that proportion of the resin component. The acronyms listed are those to be used in the current work.

I RESIN COMPONENTS

(a) Base oligomer

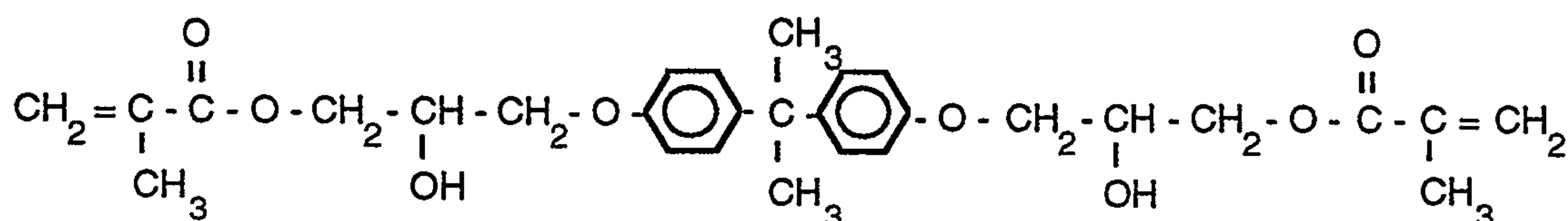
2,2-bis[4-(2-hydroxy-3-methacryloyloxy)phenyl]propane.

Synonyms: bisphenol-A-glycidyl dimethacrylate

Acronym: Bis-GMA

Proportion in Foundation: 73.63% (volume), 75% (weight). Molecular weight (MW): 512. Refractive index at 25°C (n^{25}): 1.5480 (Ruyter and Øysæd, 1982). Chemical Abstracts Number (CAS): [1565-94-2].

Generally present as three isomers (Ruyter, 1985), this aromatic oligomer confers rigidity (Craig, 1981) and increases viscosity of composite resin (Vankerckhoven *et al.*, 1982). The structure of Bis-GMA is shown in the following figure.



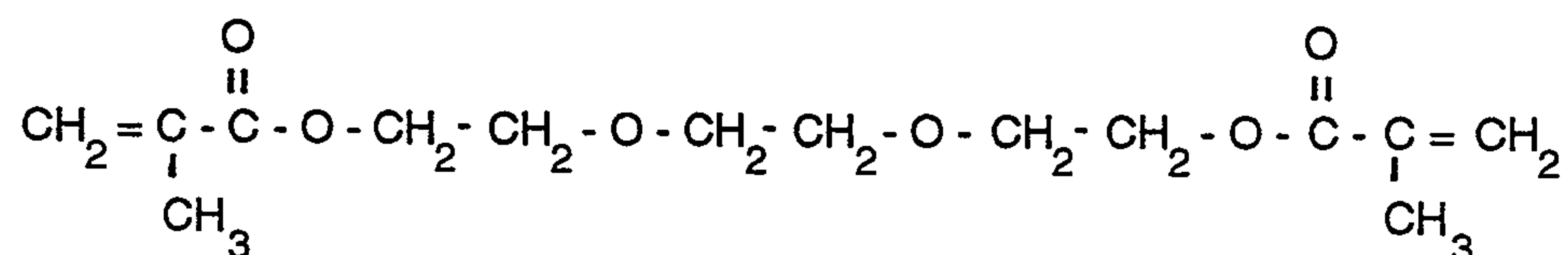
(b) Diluent monomer

Triethylene glycol dimethacrylate

Acronym: TEGDMA

Proportion in Foundation: 26.37% (volume), 25% (weight). MW: 286.3. Boiling point (bp): 171°C. Density (d): 1.092. Refractive index at 25°C (n^{25}): 1.4600 (Ruyter and Øysæd, 1982). Vapour pressure at 100°C is 0.01 mmHg (Craig, 1985). Surface tension 39 ± 0.6 dyne/cm (Dulik *et al.*, 1981). CAS: [109-16-0]. Solubility of greater than 10% in acetone and ethanol (CRC Handbook, 1985).

This aliphatic dimethacrylate dilutes the viscous base monomer and functions as a cross-linking agent (Roulet, 1987). The structure of TEGDMA is shown below.

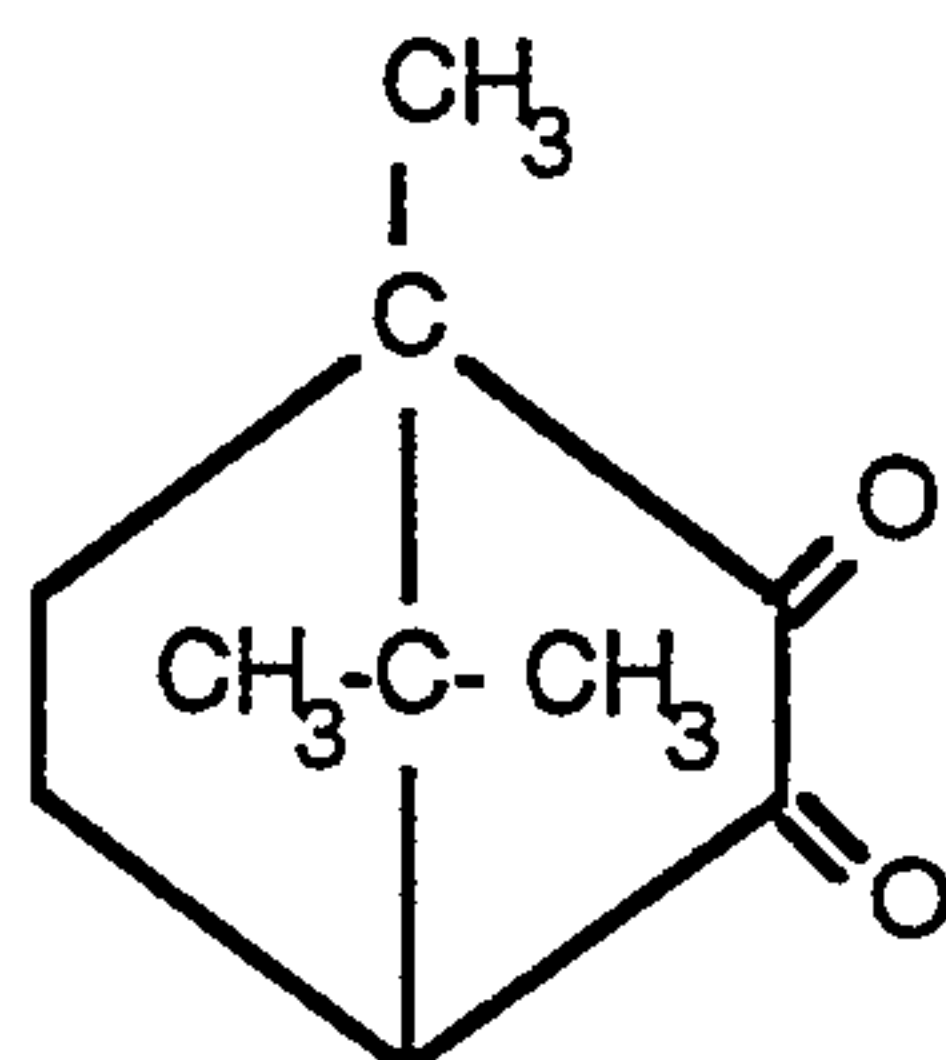
**(c) Photoinitiator**

Di-2,3-di-keto-1,7,7-trimethylnorcamphane.

Synonyms: 2,3-bornandione or camphorquinoneAcronym: CQ

Proportion in Foundation: 0.18% (weight). MW: 166.22, bp: 171°C, mp 199°C. CAS: [10334-26-6]. This bright yellow crystalline compound is a common chiral starting material and has a wavelength maximum of 468 nm (Kilian, 1979). Solubility in ethanol and chloroform is greater than 10% (CRC Handbook of Data on Organic Compounds, 1985).

Taira and co-workers (1988) found the concentration in resin portion of the composite resin to vary from 0.17 to 1.03% wt./wt. The structure of CQ is shown in the following figure.



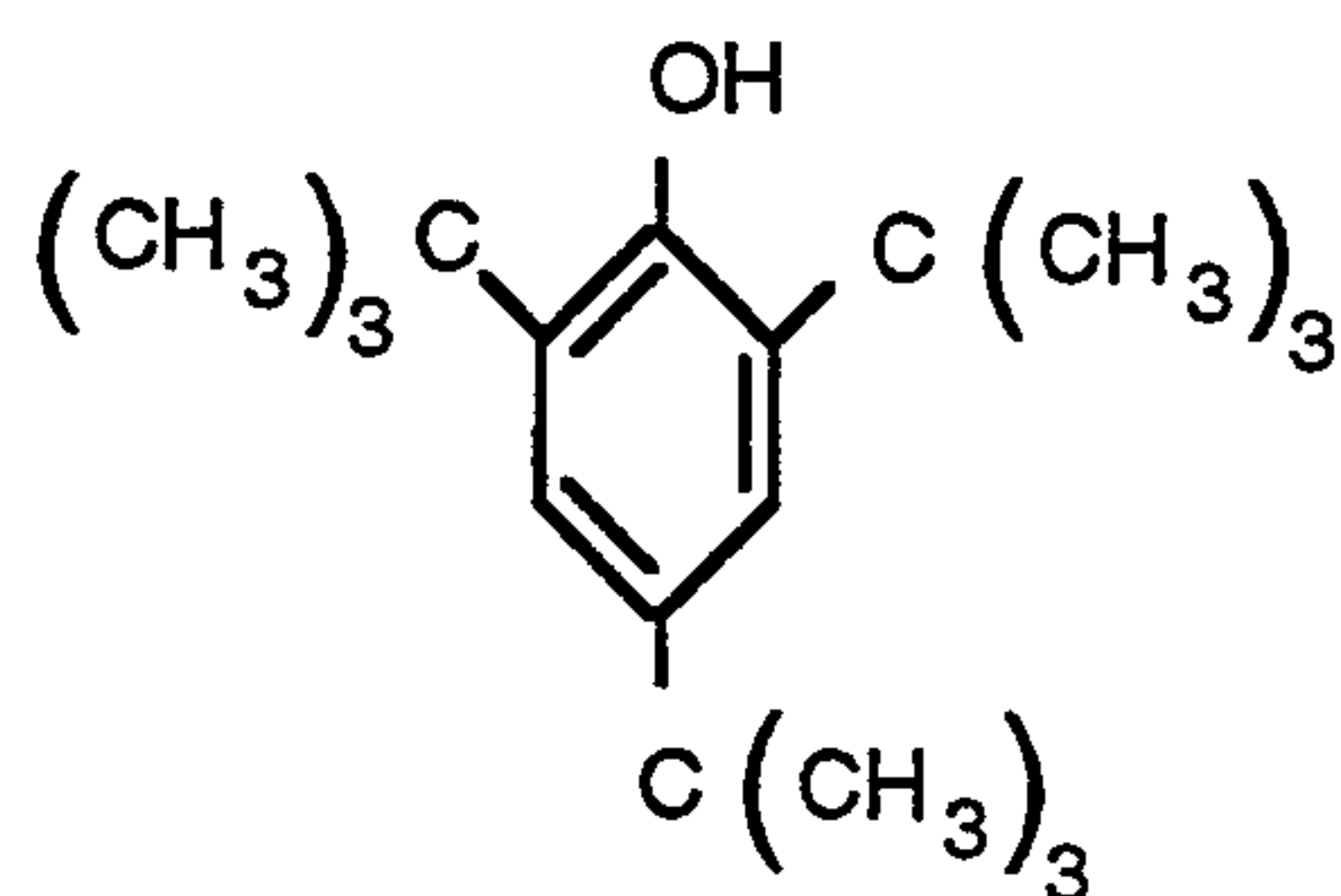
(d) Inhibitor**2,6-Di-tert-butyl-4-methylphenol**

Synonyms: 2,6-di-tert-butyl-p-cresol, butylated hydroxy toluene or 2,6-bis(1,1-dimethylethyl)-4-methylphenol

Acronym: BHT

Proportion in Foundation: 0.05% (weight). MW: 220.36, mp: 69-70°C, bp: 265°C, viscosity: 3.47cS (80°C), CAS: [128-37-0].

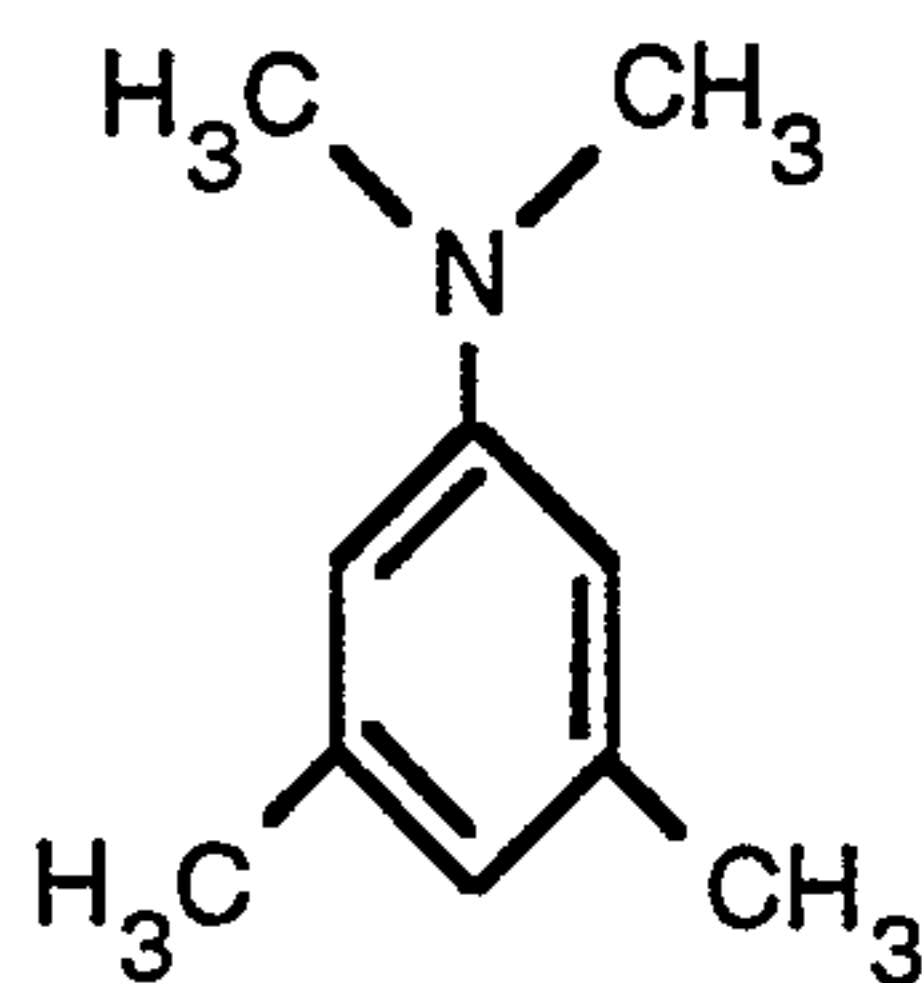
This white powder is used as an antioxidant and stabilizer by binding free radicals (Roulet, 1987). Polymerization is not initiated in the resin matrix until all the inhibitor is used (Cook, 1985). The structure of BHT is shown below.

**(e) Reducing agent****N,N,3,5-tetramethyl aniline**

Acronym: TMA

Proportion in Foundation: 0.15% (weight).

This amine is an efficient polymerization accelerator and methacrylates containing this amine exhibit good colour stability. The structure of TMA is shown in the following figure.

**II FILLER COMPONENTS**

70.56% of the composite resin volume is the filler fraction, of which 13.82% is sub-micron microfill silica glass particles (code: R709), 16.00% is T3000 barium glass particles and 7.85% is T4000 strontium glass particles with size range of 2-5 µm. 32.89% of the volume contains glass fibres with a length range of 14-16 µm.

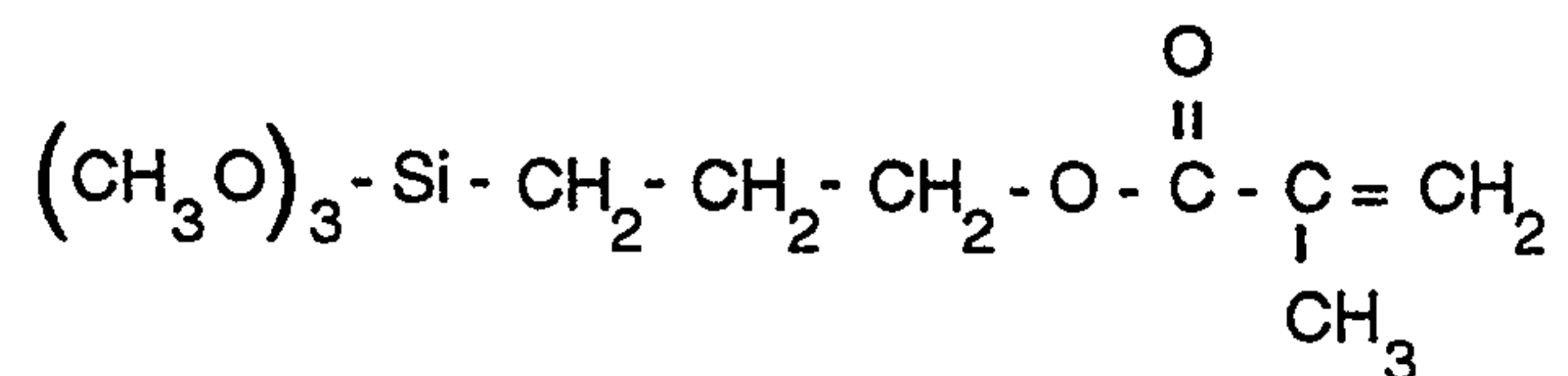
III COUPLING AGENT

γ -methacryloyloxypropyltrimethoxysilane (0.5% VTS code: RS1).

Synonyms: 3-(trimethoxysilyl)propyl methacrylate or 3-methacryloyloxypropyltrimethoxysilane

MW: 248.35, bp: 190°C, d: 1.045, n^{20} : 1.4310, CAS: [2530-85-0]

Moisture sensitive and toxic, this component contains about 1% methyl alcohol from hydrolysis (source: Aldrich). The structure of this compound is shown.



Although not components of Foundation, two other methacrylates are of importance in dental resin materials. These methacrylates are components of resin materials which will be used in the current work. The first, HEMA, is a component of the primer solution and of the dentine bonding agent in the dentine bonding system, Scotchbond Multi-purpose (3M Dental Products, St. Paul, MN, USA). UDMA is a common alternative base oligomer to Bis-GMA in current composite resins, such as the composite resin Occlusin (GC, Tokyo, Japan). Both these resin materials will be used in experimental work described in subsequent Chapters. These components are described in the following sections.

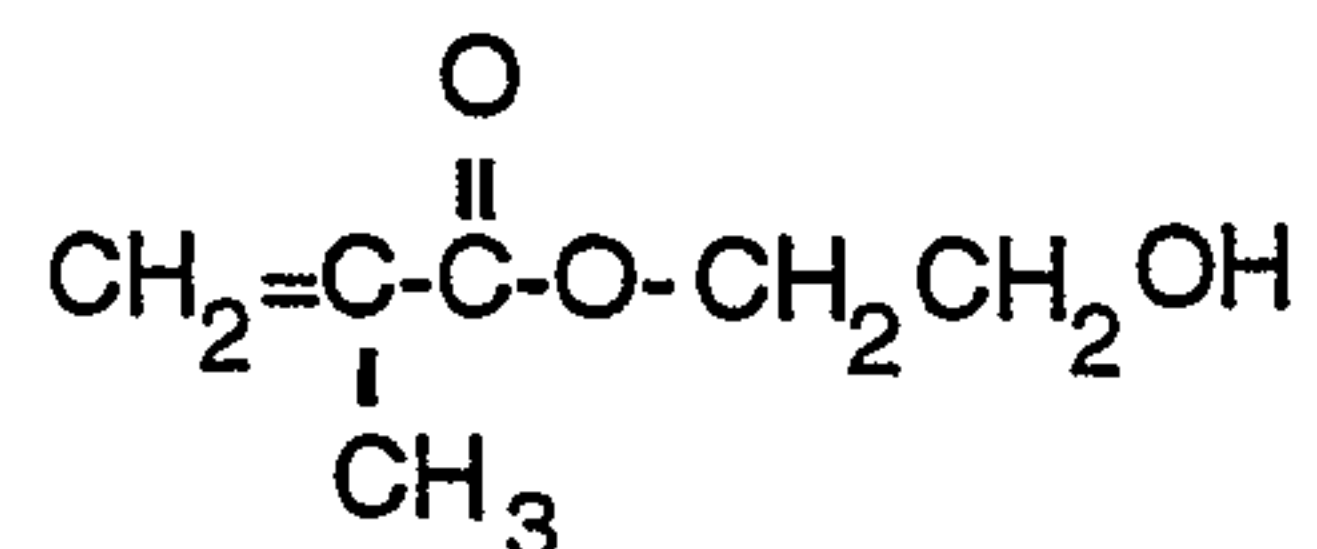
I DILUENT MONOMER

(also component of the primer in some dentine bonding agent systems)

2-hydroxy ethyl methacrylate

Acronym: HEMA

MW: 130. The structure of HEMA is shown.



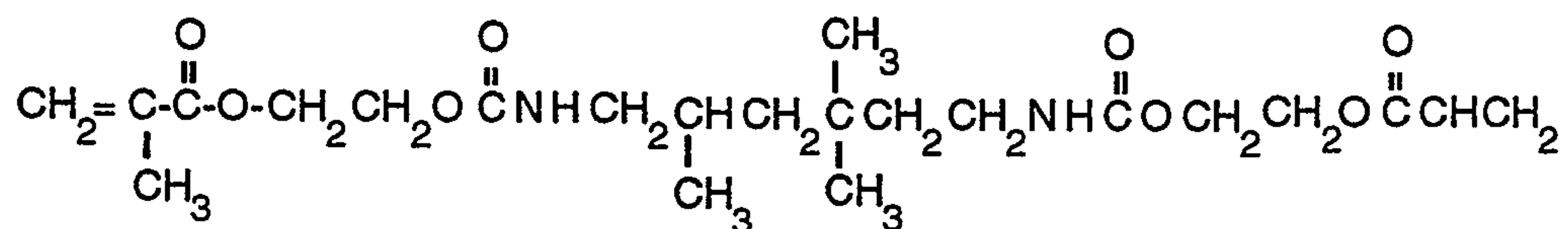
II BASE MONOMER: UDMA-based dental composite resins

1,6-bis(methacryloxy-2-ethoxycarbonylamino)-2,4,4-trimethylhexane

Synonyms: urethane dimethacrylate

Acronym: UDMA

MW: 470. The structure of UDMA is shown below.



1.4.5 Residual unsaturation in composite resins

Another aspect of composite resin which may contribute to component release is *residual unsaturation*. As polymerization proceeds, diffusion rates of radicals and unreacted dimethacrylate molecules are reduced due to steric interference and the approach of the glass transition temperature to polymerization temperature (Aso, 1959; Horie *et al.*, 1975). This means that a significant amount of the methacrylate groups remains unreacted (Cowperthwaite *et al.*, 1981; Vankerckhoven *et al.*, 1982). Degree of conversion, however, can be enhanced if a flexible dimethacrylate is chosen which can lower the glass transition temperature (Cook, 1985). The initiation system used also influences residual unsaturation. The extent of conversion near the surface of light-initiated composite resins, for example, is less sensitive to exposure time or intensity (Killian, 1979; Leung *et al.*, 1983; Watts *et al.*, 1984) and is probably different from that of the bulk of the material. Oxygen inhibition of surface polymerization can be to a depth of 5-100 μm (Ruyter, 1981) but also depends on the rate of initiation and the viscosity of the dimethacrylate resin.

1.4.6 Partly filled and unfilled resins

In view of the range of terms used by authors to describe the various elements of dentine bonding systems and other concepts relative to their use, the following terms will be used in this text. **Bonding agent** will be used to describe the unfilled adhesive resin component. This term will thus encompass *low-viscosity resin* (Asmussen *et al.*, 1991), *intermediary resin* (Asmussen *et al.*, 1991), *dentine adhesives* (Bordin-Aykroyd *et al.*, 1992), *bonding resin* (Pashley *et al.*, 1993) and *adhesive resin* (Van Meerbeek *et al.*,

1993) The term **primer** will describe the solution intended to be placed on the prepared dentine prior to the placement of a bonding agent. **Low-fill resin** will be used to describe low viscosity, relatively lightly filled resins. **Dentine pre-treatment** will describe procedures where solutions are applied to the cut dentine surface including dentine conditioners and acids. **Dentine bonding system** will be used to describe the commercial system available for dentine bonding which usually contains all or most of the following; a dentine pre-treatment agent, a dentine primer and a bonding agent. **Resin-reinforced layer** will be used to describe the layer of dentine at the pulpal wall of a cavity created following the application of primers and adhesive resins during dentine bonding procedures and will thus be used to describe the *interdiffusion zone* or *hybrid zone* (Asmussen *et al.*, 1991).

Some resin materials used in dentistry lack the filler volume of composite resins although some filler may be present. These resins may be chemically- or visible-light-initiated and a combination of these two systems (that is, "dual cure") also exists (Inokoshi *et al.*, 1993; Van Meerbeek *et al.*, 1994).

Degree of cure is greater in more diluted resins and varies between 55 and 72% (Ferracane and Greener, 1984). Generally, low-fill resins and bonding agents contain a variety of resin components, with the main monomer/oligomer being Bis-GMA or UDMA, although other monomers are also found in dentine-bonding agents such as TEGDMA, HEMA, 10-methacryloyloxy decyl dihydrogenphosphate (MDP), tri-n-butylboran (TBB), N-methacryloyloxyethyl-N-methyl formamide (NMENMF), isocyanate, glutaraldehyde, MMA, polyethylene glycol-dimethacrylate (PEGDMA) and phosphonated penta-acrylate ester (PENTA) (Van Meerbeek *et al.*, 1992a; Van Meerbeek *et al.*, 1992b; Pashley *et al.*, 1993).

1.4.7 Resin bonding

Adhesion is the force that causes two dissimilar substances to attach when brought into intimate contact (Phillips, 1973). whereas cohesive force causes similar molecules to attach to each other. The strength of the attraction is dependent on surface energy at the interface, wetting ability of one material on another, which can be determined by the contact angle, and the physical state of the interface. Adhesion in a dental context occurs between several different substrates; with enamel, with dentine, with both

enamel and dentine simultaneously and with other restorative materials. Unique difficulties in bonding to tooth structure relate to the inhomogeneity of structures presenting different chemical substrates for the adhesive, innate hydrophilicity which reduces surface energy and wetting efficiency of, generally, hydrophobic resin materials and sensitivity to pathological and physiological change which alters basic interface morphology (Bowen, 1985; Nakabayashi, 1985; Phillips, 1988; FDI technical report No. 35, 1990). Most composite resin materials are hydrophobic, so to promote adaptation of these materials to hydrophilic dentine, a bonding agent, featuring hydrophilic and hydrophobic functional areas, has proven to be useful. Substrate-modifying procedures have also reduced these inherent difficulties.

Surface energy of enamel is improved by acid-etching (Buonocore, 1955) and the effectiveness of this procedure is dependent on several factors such as pKa of the acid, duration of action and the nature of the vehicle in which the acid is carried. Acid solution (for example, 37% w/w unbuffered phosphoric acid) affects enamel by preferential removal of enamel prism structure, commonly prism centres (Silverstone, 1982). Enamel bonding agents are able to penetrate the etched surface and so establish a micromechanical bond. The bonding agents used are generally Bis-GMA or UDMA resins. This facility to "link" to tooth structure provides potential clinical advantages in the exclusion of marginal leakage, reduction in the necessity for substantial tooth structure removal, improved adaptation of composite resin materials and the dissipation of interface stress.

Dentine is a mineralized collagen matrix traversed by dentinal tubules (Pashley, 1989). The unique nature of dentine complicates bonding systems and has necessitated an evolution of dentine bonding agents and systems which has been associated with improvements in bond strengths and adaptation (Erickson, 1989a; Nakabayashi, 1989; Erickson, 1992; Van Meerbeek *et al.*, 1992a; Van Meerbeek *et al.*, 1992b; Powell, 1992; Pashley *et al.*, 1993).

Earliest dentine bonding agents attempted to achieve micromechanical adhesion by acid etching the dentine, borrowing the successful concept from enamel etching, and then applying a dentine bonding agent. However, acid etching of dentine produced a small positive counter pressure of tubular fluid which prevented wetting by hydrophobic resins.

Concurrently, several different compounds were examined to test potential as dentine bonding agents. Bowen (1965), for example, used comonomers of N-phenylglycine and glycidyl methacrylate (NPG-GMA) to promote adaptation of acrylic resins to dentine but found low bond strengths of about 2 MPa. Throughout the 1970s and 1980s other compounds were similarly tested by different workers (see Erickson, 1989). Several problems observed in clinical studies using the early systems, such as hydrolytic instability of some agents, reversible interactions with hydroxyapatite and low bond strengths, suggested a need for further development and consideration of other possible components. Nakabayashi and co-workers (1982, 1985) and others, for example, investigated a monomer, HEMA, which remains a component of most recent bonding systems.

Clearly, other mechanisms of bonding to dentine needed to be explored. Attempts at achieving "chemical adhesion" have characterized most of the later "generations" of dentine bonding systems which manipulate smear layer to some degree. Second generation systems employed acidic coupling agents (primers) based on chlorosubstituted phosphated esters of Bis-GMA, TEGDMA and UDMA (Erickson, 1989; Phillips, 1988) or isocyanates with alcohol amine solvents (Antonucci *et al.*, 1980). Bonding was considered to be mediated by phosphate bonding to calcium in apatite, hydrogen bonding to N-H groups in the organic matrix and bonding to collagen following dissolution of the smear layer. The development of resin tags in dentinal tubules may also have been of assistance. Third generation systems employed pre-treatment of dentine with either weak acids (for example, citric) or cleansing agents (for example, acidic ferric oxalate, ferric chloride) followed by the application of coupling or wetting agents which themselves chemically interacted with dentine (although the nature of the chemical bonding is ill-defined). Several compounds have been tested as coupling agents which act by chelating with calcium or by bonding to hydroxy or amino groups of collagen: NPG-GMA and PMDM (Bowen *et al.*, 1982); 4-methacryloxythyltrimellitic anhydride (4-META)-(Nakabayashi, *et al.*, 1982) and HEMA/aldehydes (Munksgaard and Asmussen, 1984). Fourth generation agents have evolved more recently employing a new strategy to achieve dentine bonding. Van Meerbeek and his co-workers (1992a) classified these strategies as (a) those which incorporate smear layer, (b) those which remove smear layer and (c) those with intermediary strategies. These materials aim to create chemical bonding with the organic or inorganic components of dentine with the presence of bifunctional organic

monomers, which contain hydrophobic and hydrophilic groups, in their individual solutions. Like composite resin, these bonding agents suffer hydrolytic degradation over time which, as suggested by Eliades and Vougiouklakis (1989), reduces their ability to bond with tooth structure. Bonding agents also exhibit thicker air-inhibited layers which enhance copolymerization with composite resins (Rueggeberg and Margeson, 1990).

1.4.8 Resin-reinforced dentine

Current clinical techniques of dentine bonding aim at establishing a resin-reinforced layer in dentine. Asmussen and co-workers (1991) have suggested that, in contrast to composite resins, some bonding agents are capable of softening or solubilizing dentine, due to their compatible solubility parameter with that of dentine. This aids in the development of a "resin-reinforced layer". Systems which use highly fluid primer solutions often contain bifunctional hydrophilic monomers in an acidic medium (Nakabayashi, 1985; Nakabayashi *et al.*, 1992). Although most of these systems remove smear layer, some of these primer solutions were designed to penetrate smear layer and dentine and to enhance dentine diffusivity and so facilitate copolymerization of bonding agent with composite resins. One such monomer used in primers is HEMA. This compound is critical to bond formation because, in the presence of demineralized dentine, HEMA facilitates or "primes" penetration of polymerizable monomer into the microporosities of the collagen network by widening collagen interfibrillar spaces; it is in these areas that monomer undergoes polymerization (Erickson, 1989; Nakabayashi and Takarada, 1992). The completeness of polymerization in these areas, however, may be questionable.

The resultant "structure" has been termed the "resin-reinforced hybrid layer" (Nakabayashi, 1982) or the "resin-dentine interdiffusion zone" (Van Meerbeek *et al.*, 1992) and its presence is associated with improved bond strengths and a possible protective potential by blocking movement through tubules. The layer also demonstrates greater resistance to demineralization by hydrochloric acids (Van Meerbeek *et al.*, 1992). The thickness of this layer is dependent on the nature of the dentine conditioning treatment, the depth of decalcified dentine, and the viscosity of the primer and bonding agents. Apart from HEMA, there are other compounds which improve the diffusion of resins into the surface layer of dentine. These compounds are usually major components of primer solutions such as acetone, 4-META, N-tolyglycine-glycidyl-methacrylate

(NTG-GMA), biphenyl dimethacrylate (BPDM), TEGDMA, PENTA, N-methacryloyl-5-aminosalicylic acid (NMSA) and PEGDMA. In fact, TEGDMA and HEMA contents in bonding agents vary from 10% to 90% and Bis-GMA from 3 to 20% (Asmussen and Uno, 1993).

As can be seen from the above discussion, the developing chemistry of dentine bonding agents has been complex. Latest bonding systems exploit dentine as a structural and chemical substrate and the most successful systems are those which provide best diffusivity of their components into dentine. It is appropriate to question this development with respect to several issues; movement of components out of the resin-reinforced layer over time, the potential for the layer to act as a reservoir for components which might have biological effects elsewhere, and the long-term stability of the reinforced dentine layer to hydrolytic and enzymatic breakdown.

1.5 Component availability from dental materials

The preceding discussion of the chemistry and kinetics of composite resins strongly suggests that there are important implications regarding component release from these materials. In the following section, work that has examined component release, from dental materials in general, and from composite resin in particular, is reviewed. Of specific interest in a consideration of composite resin, is the importance of degree of conversion to the release of components.

1.5.1 Analysis of bioavailable components from dental materials

The release of components and reaction products from many dental materials has been reported. Probably the phenomenon which has received most attention has been the release of mercury compounds and other metal ions from amalgam restorations. However, other dental materials have been shown to release components and products in higher quantities than the quantity of mercury released from amalgam. Bioavailable components from various denture base materials, for example, have included formaldehyde, MMA, methacrylic acid, benzoic acid, benzoyl peroxide, N,N-dimethyl p-toluidine, chlorhexidine, dibutylphthalate and phenyl benzoate (Ruyter, 1980; Vink, 1986; Koda *et al.*, 1989; Basker *et al.*, 1989; Lygre *et al.*, 1993; Shintani *et al.*, 1993; Wilson and Wilson, 1993). Other dental materials have also been a source of bioavailable components; peroxides from restorative resins (Asmussen, 1980b), zinc from zinc oxide eugenol cements (Ashrafi *et al.*, 1983), eugenol from zinc oxide eugenol

(Becker *et al.*, 1983), metal ions from gold alloys and chromium-cobalt alloys (Brune, 1988), additives, monomers, initiators and degradation products from orthodontic resins (Palasz *et al.*, 1994; Lygre *et al.*, 1994) and TEGDMA and Bis-GMA from resin cements (Al-Fawaz *et al.*, 1993). In some dental materials, however, such as materials used in periodontal and endodontic therapies, intentional bioavailability of their components provides desirable therapeutic effects. The components of these materials have been traced to determine the duration of efficacy and diffusion parameters, for example, triamcinolone (Hume and Testa Kenney, 1981) and tetracycline from Ledermix® (Abbott *et al.*, 1988), and fluoride from glass ionomer cements (Tsanidis and Koulourides, 1992; Forss, 1993).

Like any solid matter, composite resin is a potential source of either components or reaction products which may leave the material during service. Apart from physical factors involved in solubility of the material in aqueous solution, there are *clinical* factors which influence both the degree of conversion and polymerization kinetics of composite resins. All these factors determine the readiness with which components are released.

1.5.2 Analysis of bioavailable components from composite resin

The release of components from composite resin has been investigated in various studies to achieve several different aims. These aims have included examination of composite resin composition; examination of toxic potential; assessment of the degree of conversion and assessment of the stability of the restorative material. In most of these studies, component release has been achieved either by "aggressive" solvent dissolution of the material to actively remove all filler particles followed by analysis of the supernatant to determine composition or by "passive" submersion of the composite material in a solvent and analysis of the decanted solution to examine which components are released or to monitor the completeness of conversion.

In chemical analysis of the composition of composite resin, the filler component is removed from the composite resin and the resin fraction is then separated and analysed. The solvents used to solubilize the composite resin are often chosen to optimize filler separation and to provide good compatibility with the chosen analytical method. Sometimes the analysis has been purely qualitative, for example, to provide data withheld by manufacturers. Early reports of specific components released from dental resin filling materials, for example, described monomer release from MMA materials (Kramer and McLean, 1952; Müller and Maeglin, 1953). Asmussen

(1975a) employed NMR spectra to provide new data on monomer content in restorative resins using deuterated chloroform as a solvent. Quantitative data reported were relatively imprecise (mole%) in this early application of the analytical tool. Later, Asmussen (1980a) refined the analytical methodology to enable more precise quantitation and corroborated NMR data with spectroscopy.

Vankerckhoven and his co-workers (1981), following Asmussen, used high performance liquid chromatography (HPLC)/NMR analysis to "characterize" composite resins. Ruyter and Sjøvik, in 1981, completed a compositional analysis of 36 resin materials using IR spectroscopy, mass spectrometry (MS), HPLC and NMR and were able to discern the presence of linear and branched isomers of Bis-GMA by NMR. The composition of a range of bonding agents has been similarly examined using a combination of HPLC, MS and IR by Miyazaki and his co-workers (1981), again using solvent extraction of the components. Ban and co-workers (1982) used IR to analyse chloroform eluates of two self-cured composite resins. Munteanu and co-workers (1987b) were able to detect three Bis-GMA isomers by HPLC and NMR analysis of alcohol eluates from composite resin. Significantly, most workers have noted great variety in the composition and purity of different proprietary dental composite resins, both among materials and among batches of one material.

Components passively released from composite resin into solvents, without explicit removal of filler, have also been reported. In these studies, composite resin is immersed in a solvent and, after a variable period, the decanted solvent (or eluate) is analysed. These techniques have led to the identification of a variety of components by different workers but, often, as in compositional studies, quantitation was not possible or was imprecise due to insensitivity of the analytical method. Despite considerable variation in methods between studies, such work has often shaped the development of composite resin materials and these data are nevertheless valuable as they better simulate the release of components under conditions which may occur clinically, for example, elution into water.

HPLC analysis of aqueous methanol eluates from composite resins has revealed the release of "residual" monomers with Bis-GMA, TEGDMA and MMA predominating (Fujisawa and Masuhara, 1979; Inoue and Hayashi 1982; Miyazaki *et al.*, 1983; Yoshida *et al.* 1992). HPLC has been used to determine formaldehyde release from composite resin in a study by Øysæd and co-

workers (1988) who found that degradation of composite resin in water gave rise to formation and release of formaldehyde in the range 0.05 to 0.5 $\mu\text{g}/\text{cm}^2$ and highest yield occurred when "cure" was air-inhibited. In other studies, the filler component has also been examined. Söderholm (1990), for example, stored composite resin samples in distilled water at 60°C for 6 months and examined the decanted eluate at intervals by electron dispersive X-ray analysis; the pattern of filler leaching was observed to plateau with time.

Gravimetric data have also been reported, illustrating the release of components and allowing the calculation of solubility parameters (Pearson, 1979; Øysæd and Ruyter, 1986; Rueggeberg and Craig, 1988; Ferracane and Condon, 1990). Ferracane and Condon (1990) examined both water and 75% ethanol eluates from composite resin and concluded that component release was completed after 24 hours; little was released after that time. Gravimetric methods such as these are limited in reliability and precision but do allow good description of the phenomenon.

Most examinations of degree of conversion utilize surface spectrometric analyses such as MIR (Ruyter and Svendsen, 1977; Ruyter and Øysæd, 1982). However, Ruyter and Sjøvik (1978) were able to correlate monomer composition to degree of conversion in composite resin eluates by using HPLC and MS.

1.6 *In vitro* bioavailability studies

Bioavailability studies have been conducted for many dental materials and many of these evaluations are based on the ability of the materials to release components which are then able to contact vital cells and cause untoward responses. It is essential that this capability is investigated for any material which is used intra-orally.

Resins, as a family of compounds, are not without a long history of reported cases of cytotoxic and allergic responses. As continual improvement in these materials has also created increased usage, it is reasonable to suggest that untoward biological responses need to be well understood. In that light, it is important to the experimental work to be described to briefly review biocompatibility of components of composite resin, and how these materials are evaluated.

Comprehensive biological evaluation of dental materials generally commences with cytotoxicity testing of components or whole materials; this remains the simplest system (Stanley, 1985a). This is followed by animal implantation tests and finally animal usage tests. Human clinical trials then complete the evaluation. This hierarchical sequence is purposeful and deliberate, aiming to be conservative of resources, time and animals whilst deriving meaningful data. In the testing of restorative materials, however, apart from pre-clinical usage tests, each tier of the tests has limited validity to the ultimate clinical conditions under which the materials are used.

Cytotoxicity tests provide excellent overall screening of restorative materials and provide good data about the individual toxic potential of components (Hanks *et al.*, 1991; Rathbun *et al.*, 1991). This is well supplemented by data on the bioavailability of molecules occurring clinically. The "pulp irritation test", as described by Stanley (1985a), is a form of pre-clinical usage test and examines the response of vital pulp, determined by histological criteria, when challenged with a restorative material and/or restorative procedure. At times, however, a direct comparison between studies and different researchers is difficult and results may conflict. The long test periods are often not conservative of resources and animals, results due to systemic changes in the animals often cannot be separated easily from local effects and there is difficulty in separating the influence of confounding variables.

Cytotoxicity studies generally apply restorative materials directly to culture media. These studies supply good screening data and enable toxicity comparisons to be made between components. For example, Hume and his co-workers investigated cytotoxicity of composite resin and related this to several physical states of the material (Hume and Hood, 1990, 1991; Hood and Hume, 1990) and to diluent composition (Hood and Hume, 1991). Careful correlation of similar studies where the same test materials are subjected to different cell assays can give insight into the exact toxic mechanism (Tyas, 1979; Hanks *et al.*, 1981; Terakado *et al.*, 1984). Data from such component cytotoxicity tests are likely to be more valid when supplemented by data about component availability to tooth structures when these materials are used clinically. This has been particularly borne out by a common finding amongst *tooth model studies* that dentine has a profound influence on toxic potential of restorative materials,

In vitro tooth models utilize extracted and human, non-carious teeth or tooth structure. These forms of tests offer several significant advantages over cytotoxicity tests in the evaluation of restorative materials in a clinically meaningful way. The incorporation of human dentine as a biological interface simulates a fundamental component of the restorative material/tooth interface which can enhance the information derived from cytotoxicity testing. Many variables present in usage tests may be controlled, simulated and even manipulated, in the tooth model. Manipulation of test conditions can provide information which is either not possible or unethical to create in *in vivo* tests. *In vitro* testing also allows the examination of tooth structure using sophisticated spectroscopic or analytical technologies *during* the experiment. In the testing of restorative materials, it is suggested that the tooth model is a vital preliminary usage test, complementing and optimizing pulp irritation test data. Some of these models are reviewed in Chapter 4.

1.7 Experimental rationale

Dental resin materials are polymer formulations of several compounds purposefully blended to achieve optimal clinical performance. There is evidence, however, suggesting that these materials are sometimes associated with post-operative discomfort and ill-effects. In addition, the dynamics of polymerization and the physical demands placed on the material clinically contribute to physical and chemical changes in the material with time and one manifestation of this change is the release of components or reaction products. The current work will examine component release from composite resin to aqueous solution and the effect of variation of physical parameters on release; it will then examine the influence of dentine on this release.

First, some work in this area has already been reported in the literature. The availability of leaching components has been established for an extensive range of dental resin materials including composite resin. The variation in the purity and the exact composition of commercial resin materials has also been reported (Asmussen, 1975b; Pashley, 1979; Pashley *et al.*, 1977; Miyazaki *et al.*, 1981; Munteanu *et al.*, 1987; Ferracane and Condon, 1990). With respect to composite resin, pulpal responses exclusive of bacterial contamination can be attributed to these materials (Qvist and Stoltze, 1982; Qvist *et al.*, 1989) as can delayed hypersensitivity and allergic

reactions (Nathanson and Lockhart, 1979; Niinimäki *et al.*, 1983). Composite resin materials are therefore not biologically inert. It is pertinent then to examine the hypothesis that components from composite resin are released to an aqueous phase and can be identified and quantified over time and, further, that release can be correlated to physical variables which are either of clinical relevance or are known to influence the degree of conversion of the resin.

Secondly, in view of the diminution of pulpal response to composite resins when applied to greater thicknesses of dentine (Mitchell *et al.*, 1962; Stanley, 1968; Suarez *et al.*, 1970; Baume *et al.*, 1971; Dickey *et al.*, 1974), this work seeks to examine the hypothesis that composite resin elutes its components to aqueous phase and that dentine, as suggested by Van Meerbeek and co-workers (1992b), has a "protective potential", reducing or preventing passage of molecules through its substance when resin materials are directly applied. To examine the potential of dentine to protect against component release, the Hume tooth model (Hume, 1985) will be adapted to examine composite resin component blockage or diffusion when dentine is prepared in a way that simulates clinical techniques and procedures. The contents of pulpal space will be analysed for component presence and concentration. The function of dentine as a protective structure will be examined by comparing findings for the release of components into aqueous phase with findings obtained for diffusion of components into the pulpal space following interaction with dentine.

A third area of investigation will be the effect of the resin-dentine interface, when dentine is pre-treated or dentine bonding system compounds are applied, on component diffusion, in order to address the hypothesis that this layer influences composite resin component release and/or diffusion kinetics. Resin-reinforced dentine has been shown to improve dentine bonding strengths (Asmussen and Uno, 1992; Triolo *et al.*, 1992; Van Meerbeek *et al.*, 1992b) but the durability of the zone remains ill-defined (Van Meerbeek *et al.*, 1993). The specific effects of different dentine pre-treatments and dentine bonding systems on the release of components from bonding agents and composite resins will be examined.

The following Chapters will first provide background to the analytical techniques to be used in the experimental work, namely high performance liquid chromatography and mass spectrometry, including development of

these methods for the analysis of aqueous eluates of composite resin. Experimental studies on component release from composite resin will then be presented. As the nature of dentine largely predicts the nature of the interaction of restorative materials with dentine, this structure will be discussed with particular reference to composite resin biocompatibility. Experimental studies of the interactions of composite resin with dentine will then be described.

To begin examining the component release from composite resins, aqueous eluates of resin materials were first prepared and subjected to quantitative and qualitative analysis by high performance liquid chromatography and mass spectrometry. The following chapter describes these analytical techniques and their use in the current work.

CHAPTER 2

High Performance Liquid Chromatography and Mass Spectrometry Analysis

2.1 General introduction

2.2 High performance liquid chromatography (HPLC)

- 2.2.1 Introduction to chromatography
- 2.2.2 Basic theory: chromatographic process
- 2.2.3 Equipment for chromatographic analysis
- 2.2.4 HPLC method development
- 2.2.5 Gradient separation by HPLC
- 2.2.6 Control of HPLC separation
- 2.2.7 Qualitative analysis by HPLC
- 2.2.8 Quantitative analysis by HPLC

2.3 Mass spectrometry (MS)

- 2.3.1 Introduction to mass spectrometry
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2.1 General introduction

Many analytical techniques are available to determine the identity of compounds within complex mixtures. Separation science unites these techniques with a common objective being the adequate and efficient segregation of components of interest quickly and with maximal recovery of those components (Karger *et al.*, 1973b). Separations can be categorized as those based on *equilibrium* processes where components are distributed over different phases, and those based on *rate* processes where kinetic properties characterize different components. High performance liquid chromatography (HPLC) is an example of a separation method based on phase equilibria, while mass spectrometry (MS) is an example of a field separation based on rate processes. When the two techniques are united, it is possible to identify and quantify compounds readily and with a high degree of confidence. Both techniques have been used previously in dental research.

As these techniques were employed in the analysis of components of dental restorative materials in the current work, the method development and application of these techniques to the analysis of samples will be discussed in detail.

2.2 High performance liquid chromatography (HPLC)

2.2.1 Introduction to chromatography

The word *chromatography* stems from the Greek *chroma* 'colour' and *graphos* 'writing', and takes its origins from early dye chemists who tested their dye mixtures by dipping pieces of cloth into a dye vat and examining migration and separation of the various colours up the cloth by capillary action.

The work of Russian botanist Mikhail Tsvet in 1905, however, established the physicochemical basis of chromatography. Tsvet developed a method of separation of plant pigments, carotenoids and chlorophylls, using a technique that is used today in essentially the same form. There was little interest in the technique until between 1930 and 1940 when *thin-layer* and *ion-exchange chromatography* and *electrophoresis* were developed (Ettre, 1980). Substantial contributions were then made and amongst these was that of Martin and Synge who, in 1952, received the Nobel Prize for the invention of *partition chromatography* (Poole and Poole, 1991c). With

evolving understanding of gas chromatography in the 1960s came the identification of the limitations of a gaseous stationary phase, which included limited phase interactions, inapplicability to thermally unstable and non-volatile samples and poor sample recovery (Snyder and Kirkland, 1979). Clearly, there was need to extend chromatographic potential to the analysis of other samples which prompted the work of Giddings in 1965, Hamilton in 1966 and Kirkland in 1976, but earliest *liquid* chromatography started with amino acid analysis by Spackman, Stein and Moore in 1958.

Chromatography is "a method used primarily for separation of the components of a sample, in which components are distributed between two phases, one of which is stationary while the other moves" (IUPAC Analytical Chemistry Division, 1974). Both phases are intentionally selected to promote the distribution of components of the sample into the stationary phase to varying degrees. This distribution selectively retards the passage of sample components because components vary in their affinity for the stationary phase. The time taken for the solute (containing the sample) to pass through the chromatographic system is characteristic of that solute for those particular conditions of analysis. Whilst chromatography separates the components of the samples very well, their identification only follows spectrophotometric analysis which will be discussed in a later section.

Different states of matter can be used as the stationary phase, which is confined within a tube or column. Thus the family of techniques is often called *column* chromatography. The nature of the partitioning phases is only one feature which distinguishes the techniques, there are also differing chemical interactions, detectors and sensitivity. These distinctions enable the different techniques to be better suited to the separation of different compounds, for example, *liquid* chromatography is ideally suited to macromolecules and ionic species.

High performance liquid chromatography (HPLC) describes a form of column chromatography where the stationary phase, a solid, is confined within a small bore tube, or column, and the entire system performs under high pressure (Skoog and Leary, 1992b). High pressure is needed to achieve a flow velocity of the mobile phase through the densely packed stationary phase (Guiochon, 1980). Modern HPLC started to evolve in the 1960s, following the extension of gas chromatographic theory, and is now a commonly used form of column chromatography (Snyder and Kirkland,

1979). Further historical detail of the development of HPLC can be found in relevant texts (for example, Snyder and Kirkland, 1979). As HPLC is the principal analytic technique used in the current work, the following discussion will be confined to the theory of liquid chromatography and HPLC.

2.2.2 Basic theory: chromatographic process

The chromatographic process "occurs as a result of repeated sorption/desorption acts during the movement of the sample components along the stationary bed, and the separation is due to differences in the distribution constants of the individual sample components" (Poole and Poole, 1991b). The essential aspects of this process are *differential migration* of a sample, *distribution of sample components* over the mobile and stationary phases, *molecule diffusion and separation* through the column and finally *detection* of eluted components (Snyder and Kirkland, 1979).

Differential migration refers to the varying rates of progress of different molecules through the column. Compounds which proceed at the same rate will not be separated. This migration very much relates to differences in the equilibrium distribution of the components over two phases, the mobile phase and the stationary phase. Several factors affect this distribution, such as the composition of both mobile and stationary phases, the morphology of the stationary phase particles and the separation temperature. Thus, as different component molecules encounter stationary phase particles being propelled in a moving stream, the molecules pass around and, at times, through porous particles at different rates. In so doing, the molecules of each component of the sample are separated from each other and confined as *bands* on the column. The flow path of a particular component is also subject to forces, such as eddy diffusion, mass transfers from the stationary and mobile phases and longitudinal diffusion, which together cause spreading of the separated band over the column.

Eventually the various bands reach the end of the column and are propelled to the detector, where concentrations are determined as a function of separation time (Snyder and Kirkland, 1979) and plotted as Gaussian *peaks* on a *chromatogram*. The chromatogram is basically a graph plot of peak response on the ordinate axis and time since injection on the abscissa. The detector specifically bases distinction of eluting components on certain

chemical characteristics such as UV-spectrophotometric chromophore and electrochemical characteristics which are selected to best allow reliable and reproducible peaks in a chromatogram. Snyder and Kirkland defined four features of a chromatogram which describe the separation; the *shape of the peak* (Gaussian or standard-error curve), the *retention time* which is measured from the time of sample injection to the time the band maximum leaves the column, the *difference in retention times between adjacent bands*, which when further apart denotes easier separation and quantitation and better peak resolution, and the *band width* which is also used in quantitation. The implications of these features will be discussed further in sections on quantitative and qualitative analysis.

Several important parameters describe HPLC separations in the literature and are used to improve analysis efficiency and performance (Table 2.1). These parameters are united in the "resolution factor" equation to be presented shortly.

Table 2.1 Parameters commonly used to describe HPLC separations

Parameter	Symbol	Definition
Capacity factor	k'	measures solute retention
Selectivity	α	ability of a column to separate components
Theoretical plates	N	measures band spread throughout the chromatographic system; column efficiency
Resolution factor	R_s	measures the adequate separation of peaks
Retention time	t_R	measures the time from sample injection to time of band maximum (in minutes)

The capacity factor (k') directly measures how strongly the sample interacts with the packing material, such that a very low value shows very little interaction and the peak will appear early very close to the void volume peak on the chromatogram. Optimal selectivity of the column describes how well the chromatographic system can separate two components and is the ratio of capacity factors for the two components. The

plate count or efficiency is the number of bands theoretically possible in the column and is highest when the column is first packed.

Resolution is proportional to square root of the plate number and is related to capacity and selectivity and can explicitly evaluate a chromatogram as shown in following equation (Snyder and Kirkland, 1979).

$$R_s = \left(\frac{1}{4}\right)(\alpha - 1)\sqrt{N}\left[\frac{k'}{1 + k'}\right] \quad (2.1)$$

The optimization of the chromatographic separation during method development is accomplished by systematically manipulating the factors as described in this equation. These factors will be used to facilitate discussion in the following sections.

2.2.3 Equipment for chromatographic analysis

One of the advantages of HPLC has been to provide trace analysis from very small quantities of sample. Careful re-designing and development of liquid chromatography (LC) technology has provided this facility which requires the components of a HPLC system to have versatility, speed, reproducibility, sensitivity and the ability to reduce sample-size effects (Snyder and Kirkland, 1979). In simpler terms, the system uses a *high pressure pump/system controller* to propel the mobile phase and control flow, a *supply of mobile phase*, an *injection unit* with which the sample is introduced on to the solvent flow, a *column* packed with stationary phase material, an *in-line detector* and a method of *detector signal display*. Extra units, such as line filters, column thermostats and pulse dampers are also sometimes employed. All components of the system are united by small bore tubing and all units are chemically resistant to mobile phase solvents.

The pump functions to accurately meter a constant, reproducible flow of mobile phase through the system. Constant high pressure, up to approximately 70 MPa, is required to force mobile phase through columns which are densely packed with very small-sized particles. Since the detector is sensitive to many changes in flow, including component presence, the mobile phase flow must be continuous and pulse-less. If present, these pulses can appear as *noise* on the chromatogram which interferes with peak interpretation. Commonest pumps used feature low

volume (35-400 μ l) chambers and reciprocating pistons or flexible diaphragms which drive solvent flow against the column back pressure, minimize pumping noise and compensate for solvent compressibility. Gradient conditions of HPLC analysis (see section 2.2.6) require, in addition, the ability of the pump system to vary either pressure or flow rate during an analysis by using flow controllers or mixers. A 600E system controller reciprocating pump (Waters) was used in the current study.

The mobile phase in chromatography is selected to match the column and the sample and is generally an aqueous-organic mixture (Lindsay, 1987). Of the range of possible mobile phases (or solvents) many are eliminated from choice by inappropriate LC suitability, for example, high viscosity or low boiling point. The ideal solvent should provide a k' which falls within the optimal range of 2-5 for a two-component separation and 0.5-20 for multiple components (Snyder and Kirkland, 1979). The solvent should readily dissolve the sample, be ultra-pure and relatively unreactive and be readily miscible with other solvents used. Reproducible solvent delivery is facilitated by check valves, flow controllers, mixing chambers and pulse dampers which are often incorporated in the pump unit. When different solvents are mixed, as during gradient separations, gas bubble formation due to either air incorporation or oxidative degradation of the solvents reduces sensitivity and baseline stability (Poole and Poole, 1991a). This problem is reduced in degassing (or sparging) the solvent volumes with an inert gas, such as helium, by gaseous equilibration.

Four main intermolecular interactions between sample and mobile-phase molecules are important to efficient separation; dispersion, dipole, hydrogen bonding and dielectric (Snyder and Kirkland, 1979) and the ability of either the sample or solvent to interact in all of these four ways is referred to as the *polarity* of the compound. Polar solvents preferentially dissolve polar solutes and solvent strength is directly related to its polarity. Indices for solvent polarity have been suggested to enable solvent choice, for example, the *polarity parameter*, based on solubility data and the *Hildebrand solubility parameter* (Snyder and Kirkland, 1979). Improving solvent selectivity refers to the optimizing of solvent/sample interactions which may be indicated when bands overlap or components co-elute.

The sample is introduced as a narrow plug onto the mobile phase stream following syringe injection or through a sampling valve as part of an

automatic unit. Ideally, sample introduction onto the pressurized system occurs without disturbing mobile phase flow, without causing appreciable peak broadening and with high reproducibility. A more complete description of these units is found in relevant texts (for example Snyder and Kirkland, 1979).

The column in HPLC is a critical component of the system and is specifically produced with certain features to efficiently allow separation of components. Generally column packing materials in chromatography are either solids or gels, porous or pellicular, spherical or irregular and vary in size, although small porous particles are usually preferred for LC applications. Pellicular particles are made from glass beads coated with a thin porous layer of polymeric stationary phase. The packing is selected to provide the desired solute (component) interactions with a uniform bed free of cracks and channels. The use of smaller particles also offers higher efficiency, better sensitivity and reduced "run time". Following packing of the column, the manufacturer performs preliminary analyses with the column using standards and these data are enclosed with the column. These data include plate count, retention for sample solutes and peak symmetry on a sample chromatogram. Continued column performance is usually determined at intervals in the lifetime of the column by again determining these data and comparing these to those provided by the manufacturer. Any change in peak shape or height or in the retention time suggests ageing of the column.

The chemistry of the column packing materials provides the basis of the separation modes of HPLC separation. These modes include gel-permeation, adsorption (or normal phase), partition (liquid-liquid), ion-exchange and reversed-phase (Lim, 1986). As reversed-phase HPLC was used in the current work, only this mode will be discussed here. This mode is the preferred method for neutral or non-ionized compounds that dissolve in water or organic mixtures (Snyder *et al.*, 1988b). In reversed-phase separations, the polarity of the stationary phase (column packing) is considerably less than that of the mobile phase and polar mobile phases are used (Lim, 1986; Lindsay, 1987b). Since solute retention is mainly due to hydrophobic interactions between solute and the hydrocarbonaceous stationary phase surface, the most polar solutes (components) are eluted first and increasing mobile phase polarity increases solute retention on the column. These selective solvent-sample interactions are largely due to

dipole attraction and hydrogen bonding (Snyder *et al.*, 1988b) and selectivity of the separation can further be achieved by adding reagents to the mobile phase which modify pH and ionization (Lim, 1986). In the current work, an ammonium phosphate buffer was added to control solute ionization and limit mobile phase pH to the range 6.2-6.5; this had the effect of greatly improving component resolution in the gradient separation (see section 2.2.7). As the column used was non-end capped, this buffer also suppressed the effects of residual silanol groups formed after unavoidable incomplete bonding of the silica particles in packing manufacture (Lim, 1986).

The detector continuously monitors the column effluent for changes in either a bulk property, such as refractive index or a solute property, such as UV-absorption (Snyder and Kirkland, 1979). Commonest detectors are based on UV-absorbance, fluorescence, conductivity, electrochemical measurement and refractive index and component properties can suggest choice of detection method, for example good UV-absorbance suggests UV-absorbance detection, adequate refractive indices suggests refractometer detection, and ability to fluoresce or oxidize easily suggests fluorescence or electrochemical detection (Snyder *et al.*, 1988a). The detector output is an electrical signal which is directly proportional to the property monitored (Lindsay, 1987a). Ideally, the detector should feature good sensitivity, low baseline noise, low signal drift and wide linear dynamic range. In the current experimental study, UV-absorbance detection was used. The basis of the UV-absorbance detector is to compare UV-absorbance of column effluent in a detector flow cell to that of pure mobile phase. This comparison is dependent on the spectrophotometric "invisibility" (UV-transmittance) of the mobile phase and the non-UV-absorbance of other components in the sample at the wavelength of detection. Sample concentration in the flow cell is related to the fraction of light transmitted through the cell according to Beer's law, so that the signal output or *absorbance* is linearly proportional to the sample concentration in the flow cell (Snyder and Kirkland, 1979). Highly sensitive photometers (such as those with about 0.002 AUFS or "absorbance units full scale") allow detection of components with very low absorptivities and for quantitative analysis, high detector sensitivity and low detection limit are preferred. Detector limit (sensitivity) is taken as the concentration or mass of the solute entering the detector per unit time that will provide a signal-to-noise ratio of 2, but this can be dependent on the chromatographic system.

Variable detectors allow the detector to survey the sample at different wavelengths during the same analysis to aid comprehensive detection and to remove the detection of known contaminants. In a multichannel or diode array detector, polychromatic radiation emerging from the flow cell is diffracted onto a collection of photodiodes, each of which receives a wavelength. Microprocessing of the collection of signals enables a three-dimensional scan of the column effluent (Lindsay, 1987a).

2.2.4 HPLC method development

Prior to planning method development it is pertinent to define the goals of the specific experiment, such as component identification only, necessity to resolve all components, component quantitation or the necessity for the establishment of a routine laboratory method. Once this is defined, *systematic* method development to achieve these goals is commenced by accumulating data about sample characteristics, establishing the need for sample pre-treatment, choosing detection conditions, performing the first separation, optimizing and controlling separation (see section 2.2.6) and problem solving. These are discussed in the following sections.

Despite the comment of Snyder and his co-workers (1988a) that an alternative approach to HPLC method development is to "minimize the use of information on sample structure and carry out method development for most samples in the same general way", some sample data are probably imperative for the novice chromatographer. Apart from an idea about the likely number of components in the sample, the nature of the sample matrix and the sample solubility, accurate information required about each component includes chemical structure, molecular weight, pK_a values, UV spectra and possible concentration range in the sample (Snyder *et al.*, 1988a). Component structure, for example, reveals the presence of UV-absorbing functional groups (chromophore) and therefore can suggest both the use of UV detection and the appropriate wavelength for detection. Data for the other characteristics already listed then allows entry to several method development flowcharts in the literature and in manufacturer databases which ultimately simplify method choice (for example, Snyder and Kirkland, 1979).

Sample preparation is required to remove interfering elements and particulates in the sample solution, enhance separation sensitivity, to prevent damage to the chromatographic system and, at times, to concentrate

weak samples. The method selected is usually dependent on characteristics of the sample solution. Samples are well prepared for analysis when they are representative of the entire sample volume, are dissolved in solvents compatible with the mobile phase or are stable, and can be accurately recovered quantitatively in minimum volume (Poole and Poole, 1991b). Many options for sample preparation exist including filtration, centrifugation, ultrafiltration, selective precipitation, liquid-liquid extraction, dilution and derivatization (Liteanu and Gocan, 1974a; Snyder and Kirkland, 1979; Engelhardt and Elgass, 1980; Karger *et al.*, 1980; Lindsay, 1987c; Snyder *et al.*, 1988; Poole and Poole, 1991a).

In HPLC, equipment malfunctions are often a direct cause of poor results and the many types of malfunctions reported preclude discussion here. In brief, when equipment operation is satisfactory, poor chromatography is likely due to either incorrect operator technique (and troubleshooting trees enable correction) or separation artifacts. This latter group of causes is especially important as they directly affect chromatogram accuracy and can occur suddenly. *Band tailing*, causing peak asymmetry, can be due to slow retention kinetics, column overload or poor column packing, micelle formation and extracolumn effects (Snyder and Kirkland, 1979). *Slow retention kinetics* is the slow breaking of bonds between sample molecules and functional groups within the stationary phase. *Column overload* occurs when the amount of sample loaded onto the column exceeds 1 mg/g of column packing material and causes both retention times and plate numbers to decrease. *Extracolumn effects* refer to changed volume in the sample injector, detector cell or tubing between HPLC units which then causes band broadening and decreased plate number. *Sample reaction*, such as isomerization or degradation during assay causes the appearance of the isomers or reaction products as spurious peaks on the chromatogram. *Negative peaks* are due to several causes, such as a greater detector response for the mobile phase than for the sample, pressure fluctuations at time zero that accompany sample injection (sometimes called the solvent front) and temperature changes in the mobile phase. *Vacancy or "ghost" peaks* are associated with the use of a mobile phase containing a moderator which can spontaneously appear.

A method development flowchart is presented in Fig. 2.1. The cascade on the left hand side is a general guide to HPLC method development. The right hand side cascade, specific for reversed-phase HPLC, shows the

recommended steps to improve unacceptable separations. In this figure ACN refers to the solvent acetonitrile, MeOH to methanol and THF to tetrahydrofuran.

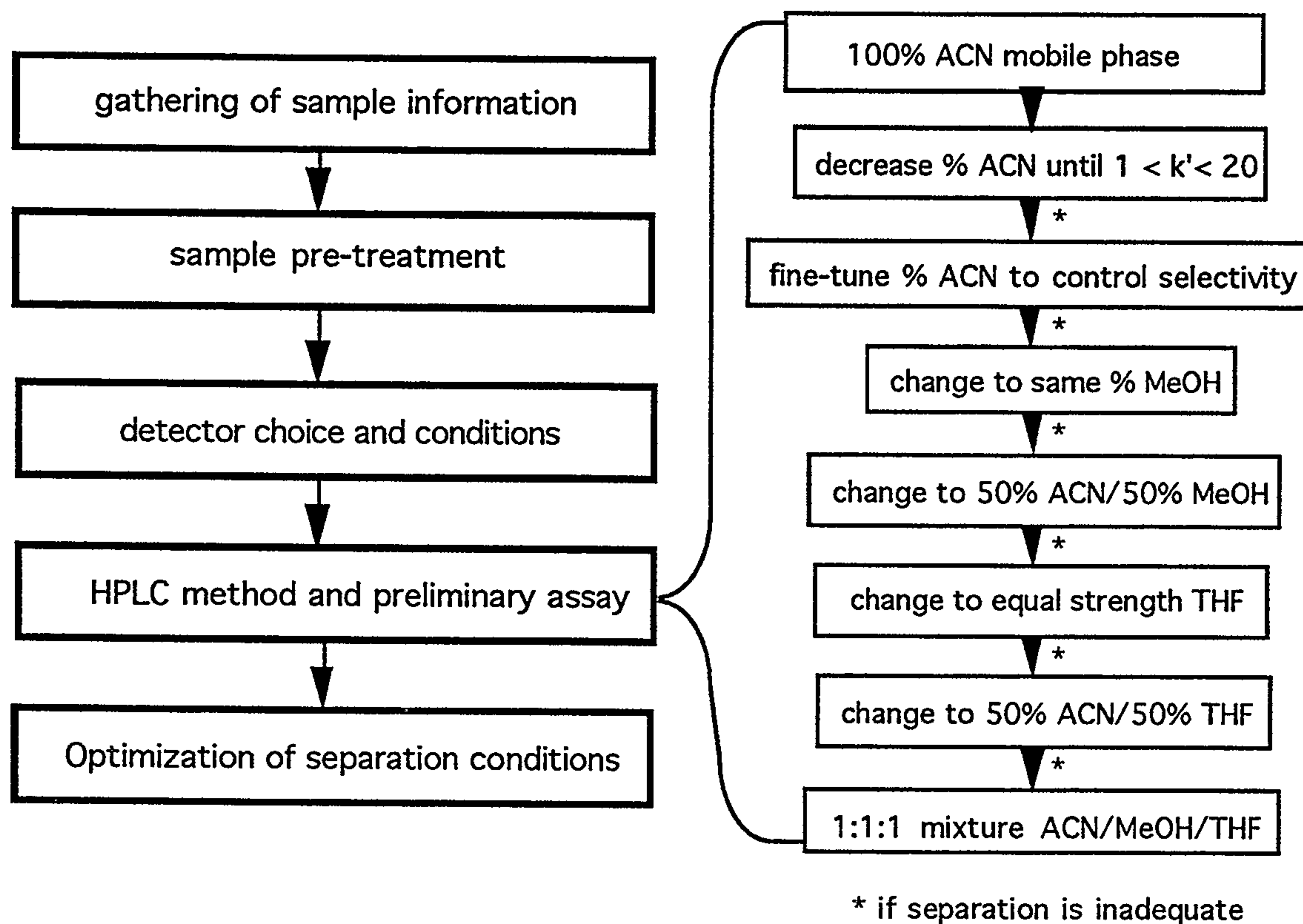


Figure 2.1 HPLC method development flow chart
(Adapted from Snyder and Kirkland, 1979 and Snyder and co-workers, 1988b)

General separation goals for simple mixtures in HPLC include: $R_s > 1.5$, separation time < 5 -10 minutes, narrow peaks for large signal/noise ratio and minimal mobile phase usage per assay (Snyder *et al.*, 1988b).

2.2.5 Gradient separation by HPLC

Thus far, *isocratic* HPLC elution has been described, where analytical conditions do not vary during the assay. Sometimes, when the sample is more chemically complex or widely varying k' values have been discerned for different peaks or peaks fail to appear, a general elution problem arises which may be solved by varying conditions *during* the separation (Snyder and Kirkland, 1979). A common non-isocratic method is *gradient elution*. The objective in this form of HPLC is to simultaneously optimize resolution and sensitivity for every component in a complex sample. Gradients may be

based on mobile phase variation, stationary phase variation or combination gradients. Since the gradient used in the current work was based on mobile phase changes, the following discussion is restricted to that form. A very complete coverage of gradient elution is found in a text by Liteanu and Gocan (1974b).

Equipment differences for mobile phase gradient conditions include additional mobile phase reservoirs and a gradient former (often part of the pump) which controls mobile phase mixing. Basically, solvent blend is varied throughout the assay according to a gradient plan which describes the relative proportioning of two (or more) pure solvents in a continuous or stepwise fashion which may be linear, concave or convex over time. The solvents used are usually described as A and B and the gradient plan generally plots %B vs time course. The mobile phase composition varies throughout the assay by increasing the amount of A added to B, an example is "10% B to 75% B over 35 minutes" or as a "2% / min linear change". Common solvent pairs in reversed-phase HPLC are water/methanol and acetonitrile/tetrahydrofuran (Snyder, 1980). Since solvent strength commonly increases over the gradient, late eluting peaks in isocratic mode are hastened in their passage through the column as the progressively stronger mobile phase decreases the retentive interaction of the component with the stationary phase (Liteanu and Gocan, 1974). This acceleration of the peak may also reduce its retention time and improve its symmetry. This form of gradient HPLC has been successfully used for the separation of a wide range of compounds including organic acids, carbohydrates, aromatic hydrocarbons, amino-acids, proteins, nucleic acids, steroids, antibiotics and lipids.

Since the solvent blend is constantly changing in gradient HPLC, the potential for the UV characteristics of the mobile phase to differ over the time course (for example, as the concentration of UV-absorbing impurities in one mobile phase builds up) is high and is manifest in systematic baseline drift. This effect is more apparent for detection wavelengths below 220 nm. The use of ultra-pure water (as one of the solvents) can reduce this tendency. Injectionless assays using the gradient can establish the magnitude of the drift by acting as a "system control" and in some computer-based programming chromatography units, the baseline generated in one of these assays can be "subtracted" from the baseline of sample chromatograms leaving a far simpler "net" chromatogram. Another

cause of baseline drift can be dissolved oxygen bubbles formed during solvent blending. Continuous degassing (or *sparging*) of the solvents (particularly water) with an inert gas (for example, helium) can reduce this effect. Helium molecules displace dissolved oxygen in the mobile phase and, if permitted to collect as a "blanket" of gas over the mobile phase surface during the assay, can both prevent re-dissolution of oxygen and degradation of oxidizable samples.

A vital aspect unique to gradient elution is the necessity for column regeneration (or re-equilibration) which restores the column to pre-gradient conditions before the next sample can be assayed. This period of regeneration is built into the gradient assay (although not described in conditions) and is generally a stepwise downward change in solvent blend until initial blended conditions are restored. It is recommended, following column re-equilibration, that a period of column conditioning also be completed to allow thorough resumption of pre-analytical conditions.

The solvent blending throughout the gradient separation is not without hazard, as at some concentrations the mixing of two solvents can cause exothermic degassing, non-wetting of column packing or slow degeneration. All of these may disturb the assay. Other aspects of gradient elution such as column switching, stationary-phase and flow programming, coupled column operation and temperature programming during gradient elution are considered in greater detail in the references. In the current work under the sections describing the experimental analysis, gradient elution reversed-phase HPLC is discussed separately as it was the technique used for sample separation and identification. This mode, however, is arguably less associated with reproducible and precise quantitative potential (Snyder *et al.*, 1988) and for this reason, quantitation of identified components was carried out following isocratic separation.

2.2.6 Control of HPLC separation

Control and optimization of the separation is critical to the ultimate accuracy of the analysis and underscores the general goal of chromatographic analysis which is "adequate resolution with minimum separation time and with minimum effort in the initial development of a HPLC separation" (Snyder and Kirkland, 1979). Optimization, as depicted in Fig. 2.1, is usually considered following preliminary method development and the generation of the first chromatogram. The necessary steps include

estimating the resolution factor, R_s , for two or more peaks and determining what improvement is possible and necessary. Fine-tuning of method conditions for the necessary resolution and solving any special problems is then carried out.

R_s is estimated by comparing the initial chromatogram to a standard set of resolution curves (such as those presented by Snyder, 1972) each of which illustrate a specific R_s . When a complex chromatogram is examined, R_s for the assay is estimated for the most poorly resolved peaks, otherwise only the peaks of interest are used. Having estimated R_s , conditions are varied to increase R_s if peaks overlap or tail appreciably.

Variables presented in equation 2.1, namely selectivity (α), plate count (N) and capacity factor (k'), can be manipulated to control separation. Predicting the experimental conditions which will cause necessary changes in the parameters, that is, optimization of the separation, is rarely straightforward and perseverance usually rewards the systematic and persistent chromatographer. However, some general guides exist in the literature. Increase in α , for example, changes the position of one peak relative to another and rapidly increases R_s . Increase in N narrows peaks and increases height. An increase in k' can dramatically reduce peak height and increase separation time between peaks. When R_s must be increased, the quickest way to do this is to increase k' . In very general practical terms, N is increased by increasing the length of the column or by decreasing flow rate. k' can be increased by using a weaker solvent (Snyder and Kirkland, 1979). Increasing α for improved resolution can be accomplished by changes in mobile phase, mobile phase pH, stationary phase, temperature of analysis or the use of selective chemical additives to the mobile phase. Finally, some chromatography computer software is capable of providing some assistance in separation optimization, and even in method development.

2.2.7 Qualitative analysis by HPLC

The identification of a separated component using HPLC can be accomplished in several ways. As a starting point, a rough approximation for the retention time of a specific compound may be available in the literature or manufacturer databases. Accurate and definitive identification is particularly important when trace analyses are carried out. *Retention time match* of a compound (standard) to that of an unknown band run under

the same analytical conditions suggests that the two compounds are the same (Snyder and Kirkland, 1979). The *standard addition method* uses spiking of the unknown and *chromatographic cross-check* employs another chromatographic technique to confirm peak match. These two techniques are described below. *Relative detector response* involves the placement of two different detectors in series following the column and comparing the ratio between signals for the two detectors for the unknown and the standard. *On-line hyphenated analytical techniques* uses assay of collected HPLC fractions by mass spectrometry or infrared spectroscopy and probably offers conclusive identification.

The *standard addition method* (or spiking) has been described by Snyder and Kirkland (1979). In this method the sample is submitted for HPLC analysis and the peak height is measured. Then a known quantity of the component is added to the sample and this new "spiked" sample is reassayed by HPLC giving a new peak height. The difference in peak heights divided by the weight of component added gives a calibration factor. Assaying of unknowns spiked with different amounts of the component can then provide several calibration factors which can be plotted for the component, as a linear plot, if the conditions of analysis have remained constant for all assays.

Chromatographic cross-check involves the assaying of the standards and unknowns by a different chromatographic technique and the different separation data are compared. This is only reliable if the two techniques involve different solute/stationary phase interactions. LC data, for example, could be corroborated by GC data but liquid-solid chromatographic data can not be validly supported by thin layer chromatography assay.

2.2.8 Quantitative analysis by HPLC

The improvement in efficiency of HPLC instrumentation has led to an improvement of the reliability of quantitative data derived. However, several sources of error identified by Snyder and Kirkland (1979) can prevent accurate quantitation. *Sampling errors* occur if the injected volume is not representative of the sample (particularly if the sample is not homogeneous), if sample preparation is inaccurately carried out or if the injection volume is imprecise. *Errors in chromatographic separation* are often due to degradation of some component of the system such as ageing columns, inappropriate chromatographic parameter choice or even sample

decomposition. Diminishing peak height, tailing, peak overlaps and appearance of spurious peaks all suggest system error. *Detection errors* occur if the detector output varies (for example, non-linearly) or if detector calibration has not been carried out for each component. *Errors in peak measurement* relate to the integration and interpretation of peak shape during handling of data from the detector.

Accurate quantitation, therefore, requires attention to prevention of all these errors where possible. Since quantitation in trace analysis is of particular interest to the current work, the following discussion is restricted to this area.

Quantitation in LC is made possible through Beer's Law which describes a direct relationship between the quantity of light of a particular wavelength transmitted through a detector flow cell and the sample concentration (Snyder and Kirkland, 1979). In this way the peaks on a chromatogram can be utilized to determine sample concentration. However, there are two important and specific aspects to quantitation which must be considered—the *selection of the most accurate quantitation method* and the *importance of calibration* in quantitation.

Valid quantitation is dependent on the establishment of a relationship between the magnitude of the detector signal and the sample amount (Poole and Poole, 1991c). The detector signal or response is interpreted on a chromatogram as peak height or area. Peak height measurement is the perpendicular height from baseline to peak maximum and this measurement is less subject to interference by overlapping peaks and less resolution is required. Peak area measurements although less influenced by changes in instrument parameters cannot be performed accurately on asymmetrical peaks and are more influenced by adjacent peaks. An effective measure of a quantity proportional to peak area uses the product of the peak height times width at one-half the peak height. After manual peak integration on well-behaved peaks, computer integration uses chromatography software which can calculate peak height following necessary mathematical tasks such as interpolation or extrapolation on drifting baselines, and fused peaks.

The calculation of a *calibration factor*, which relates peak size (by height or area) and concentration or peak size and weight, ultimately enables sample

quantitation. Two calibration methods are recognized; these utilize *external standards* and *internal standards*. In the external standard method, standard solutions of increasing concentrations, which cover the range of interest of the sample, are assayed under the same separation conditions as those under which the unknown sample is assayed. This standard is usually a mixed solution of 'pure' components likely to be present in the unknown sample. Component concentration or weight for each dilution is plotted against detector response for at least three dilutions to generate a *calibration curve*. A valid calibration curve will be linear and will transect the origin. The slope of this line is called the *calibration factor* and this is unique to that separation and to that component. The amount of a component in a sample is then equal to the peak height for that component divided by the calibration factor. An example of a calibration curve is shown in Fig. 2.10.

Calibration using an internal standard is used to compensate for various analytical errors and can gauge sample recovery during pre-treatment procedures, although its absolute usefulness in quantitation is controversial. In this method, a fixed amount of a structurally similar compound is added to the unknown sample and to the external standard dilutions and the compound to internal standard peak-height ratios are plotted versus the concentration of the compound. Accurate preparation yields a linear plot which transects the origin.

As stated previously, the analytical method used in the current experimental work is reversed-phase HPLC with component identification by MS. In the following sections the theory and practice of mass spectrometry are briefly described.

2.3 Mass spectrometry (MS)

2.3.1 Introduction to mass spectrometry

Mass spectrometry is a field separation based on the electromagnetic characteristics of charged molecules (Karger *et al.*, 1973a). Originally applied to determine the masses of isotopes and the composition of isotope mixtures this technique is now used for the identification and structure determination of a wide variety of complex compounds, for the determination of isotopic ratios of atoms in samples and for the determination of structure and composition of solid surfaces (Skoog and Leary, 1992a). Other techniques for analyzing molecules are infrared,

ultraviolet/visible and nuclear magnetic radiation (Davis and Frearson, 1987) which like MS are all based on a similar principle.

2.3.2 Basic theory: mass spectrometric process

The science of MS started in 1908 when Thompson was able to measure the specific charge/mass electrons of the "cathode rays" generated when electricity was conducted through gases at low pressure in a vacuum (Majer, 1977). In MS, the reciprocal of the specific charge, that is, mass/charge or the mass-to-charge ratio, is used. Thompson found that this beam, generated from a discharge tube ion source, was able to be deflected in a magnetic field. Goldstein similarly showed that "positive rays" accompanied the electron streams but were deflected in the magnetic field in a different direction. Ionization of the molecules of the specific gas by bombardment with the electron stream, resulted in the characteristic group of ions of different masses. When the ions were separated, a plot of their relative abundance (abscissa) compared to their mass (ordinate) constitutes the mass spectrum from which the molecule can be identified.

2.3.3 The mass spectrometer

Thompson, in 1912, developed a *mass spectrograph* which projected the deflected parabolic beam onto a photographic plate. Thompson found that the extent of the deflections was related to the mass-to-charge ratio (or value) of the positive ions. Several workers following Thompson, such as Aston, Dempster, Nier and Hipple, further refined the technology (Majer, 1977). There are several major components of a *mass spectrometer* (Skoog and Leary, 1992a). These may be described in the order in which the sample is processed. The *sample inlet system* introduces a very small amount of sample into the spectrometer mass analyzer. The *ion source* then accelerates a stream of negative or positive ions into the *mass analyzer*. The stream bombards the sample thereby converting the sample components into ions. Dispersion of the ions occurs in the mass analyzer which distributes the ions based on their mass-to-charge ratio. The magnetic field causes the ions to take a circular path, 180°, 90° or 60° with heavier ions falling away first. Ions then exit through an exit slit, fall on to a collector electrode in a *detector* which then amplifies and converts the signal into an electrical signal which is then processed by a *signal processor* and then displayed or recorded. Several units of the mass spectrometer function under vacuum and 10^{-5} - 10^{-8} torr of pressure, namely the inlet system, the ion source, the mass analyzer and the detector.

Mass spectrometers now use ion sources other than electrons. The sources used are usually from two main categories; *gas-phase*, where the sample is volatilized, and *desorption*, where energy is imparted to a solid or liquid sample without volatilization. The nature of the sample is important in determining the type of ion source which is appropriate. Gas-phase sources are restricted to thermally stable compounds that have a boiling point less than 500°C, which corresponds to molecular weights less than 10^3 dalton (Skoog and Leary, 1992a). Chemical ionization mass spectrometry (CIMS), which is that used in the experimental work, uses gas phase ion sources.

Mass spectrometers can also be classified according to their method of ion separation: magnetic or quadrupole (Majer, 1973; Karasek and Clement, 1988; Skoog and Leary, 1992a). *Magnetic* separation is based on the deflection of an ion path through a magnetic field and is the more common method used. In *quadrupole* separation, ions are passed through the centre of four parallel rods to which radio frequency and A.C. and D.C. voltages are applied. Ion mass is directly related to amplitude of voltage, so that the ion trajectory can be mathematically expressed.

2.3.4 Fragmentation in MS

In electron ionization, featuring an attracting electrode potential of 70 V, each focussed electron gains an energy of 70 eV. This energy is far in excess of that required to simply ionise a sample molecule following impact of the electron with the sample molecule (Davis and Frearson, 1987). Some portion of the energy is imparted to the ion which is then able to further dissociate or fragment into new ions and a neutral particle. These new ions comprise all the conceivable positive ions that can be derived from the sample molecule when every bond can be broken. The principal ions formed from the electron impact of methane (CH_4), for example, are CH_4^+ , CH_3^+ and CH_2^+ . These ions then react with other methane molecules to give CH_5^+ , C_2H_5^+ and C_3H_5^+ . Each of the fragments appears on the *mass spectrum* as a vertical line or peak. The group of fragments also provides structural information about the sample as the cleavage follows functional group patterns which are characteristic of that compound. The relative abundances of the fragmentation ions can be used to establish compound identity. Patterns have been discerned for many compounds and are compiled in references handbooks (for example, FW McLafferty, Registry of Mass Spectral Data, 5th ed. New York: Wiley, 1989). The degree of

complexity of the fragmentation pattern is important as it can complicate compound identification.

2.3.5 Analysis by MS

Because pressures are low, only about 1 molecule in 10^6 undergoes collision which ensures that the mass spectra produced are reproducible and characteristic of the sample molecule (Karasek and Clement, 1988). The ions produced by electron impact act as Brønsted acids, which then react with sample molecules and in proton transfer, a *quasi-molecule* is formed. The presence of these molecules is essential to the correct identification of the compound as their molecular weight represents the simple addition of molecular weights of the sample molecule and that of either a proton, the reagent ion or water. Often all these molecules are present as peaks on the spectrum.

Quantitation of the sample compound depends on the specific physical laws which determine the trajectory and speed of the ion stream. The ions traversing the mass analyzer are influenced by the magnetic field according to the following equation:

$$m/z = H^2 R^2 / 2V \quad (2.2)$$

where m is the ion mass, z is its charge, H is the strength of the magnetic field in gauss, R is the radius of the curvature of the magnet and V is the accelerating voltage applied. Manipulation of these variables enables quantitative data for each ion to be determined.

Molecular weight determination requires the identification of the molecular ion peak; its location on the spectrum abscissa then gives the molecular weight. Comparison of spectra with reference spectra can also be helpful, but a more reliable practice is the comparison of spectra of the "pure" compound run under the identical conditions of analysis with those of the unknown samples. Structural information and molecular formula can also be derived from the fragmentation pattern.

2.3.6 Chemical ionization mass spectrometry (CIMS)

Chemical ionization was first introduced in 1966 and is commonly used in structure identification (Harrison, 1982). In CIMS, neutral gaseous atoms of the sample are ionized by collision with ions produced by electron

bombardment of a *reagent gas* (Skoog and Leary, 1992a). Unlike electron impact MS, in CIMS ion-sample reactions occur and only few electron-sample reactions. CIMS is particularly valuable in establishing compound identity when used with the retention indices of chromatography (Karasek and Clement, 1988).

Several essential reactions take place in CIMS (Harrison, 1982). The first reaction is that of the electrons with reagent gas molecules to yield *reagent ions* or a reagent ion array. This reaction is facilitated by the large pressure difference between the reagent gas (1 torr) and the analyzer (10^{-5} torr) which causes the electron beam to interact exclusively with the reagent molecules. The sample molecule is usually present in a very low concentration, for example, less than 1% of the reagent gas. Collision of the reagent ion with the sample molecule causes proton or hydride transfer to the sample molecule.

Several reagent gases are used including methane, propane, isobutane and ammonia. In the current work, ammonia (NH_3) was used as reagent gas. This molecule produces a reagent ion, $\text{N}^+(\text{NH}_3)_n$, which has a high proton affinity and low hydride affinity. Sample molecules would, therefore, be protonated when this reactant gas is used.

Many of the peaks in a mass spectrum are due to ions produced by the fragmentation of the sample molecule ion. This ion is in a highly excited state and may participate in the forming of new bonds creating more stable species (Majer, 1973). The ion-sample reactions produced in CIMS are much lower in energy than electron-sample reactions produced in electron impact MS and so abundant sample molecule ions and simple fragmentation patterns are seen (Karasek and Clement, 1988).

A CI mass spectrum is generally plotted as a normalized bar graph showing the relative abundance of ions (%) as the ordinate and the molecular weight as the abscissa. The most abundant ion, which may not be the compound of interest, is plotted at 100%.

Three basic approaches are used for compound identification by CIMS (Harrison, 1982). First, separation of the sample is achieved by a chromatographic method with direct introduction of the effluent into the mass spectrometer source (that is, *hyphenated method*). Secondly, as an

alternative to chromatographic separation, the components are both separated and analyzed by mass spectrometric methods; this is a *double MS analysis*. Thirdly and less reliably, the compound of interest is selectively ionized by a suitable reagent with no or minimal separation of the other components.

Quantitation by CIMS may be achieved by the independent estimation of the component in a standard sample. Accuracy and reproducibility for this method has been reported as $\pm 10-25\%$ and sensitivity to sample concentrations as low as 10 ppb is possible. CIMS also allows good applicability to a range of biological matrices (Milne, 1971).

2.4 HPLC and MS tandem analysis

Whilst MS is a powerful technique for the identification of a compound, its usefulness is limited when the sample contains a large number of compounds or is dissolved in a complex matrix. The uniting of MS with separation techniques, such as chromatography, vastly improves the power of the analysis. Skoog and Leary (1992a) have termed these unions *hyphenated methods*. In these methods, the separation technique (for example, HPLC) may be directly "on-line" with the mass spectrometer or the separated compounds are retrieved and injected into a mass spectrometer. In the current discussion, *tandem analysis* is used to refer to this latter type of hyphenated method and is the analytical technique used in the current experimental work.

Several analytical techniques, such as chromatographic techniques, spectrophotometric techniques and infrared spectroscopy, have been used in dental materials research. Some of the applications have included determination of material composition, the monitoring of chemical reactions and processes, such as polymerization conversion in composite resins, and the analysis of water eluents or tissue samples for components of dental materials.

HPLC, for example, has been used to examine component release from several resin-based dental materials such as denture base polymers (Ruyter, 1980; Koda *et al.*, 1989; Shintani *et al.*, 1993), and from composite resins (Fujisawa and Masuhara, 1979; Inoue and Hayashi, 1982; Miyazaki *et al.*, 1983); and to examine composition of composite resins (Miyazaki *et al.*, 1981;

Ruyter and Sjøvik, 1981; Munteanu *et al.*, 1987b; Ruyter and Øysaød, 1987). HPLC has also enabled separation of isomers of Bis-GMA (Munteanu *et al.*, 1987b) and allowed the detection of formaldehyde formation in composite resins (Øysaød *et al.*, 1988). Tandem analysis by gas chromatography and mass spectrometry has been applied to determine residual monomer content of acrylic or PMMA resins (McCabe and Basker, 1976; Basker *et al.*, 1989), components in alcoholic eluents from composite resins (Rathbun *et al.*, 1991), leaching of organic additives from dentures into saliva (Lygre *et al.*, 1993) and leaching of additives from orthodontic luting resins into saliva (Lygre *et al.*, 1994). Infrared spectroscopy has been used to examine composition or conversion in resins (Caul *et al.*, 1956; Ruyter and Györösi, 1976; Ban *et al.*, 1982; Ruyter and Øysaød, 1982) and other spectrophotometric techniques have been used to analyse component release from glass ionomer materials (Forss, 1993), chlorhexidine release from acrylic resin (Wilson and Wilson, 1993), mercury from amalgam (Marek, 1990), metals from dental alloys (Brune, 1988), eugenol from zinc oxide/eugenol mixtures (Becker *et al.*, 1983) and for the analysis of degradation products following esterase action on methacrylates (Bean *et al.*, 1993).

2.5 Use of HPLC and MS analysis in the current work

2.5.1 Experimental goals

The general experimental goals of any analysis are to achieve accuracy and precision, to identify and reduce sources of error and to establish confidence levels (Reynolds, 1966). In addition to these, the experimental goal in the current work was to develop an analytical technique which would accurately analyse water samples, generated under the conditions of the experiment, for the presence and quantity of all resin components. An additional goal was to optimize the conditions of analysis to facilitate efficient analysis of many samples and thereby reduce the loss of reproducibility which may occur in long term equipment usage.

2.5.2 Sample information and characteristics

Reynolds (1966) suggested that the basic steps of any analytical procedure should follow a definite sequence involving the choice of a method of analysis and then obtaining a representative sample, conducting preliminary separations and obtaining final measurements. In order to follow this sequence, a choice of the analytical method can be simplified by considering the nature of the components likely to be found in the samples.

All components of the composite resin Foundation were obtained from the manufacturer (Nulite Systems International, Hornsby, NSW, Australia) and were in the form and physical state in which the components are used in the material. Other components were obtained from Sigma Aldrich Pty Ltd, (Castle Hill, NSW, Australia). The estimated purities, according to manufacturer data, of the components of Foundation and other compounds used in the analytical methods for the current experimental work are shown in Table 2.2.

Table 2.2 Supply sources and purity of compounds used in the study

Component	Supply source	Purity
Bis-GMA	Nulite Systems International (Hornsby, NSW, Australia)	99.5% (balance inhibitor)
TEGDMA	Nulite Systems International	99.5% (balance inhibitor)
CQ	Nulite Systems International	99.9% (balance trace impurities)
BHT	Nulite Systems International	99.9% (balance trace impurities)
TMA	Nulite Systems International	99.9% (balance trace impurities)
HEMA	Sigma Chemicals, Australia	99.9% (inhibited with 2000 ppm hydro monomethyl ether)
glasses	Nulite Systems International	99.4% (balance silica and ferrous oxide)
silicas	Nulite Systems International	99.8% (balance alumina)
UDMA	Sigma Chemicals, Australia	98%

The structures and physical and chemical characteristics of the components were presented in section 1.4.4 in Chapter 1. The material consists of three main components, polymer resin matrix, filler particles and silane filler coating. Data about the fundamental characteristics of each of these components were required to assess a possible analytical method. These characteristics include solubility in water and organic solvents, molecular weight and chemical structure, spectrophotometric characteristics and sample characteristics. In the current work, these characteristics were

determined experimentally or data were obtained from reference handbooks.

2.5.2.1 Solubility in water and organic solvents

In order to allow a choice of HPLC solvent concentration with which to commence preliminary HPLC assay, simple qualitative information about the solubility of the main components of Foundation in water and blends of methanol and water was necessary. In addition, data for HEMA and UDMA were also determined, as examples of resins containing these components were also examined in the current work. To test solubility, a drop of each liquid component (TEGDMA, TMA, HEMA, UDMA) or a few grams of each solid (CQ, BHT, Bis-GMA, filler particles) was placed into an individual vial containing approximately 2 ml of the solvent tested. The solutions were agitated in a vortex mixer for 5 min and supernatant solutions were visually inspected for degree of turbidity and an estimate was made of the proportion of component which had entered solution. All components tested were readily soluble in methanol and water mixtures beyond 30% methanol but were generally only sparingly soluble in 100% water. It must be noted that these solubility tests were performed as guides to the analytical method only and although most components exhibited low solubility in water it cannot be assumed that this represents the likely solubility in oral fluids. Filler particles appeared to be insoluble in all solvents tested.

2.5.2.2 Molecular weight and chemical structure

The molecular weight of all components of Foundation (apart from filler) fell within the range of 149 to 512. An examination of the chemical structures shown in section 1.4.4 shows that components (apart from filler) are all organic compounds. The two major components TEGDMA and Bis-GMA are long chain methacrylates but Bis-GMA features a central aromatic moiety. The length of chain suggests that these molecules are non-polar and unlikely to ionize. HEMA (MW=130) and UDMA (MW=470) are present as major components in dental resins. Like TEGDMA and Bis-GMA, these methacrylates are only weakly ionic.

2.5.2.3 Spectrophotometric data

When light is projected onto a solution, one of the wavelengths of the light is selectively absorbed by substances dissolved in the solution; the energy of light which is absorbed is conferred on the molecules within the solution. If the energy of the transmitted light is compared to that of the incident

light, the difference can be applied to Beer's law to determine the concentration of the component in the solution (Reynolds, 1966). This is the basis of spectrophotometric analysis and Beer's law enables the calculation of a constant, *absorptivity*, which can be used to characterize a compound. The ultra-violet (UV) range of the electromagnetic radiation spectrum defines the wavelength of 5 to 400 nm and is a common detection parameter amongst spectrophotometric analyses. In organic compounds, each functional group within the compound has a characteristic chromophore which has a high absorptivity for a given wavelength range. A consideration of the component structure, with regard to these functional groups, can assist in determining the likelihood of UV absorbance and the wavelength of choice for detection using spectrophotometric analysis. A useful wavelength range to determine the absorptivity of the individual compounds for HPLC is 190-330 nm. In the current work, all components were examined spectrophotometrically for absorptivity in this wavelength range. All components were good UV absorbing compounds and a wavelength of 215 nm was selected as the most efficient wavelength with which all compounds would be detected satisfactorily.

2.5.2.4 Characteristics of the experimental sample

All unknown samples consisted of solutes in distilled water. At the start of each experimental interval, fresh type-1 water was obtained (pH range 6.2 - 6.8) and used as the elution medium into which the composite resin was placed. In the first instance, it was assumed that unknown samples would be assayed at full strength and preparation would only consist of filtering (0.22 μ l filter, Millex-GV, PVDF membrane, Waters, Milford MA, USA).

2.5.2.5 Choice of analytical technique

All resin components were strong UV-absorbers and UV detection at 215 nm was selected. The good solubility in organic solvents and low volatility of the resin components suggests liquid chromatography would be an appropriate form of analysis. The low molecular weights of the resin components supports the choice of HPLC. As most resin components were non-ionic, reversed-phase HPLC was indicated, although a phosphate buffer could be used to suppress any ionization which might occur during the assay. Resin components were readily soluble in methanol and water blends above 30% so this was chosen as the lower limit for the mobile phase. Most of the resin components were from the methacrylate family of compounds; isocratic conditions to suit this family were used as a starting point during

method development. Distinguishing the Bis-GMA isomers (Munteanu *et al.*, 1987) in the analysis was also considered a possibility.

2.5.3 HPLC method development for the current experimental analysis

2.5.3.1 Equipment and detection conditions

The HPLC equipment used in all experimental work consisted of: a 486 tunable UV-absorbance detector, a 600E system controller and a 717 autosampler (all units from Millipore Corporation, Waters Chromatography Division, Milford MA, USA). Following preliminary set-up of the individual units during equilibration, instrument control and data acquisition, storage, retrieval and reduction were performed using chromatography computer software (Maxima/Baseline 810 Chromatography Workstation, Dynamic Solutions, Division of Millipore, CA, USA and Millenium Chromatography Manager, Millipore Waters Chromatography, MA, USA) through a 386 SX-20 Compoint Computer with 3C875X-16 co-processor and 256K VGA card (Compoint, Australia).

A UV-absorbance detector was used (486 Tunable Absorbance Detector, Waters Chromatography Division, Milford, MA, USA). Manufacturer data state that this unit featured a wavelength range of 190-600 nm, a bandwidth of 8 nm, accuracy of ± 2 nm, reproducibility of ± 0.25 nm, noise level of $\pm 1.5 \times 10^{-5}$ AUFS, drift of 2.5×10^{-5} AUFS, sensitivity of 0.001-2.0 AUFS, flow cell pressure of 6.9 MPa and a filter constant of 0.1-5.0 s. The unit also provided automatic calibration at start-up and ability to autozero for baseline initialization.

Mobile phase mixing and delivery were controlled through a 600E Multi Solvent Delivery System with a pump head capacity of 225 μ l which allowed both isocratic and gradient mobile phase mixing. A 700 WISP (Waters Intelligent Sample Processor) autoinjector was used to introduce samples onto the solvent stream.

A non-end capped, stainless steel column, 150 mm in length and internal diameter of 3.9 mm, was used (Resolve, Waters, Milford MA, USA). According to manufacturer literature, this column was packed with porous, spherical, C18 silica particles (5 μ m in diameter) and contains 10% carbon loading. The surfaces of these porous particles were very hydrophobic and mean pore size was 9 nm so that the surface area available for solute interaction was

200 m²/g and the column was classified as "non-polar" (manufacturer data literature, Waters Chromatography, Milford, MA, USA). This relatively large pore size offered little size exclusion to molecules in the solute, which were thus able to freely diffuse into the pores and in so doing were delayed in their passage through the column. This type of packing is ideally suited to poorly-retained compounds. These columns are stable at pH 2-7 but are susceptible to rapid silica dissolution at alkaline pH.

Mobile phases used during method development and chromatographic assays were prepared from ultra-pure HPLC-grade solvents including methanol, acetonitrile and tetrahydrofuran (Waters Chromatography Division, Marsfield, NSW, Australia). Type 1 water freshly obtained from a purification unit (Milli-Q Plus, Millipore Corporation, Milford, MA, USA) was used for mobile phase mixing, HPLC system purging and as the sample elution medium. Ultra-pure grade (99.99% pure) helium (CIG, Sydney, NSW, Australia) was used to degas all solvents during equilibration and during assays.

2.5.3.2 Establishment and optimization of analytical conditions

A goal during method development was to establish the conditions of analysis which would simultaneously separate all the components in the unknown sample in one assay. To do this, several conditions of separation were systematically tested using a test solution. This solution contained all the resin components and was derived from 100 mg of each component dissolved in 100 ml of acetonitrile (ACN). A long, slow gradient assay, which would allow separation and recovery of all the components, was first applied. This gradient was linear, 30% ACN to 90% ACN, flow rate 0.8 ml/min, UV detection of 215 nm. A chromatogram of the test solution assayed under these conditions showed good resolution of TEGDMA and Bis-GMA but CQ showed marked tailing, BHT was excessively retained (appearing late in the assay) and TMA was completely retained on the column. Clearly, several assays would be required to optimize separation. Although acetonitrile is known to exhibit good baseline stability and low noise level and is the recommended solvent used during reversed-phase HPLC method development (Fig. 2.1), methanol/water was an economical alternative with only slightly inferior chromatographic properties in the current work and so further method HPLC optimization utilized these solvents.

The efficacy of subtle and systematic changes to the chromatographic conditions was followed by monitoring the chromatograms over many assays. Eventually, gradient conditions were optimized to the point where all components were well resolved under 50 min and the addition of ammonium phosphate buffer to the water reservoir enabled appearance of the component TMA which had been strongly retained on the column in all previous gradients. These initial gradient conditions are shown in Table 2.3.

Table 2.3 Initial reversed-phase HPLC chromatographic conditions used

Column:	150 x 3.9 mm silica C18, 5 μ m Resolve
Mobile phase:	A. 100% water with 0.01 M $\text{NH}_4\text{H}_2\text{PO}_4$ B. 100% methanol Isocratic: 55%B Convex gradient 20% B to 100% B over 35 min
Flow rate:	Isocratic: 1.2 ml/min Gradient: 0.8 ml/min
Detection:	UV 215 nm

These conditions allowed good resolution of some components but some components exhibited poor peak shape or symmetry (for example, CQ), or retention times were impractically long (for example, TMA) for routine assays. Further optimization of the chromatographic conditions enabled development of satisfactory final conditions shown in Table 2.4. These conditions were used for all subsequent HPLC assays in the experiments.

Table 2.4 Reversed-phase HPLC chromatographic conditions used

Column:	150 x 3.9 mm silica C18, 5 μ m 9 nm (mean pore size)
Mobile phase:	A. 100% water with 0.01 M $\text{NH}_4\text{H}_2\text{PO}_4$ B. 100% methanol 1. Isocratic 70% B 2. Linear Gradient: 40% B to 85% B over 26 min
Flow rate:	Isocratic, 1.2 ml/min Gradient, 0.8 ml/min
Detection:	UV 215 nm

Having established gradient conditions for all the components, it was then important to find a suitable internal standard which would be necessary for validation and quantitation purposes. Ideally, this compound should be structurally related to the components, elute under the same chromatographic conditions and hopefully appear in a "vacant" area of the chromatogram. Four possible internal standard compounds were tested; 0.1% methyl-para-hydroxy benzoate, 0.1% dimethyl phthalate, 0.025% ethyl-para-hydroxy benzoate and 0.025% propyl-para-hydroxy benzoate. Of these, methyl-para-hydroxy benzoate was selected. It was then of interest to assay several unknown samples from a pilot experiment under these established separation conditions.

During gradient method development, the isocratic conditions for each component were established. Generally, isocratic runs at 55%, 65% and 75% methanol in water enabled each component to be separated in an efficient time and display a reasonable "peak" shape. This difference amongst the methanol concentrations necessitated the consideration of gradient conditions which would vary the methanol concentration over time and thereby efficiently elute all components in the mixed standard in one assay. Isocratic conditions were used in quantitation as the unchanging assay conditions control peak shape which are then more reproducible and more reliably measured. The isocratic conditions used are also shown in Table 2.4.

All components were readily soluble in methanol so amounts of each of the components of the resin matrix were weighed individually and dissolved in methanol. An internal standard, methyl para-hydroxy benzoate, was similarly prepared. Concentrations of each of the individual standards and retention times of components were shown in Table 2.5. Each standard solution was freshly prepared for each day's analyses and was stored at 4°C until used. A mixed standard solution was prepared using these individual standards in the ratio 10:10:5:10:5:2 respectively. Dilutions of this mixed standard were used to generate calibration curves for each component.

Table 2.5 Concentrations of individual standard solutions and retention times by reversed-phase HPLC gradient conditions

Component	Concentration ($\mu\text{g/ml}$)	Retention time (min)
Bis-GMA	572	31.53
TEGDMA	524	20.12
CQ	49340	12.47
BHT	524	35.81
TMA	26020	30.00
Internal standard	5640	7.97
HEMA	530	3.62
UDMA	560	27.35

Chromatograms from control assays were first generated. These included a system chromatogram (no sample) assayed using gradient conditions shown in Table 2.4. This chromatogram is shown in Fig. 2.2. This was generated after equipment equilibration and established the absence of peaks and disturbances likely to confound interpretation of chromatograms of standards and unknowns. Chromatograms generated from resinless controls from each time interval used in each of the experiments were also assayed. A typical chromatogram of a control (resinless) used in the work to be described in Chapter 3 is shown in Fig. 2.3.

These chromatograms displays several features which are typical of chromatograms. Absorbance Units (AU) are used as an empirical index of signal height plotted on the ordinate axis. In these chromatograms, the ordinate units are quite small, which is not unexpected for "control" assays. Retention time, in minutes, is plotted on the abscissa.

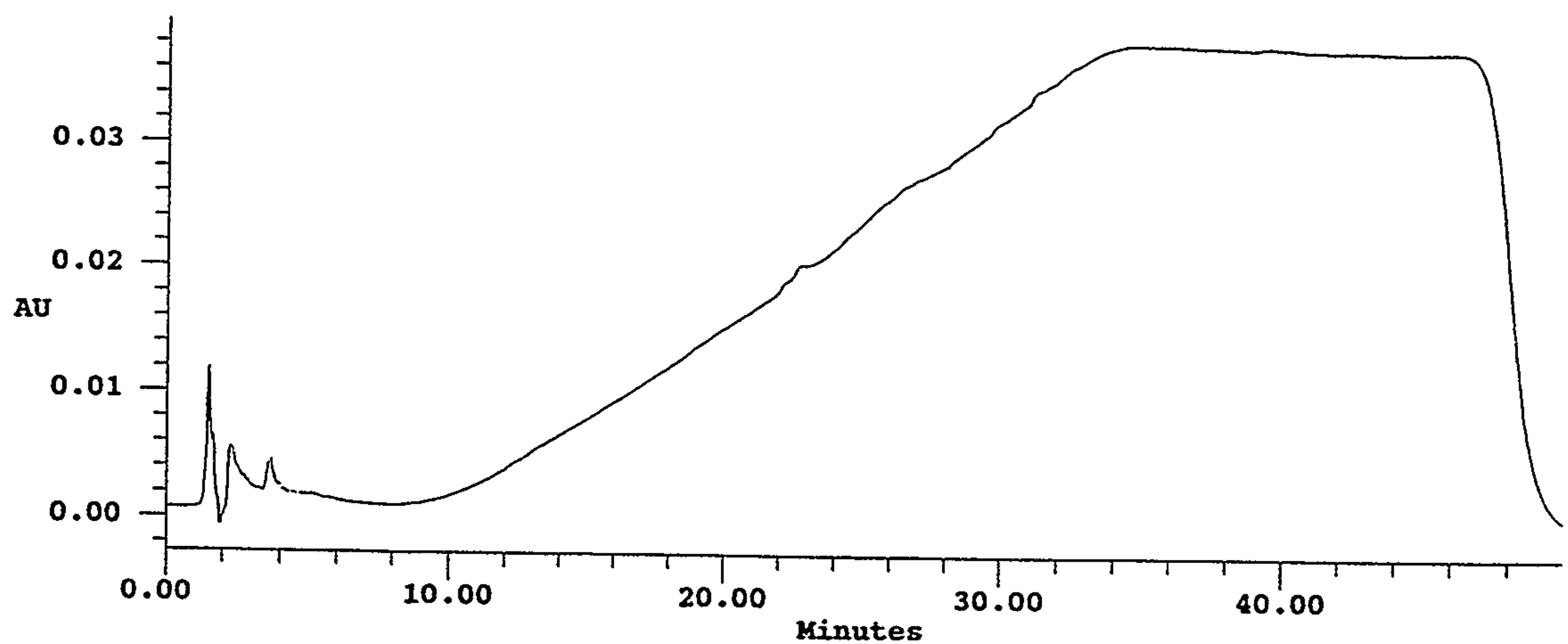


Figure 2.2 System chromatogram (no sample) assayed under reversed-phase gradient HPLC conditions

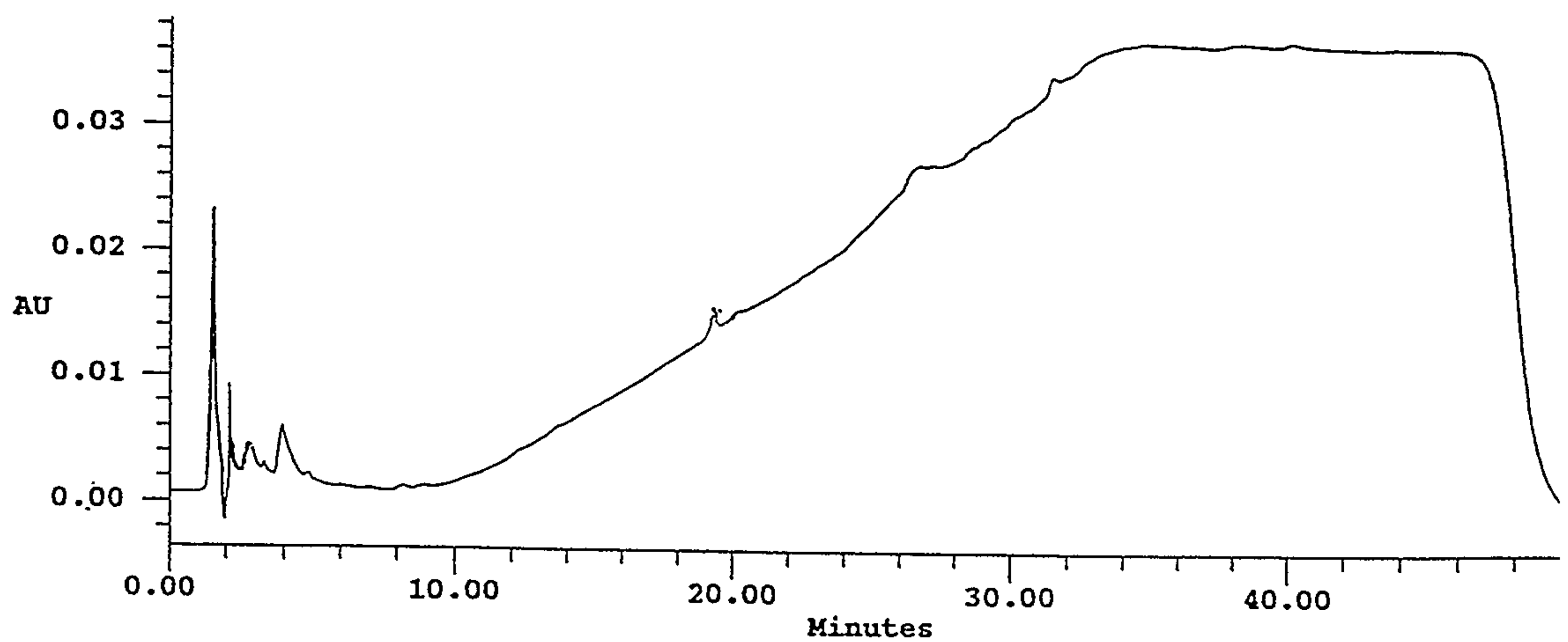


Figure 2.3 Control (resinless) chromatogram assayed under reversed-phase gradient HPLC conditions

Control and system chromatograms were important first assays and enabled valid assaying of unknown samples to be completed. These chromatograms show distinct features; the numerous early peaks, under 5 min, include the disturbance as the solvent front first encounters the packed column contents and is known as the *void volume*. *Baseline rise* or drift in both chromatograms is consistent with the changing optical properties of the increasing solvent strength as the gradient proceeds and is typical of gradient assays. Several small peaks (*noise*) seen at retention times of approximately 20, 26.5 and 32 minutes in Figure 2.3 are likely to have been water impurities. The "plateau" seen from 32 minutes reflects the unchanged solvent blend (and hence constant optical characteristics) of the *equilibration period* where the system is undergoing regeneration in preparation for the next assay. The abrupt baseline drop between 46 and 48 minutes terminates the assay and indicates that the system has effectively returned to initial conditions. The very small AU units used to plot these assays suggested that "disturbances" on the chromatograms, such as baseline drift and noise, are exceptionally weak effects in these assays. A consideration of the features in Figs. 2.2 and 2.3 confirmed the purity of the elution medium and the acceptability of the controls with respect to analytical reproducibility. Most importantly, these control chromatograms enabled the valid comparison of chromatograms of both standard solutions and unknown samples. In all experiments, assaying of standards and unknowns were only commenced when the control and system chromatograms showed features similar to those just described.

A typical chromatogram of the mixed standard solution assayed under *gradient* conditions is shown in Fig. 2.4. Each of the components in the sample is labelled. Baseline rise is comparable to that seen in the control chromatograms (Figs 2.2 and 2.3). *Peak starts and ends* are marked with small triangles; the tangent line which unites these marks encloses the *peak area* and the *peak height* (in AU). These parameters are used in quantitation. Retention times for each peak are marked at the checkmark above each peak apex. This chromatogram shows good resolution (R_s between peaks was approximately 1.8) of the resin components of the composite resin material (Foundation) used in the basic experiments and includes the internal standard at 7.967 min. Peaks generally showed good symmetry, practical retention times (under 40 min) and the buffer used successfully allowed TMA peak appearance within the assay time. CQ,

however, continued to exhibit considerable tailing despite gradient optimization.

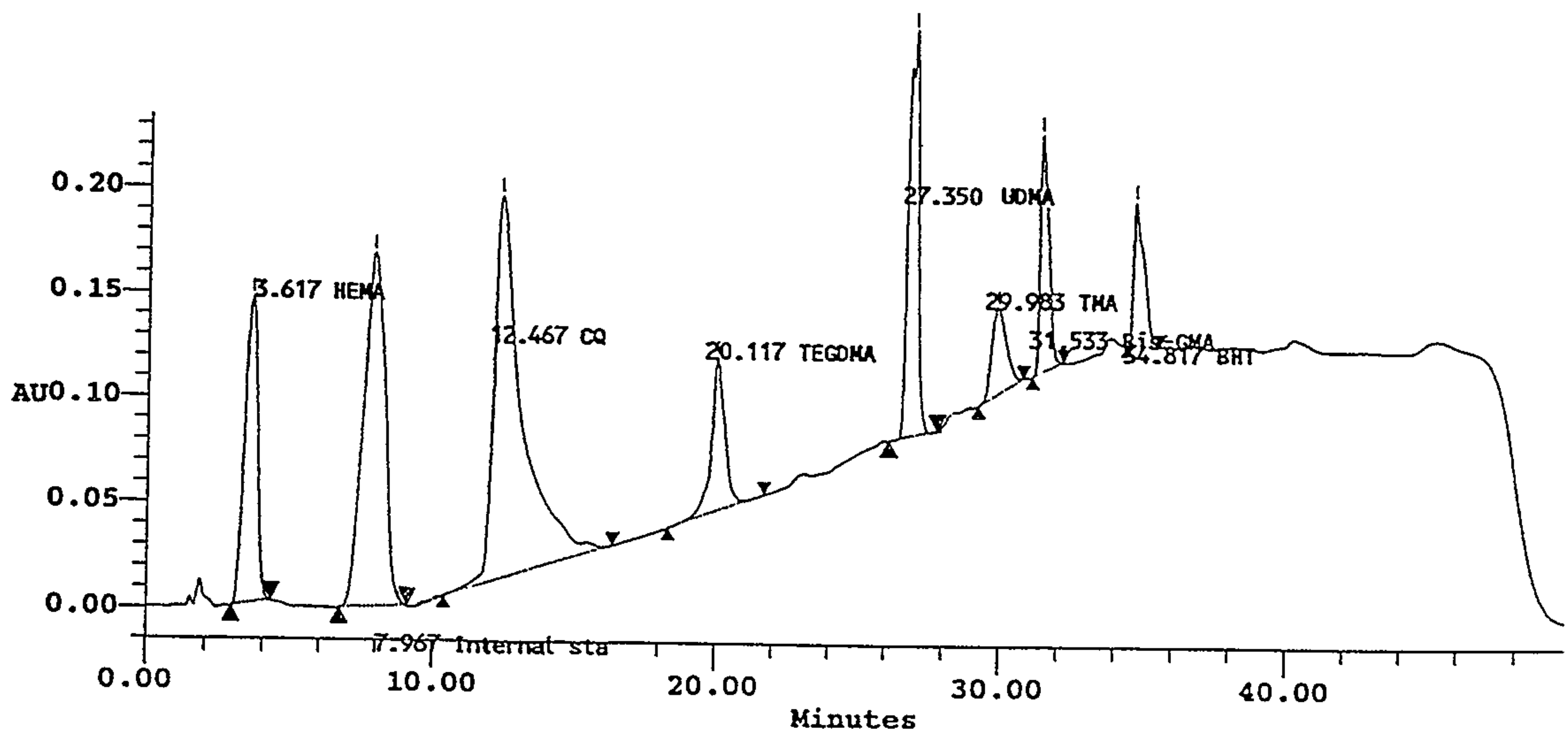
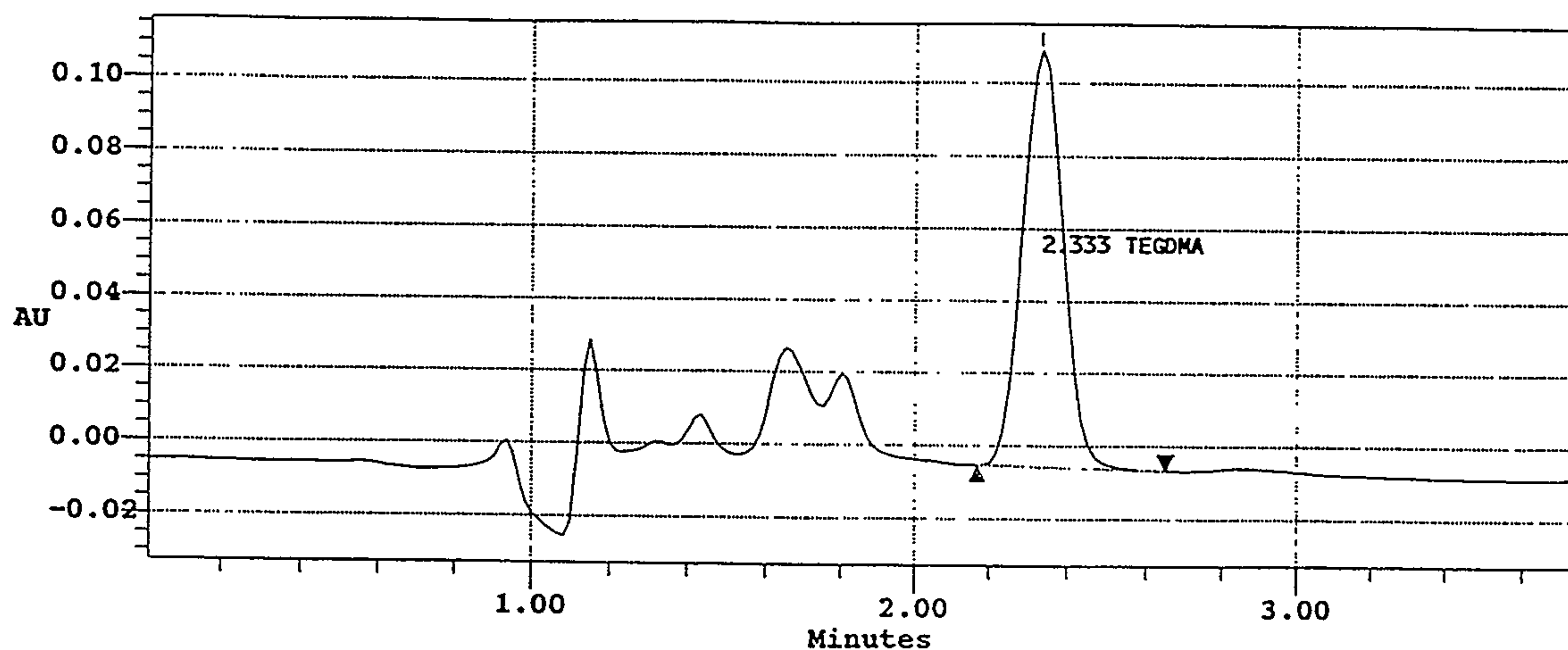


Figure 2.4 Chromatogram of components of a composite resin assayed under reversed-phase gradient HPLC conditions (mixed standard solution)

Fig. 2.5 presents a typical chromatogram assayed under *isocratic* conditions for one of the resin components, TEGDMA. This figure also includes the peak data table taken from that chromatogram. This data includes retention time, area and height of the peak and an indication of the integration parameter applied to the peak, in this case BB, which denotes "baseline resolved" and indicates that the peak start and end was at the baseline and that peak measurements were taken from a tangent uniting these points. The retention time was conveniently short (under 3 min) and the interference peaks were well distinct from the peak of interest at 2.33 min. The absence of baseline drift and optimal peak resolution and shape show this chromatogram to be suitable for measurement and quantitation. The construction of a calibration curve (see Fig. 2.10) from standard dilutions of

peaks of interest assayed under isocratic conditions required the use of peaks of similar appearance and features to that shown in Fig. 2.5.



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1	TEGDMA	2.333	827462	115195		BB

Figure 2.5 Chromatogram of a component of a composite resin, TEGDMA, assayed under reversed-phase isocratic HPLC conditions

2.5.3.3 Validation procedures for HPLC analysis

Component identification by HPLC was carried out by comparing retention times and shapes of peaks in the unknown chromatograms with those of known components in the chromatogram of the mixed standard. The accuracy of this was dependent on chromatographic separations (of both mixed standard and unknown) being conducted under identical conditions so that retention time match fell within ± 0.3 mins. This identification was corroborated by standard addition (see section 2.2.7) or "spiking" the unknown with pure component with more certain identification evidenced by compounding of the unknown peak.

2.5.4 CIMS conditions

2.5.4.1 Operating conditions for CIMS

CIMS conditions used for analysis of the fractions collected from the HPLC at times corresponding to peaks of interest commenced with fraction

concentration under a pure N_2 stream. Analysis by ammonia CIMS used a scan of m/z 50-650 in 1.5 s/decade (V670 Mass Spectrometer, VG Analytical, UK).

2.5.4.2 Spectra for components of composite resin

CIMS spectra were generated for the components of the composite resin used in the current study using ammonia as the reagent gas. By conventions used in MS, "M" refers to the molecule of interest and the protonated condition of this molecule is therefore " MH^+ ". As ammonia was used as the reagent gas, the quasi-molecular ion of M is " $(M+NH_4)^+$ ". An additional fragment, where a water molecule links to the ammonium quasi-molecule or $[M+NH_4]^+-H_2O$, may also occur. The presence of these particular peaks at the molecular weights of these ions confirms molecule identity. As in conventional practice in MS techniques, the spectra have been normalized so that the fragment of greatest abundance is plotted at full scale.

Mass spectra for several resin monomers and oligomers are presented in Figs 2.6, 2.7, 2.8 and 2.9. In each of these figures, the molecular structure of the molecule analysed is also shown and some possible fragment divisions are noted. The presences of these specific fragments in the spectrum, as peaks at these molecular weights, aid in confirmation of the molecule.

In Fig. 2.6, the lower figure shows the spectrum of TEGDMA, the peak at m/z 287.1 corresponds to the protonated ion of TEGDMA, MH^+ , and that at m/z 304.2 is the quasi-molecular ion of TEGDMA with ammonia $(M+NH_4)^+$. Peaks at m/z 69.0 and m/z 113.1 are possible fragments of TEGDMA. Possible fragments are seen at m/z 127.1 and m/z 172.1. Other peaks are likely to be known and unknown contaminants, for example EGDMA (MW=198).

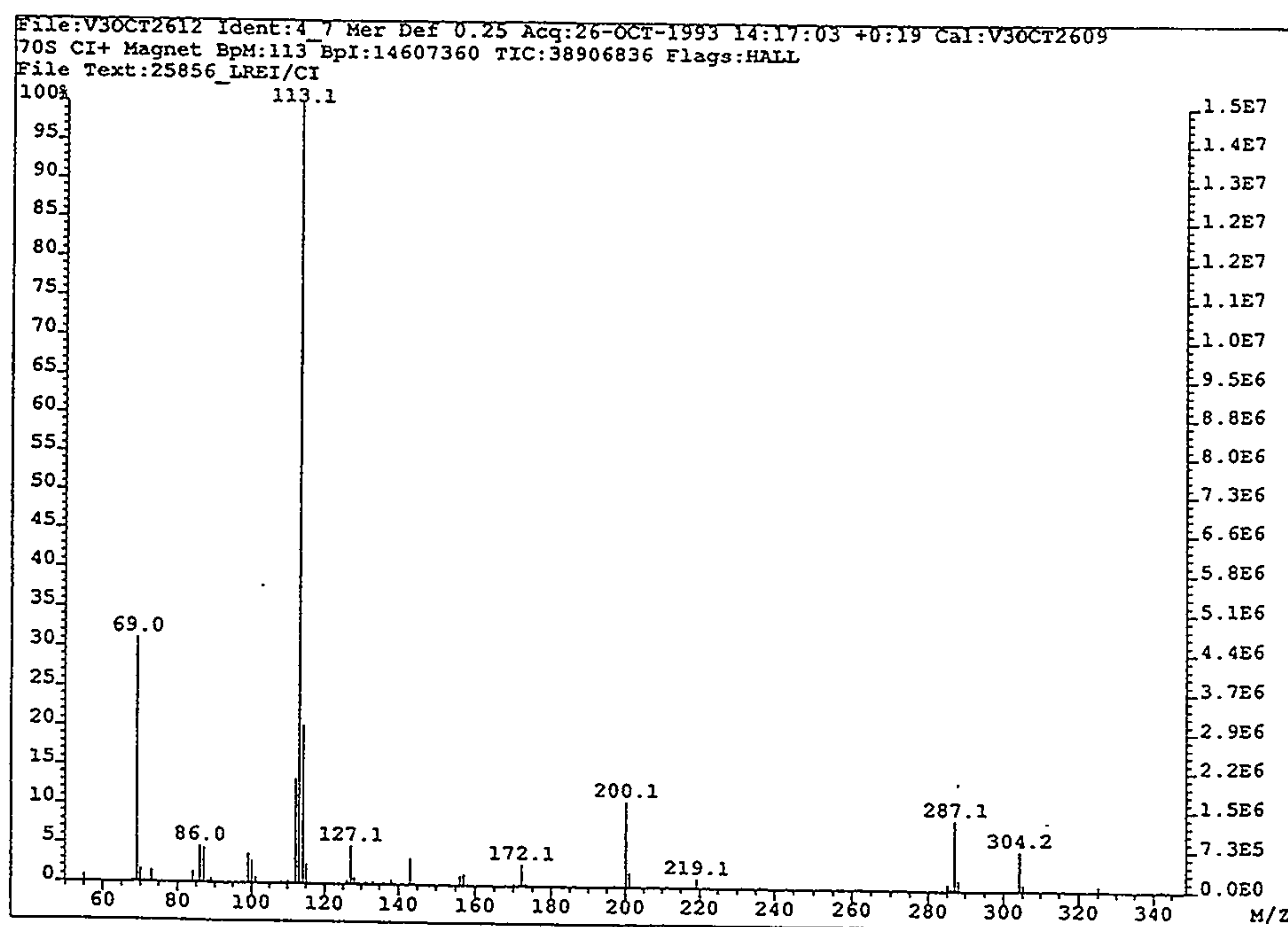
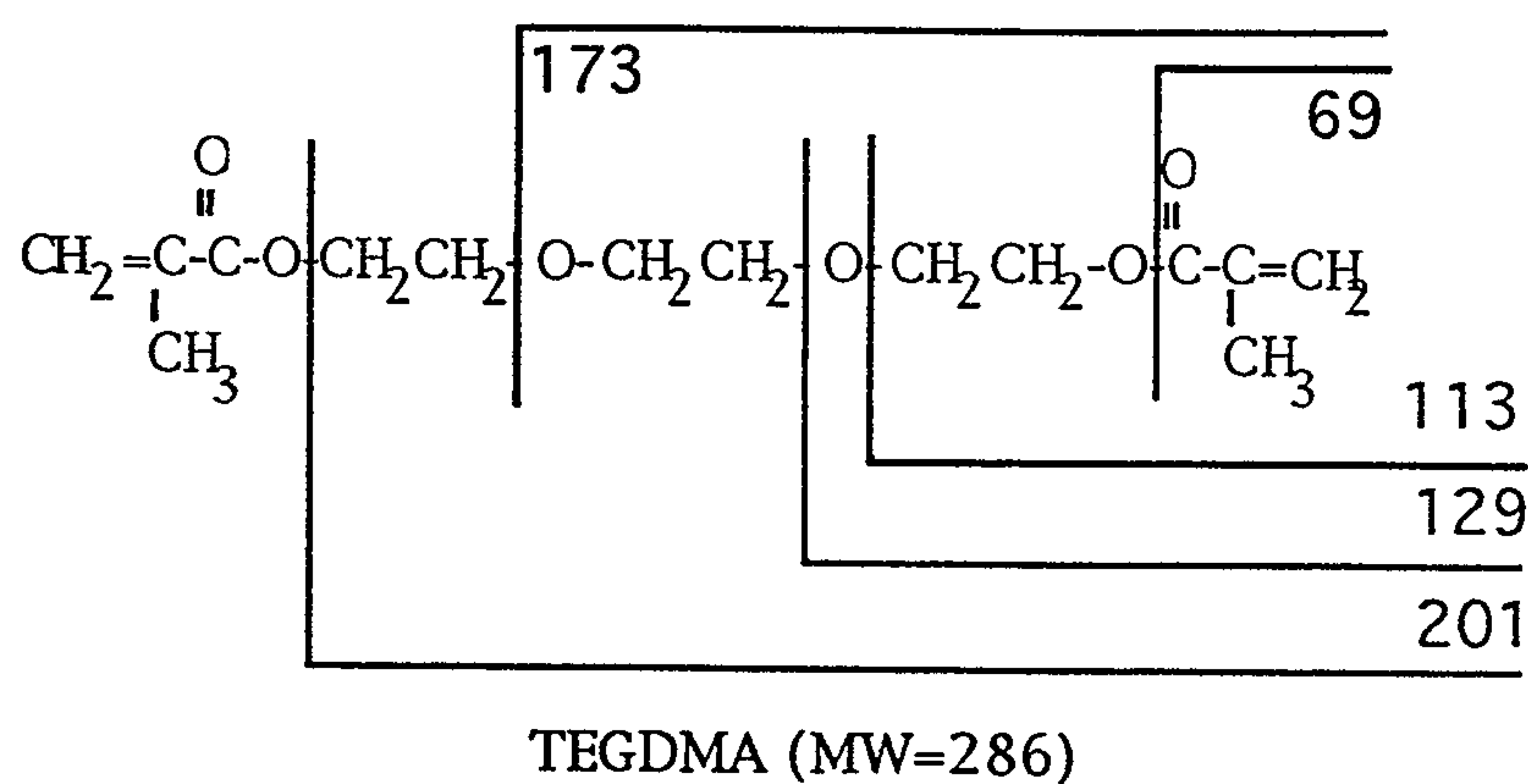


Figure 2.6 Mass spectrum for TEGDMA

In Fig. 2.7, the spectrum for Bis-GMA, numerous peaks are seen. The peak at m/z 530.3 corresponds to $(M+NH_4)^+$ and the peak at m/z 512.2 is probably the ion $[M+NH_4]^+-H_2O$. Fragments of Bis-GMA are seen at m/z 69.0, m/z 113.1 and peaks at m/z 86.0, m/z 131.1 and m/z 161.1 are also possible fragments. The additional peaks are possibly contaminants.

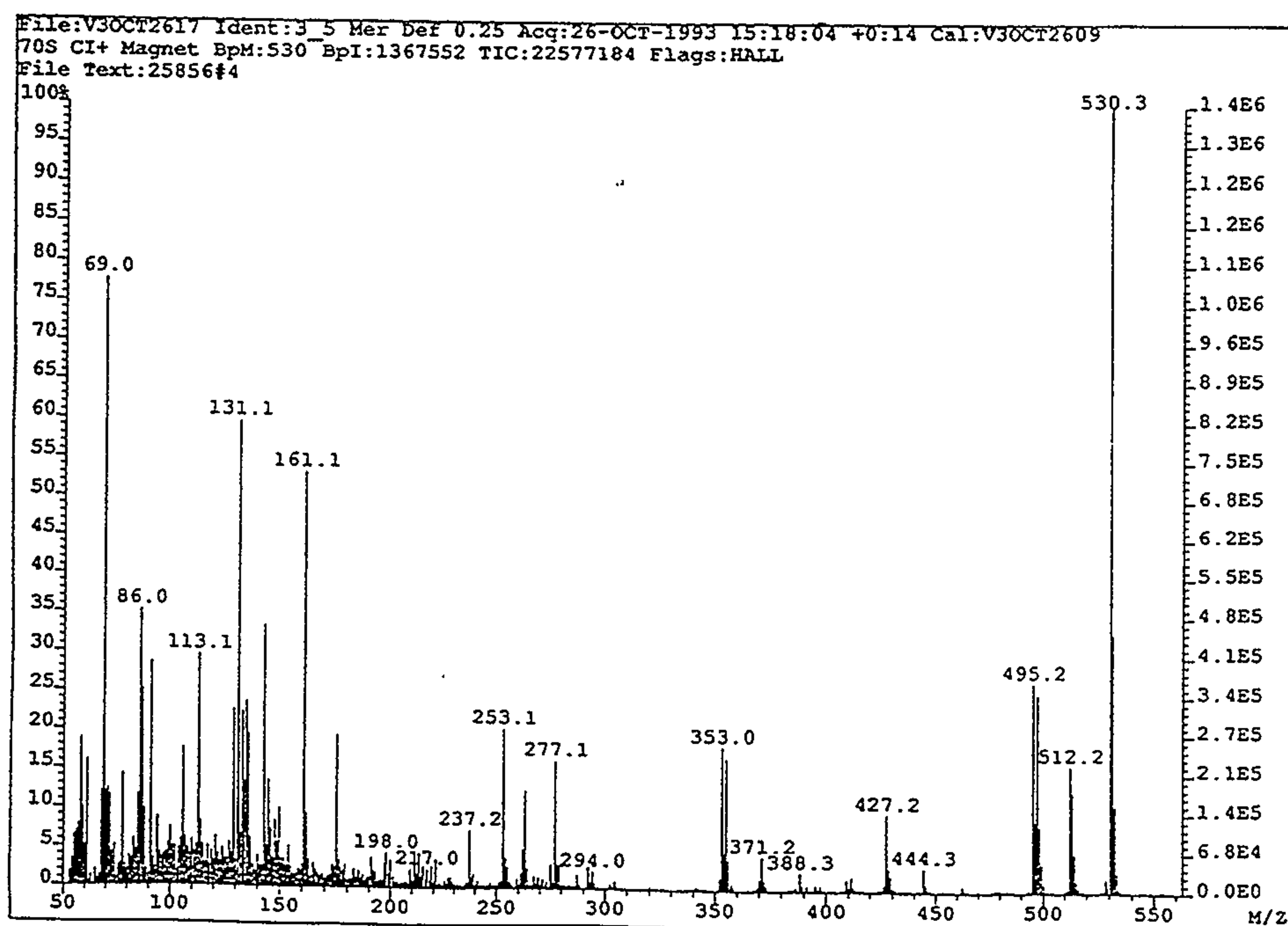
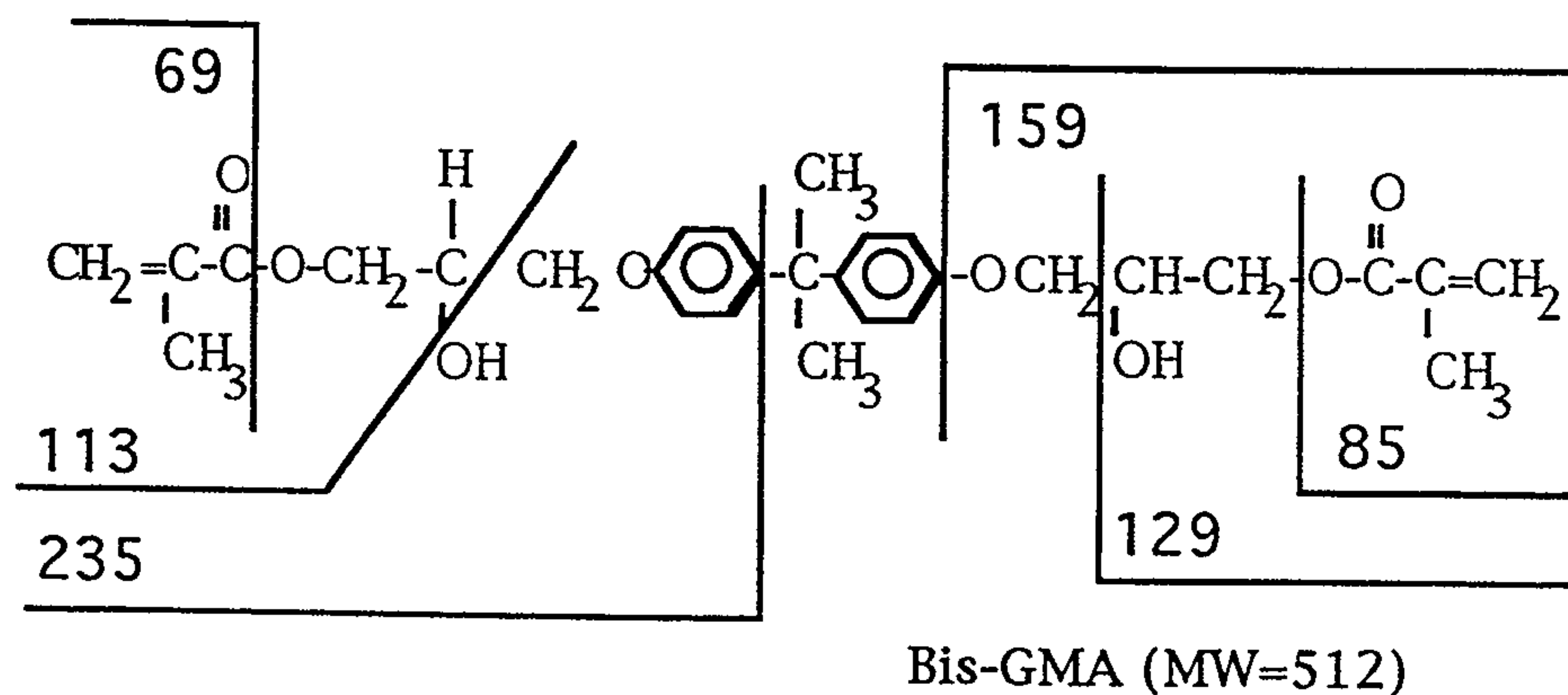


Figure 2.7 Mass spectrum for Bis-GMA

Figs. 2.8 and 2.9 present the spectra for HEMA and UDMA respectively. In Fig. 2.8, the MH^+ ion is shown at m/z 131.1 and the peak at m/z 148.1 corresponds to $(M+NH_4)^+$. The peak at m/z 113.1 is likely to be the MH^+-H_2O fragment. Another possible fragment appears at m/z 100.1.

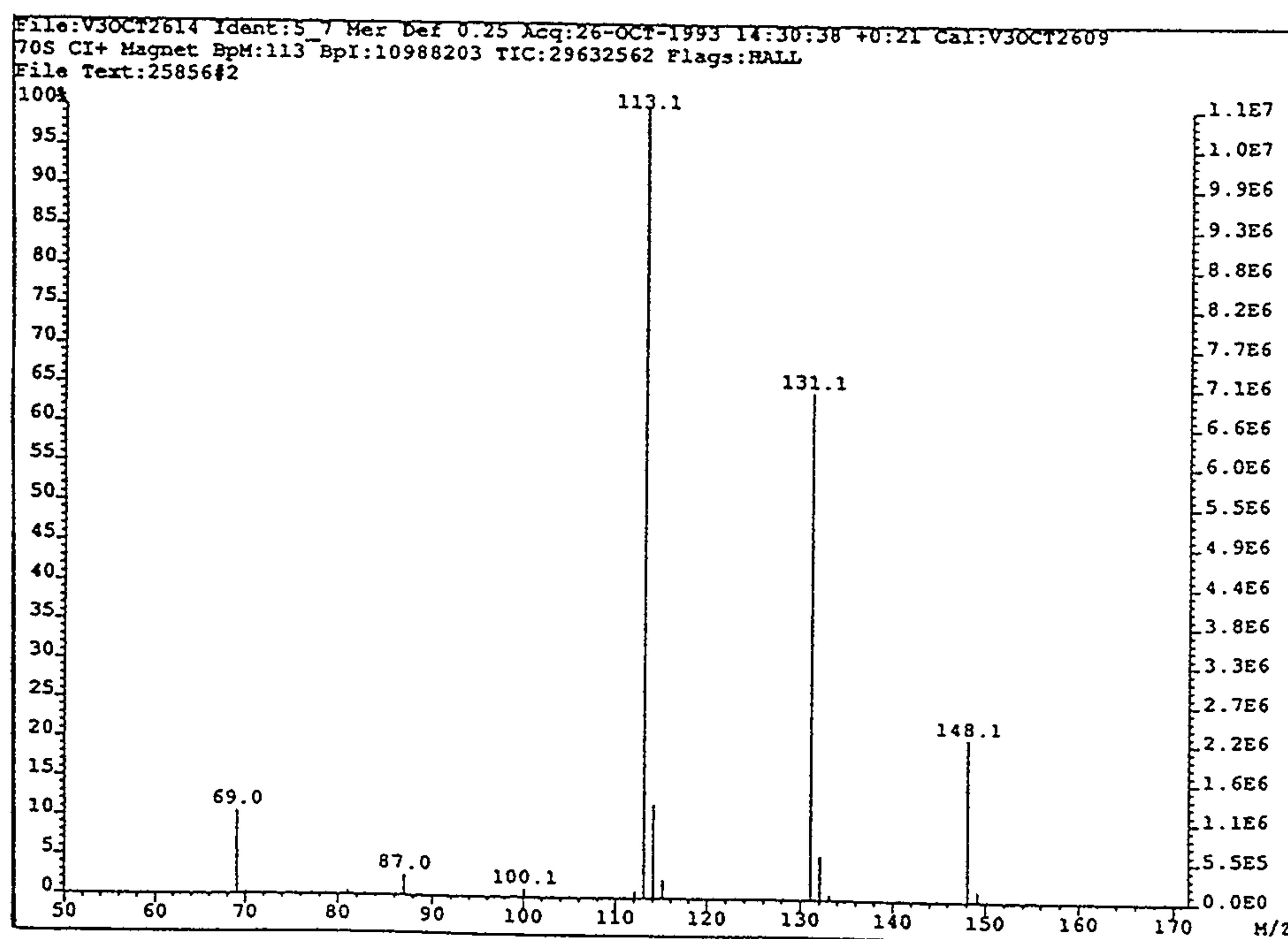
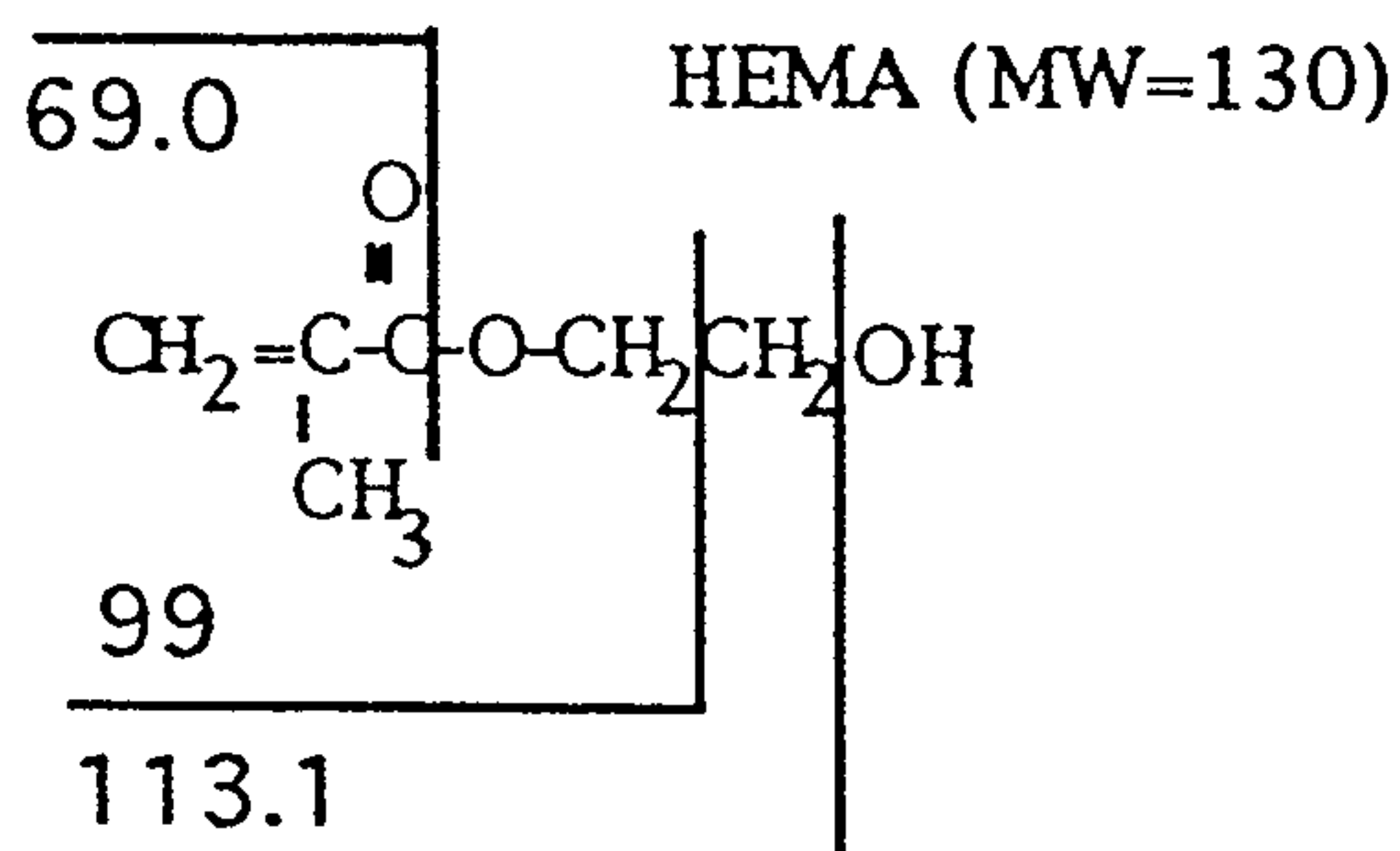


Figure 2.8 Mass spectrum for HEMA

In Fig. 2.9, the spectrum of UDMA, the peak at m/z 471.3 corresponds to MH^+ and that at m/z 488.3 to $(M+NH_4)^+$. Other likely fragments are also present, at m/z 69.0, m/z 185.2, m/z 228.3 and m/z 255.2.

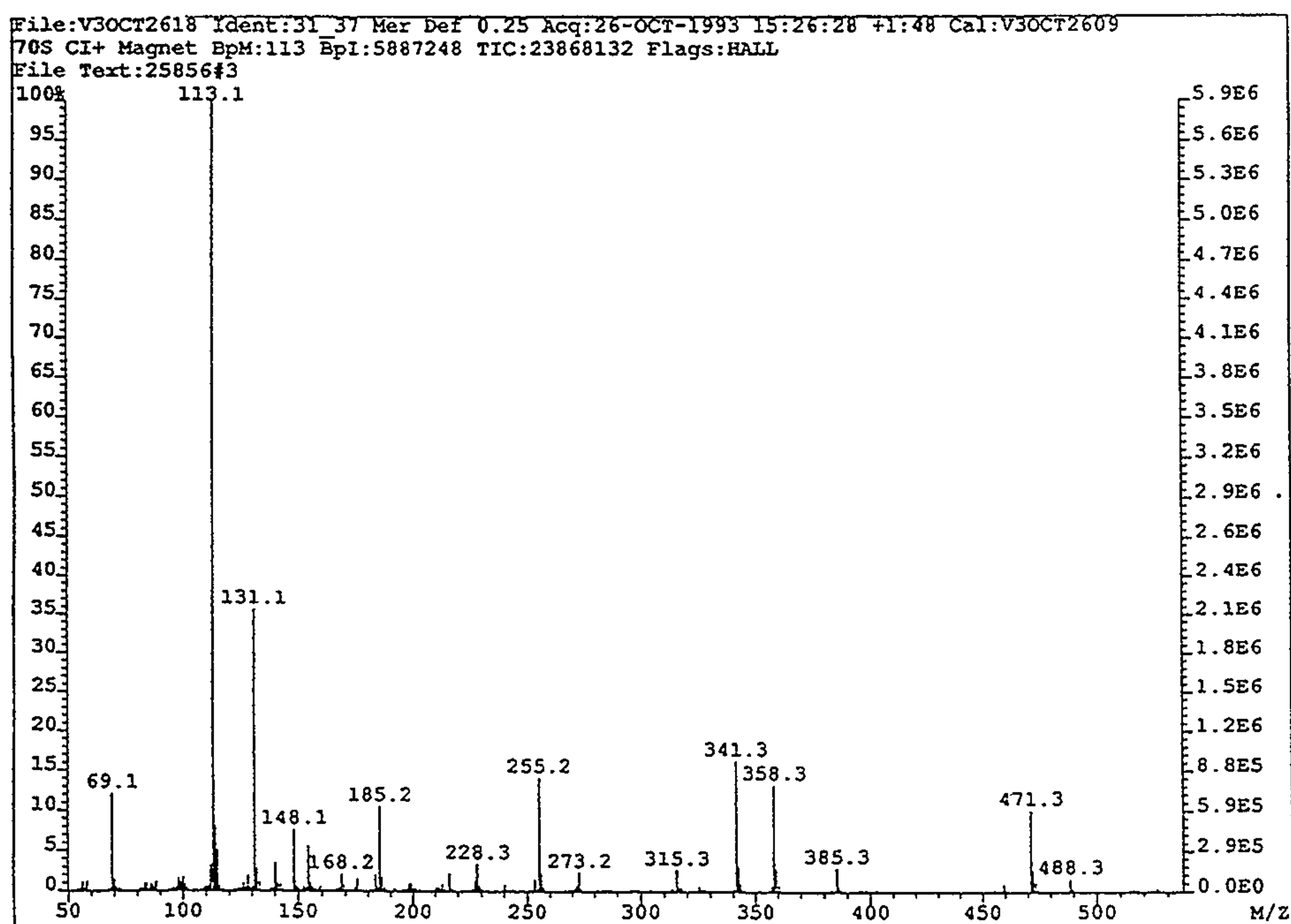
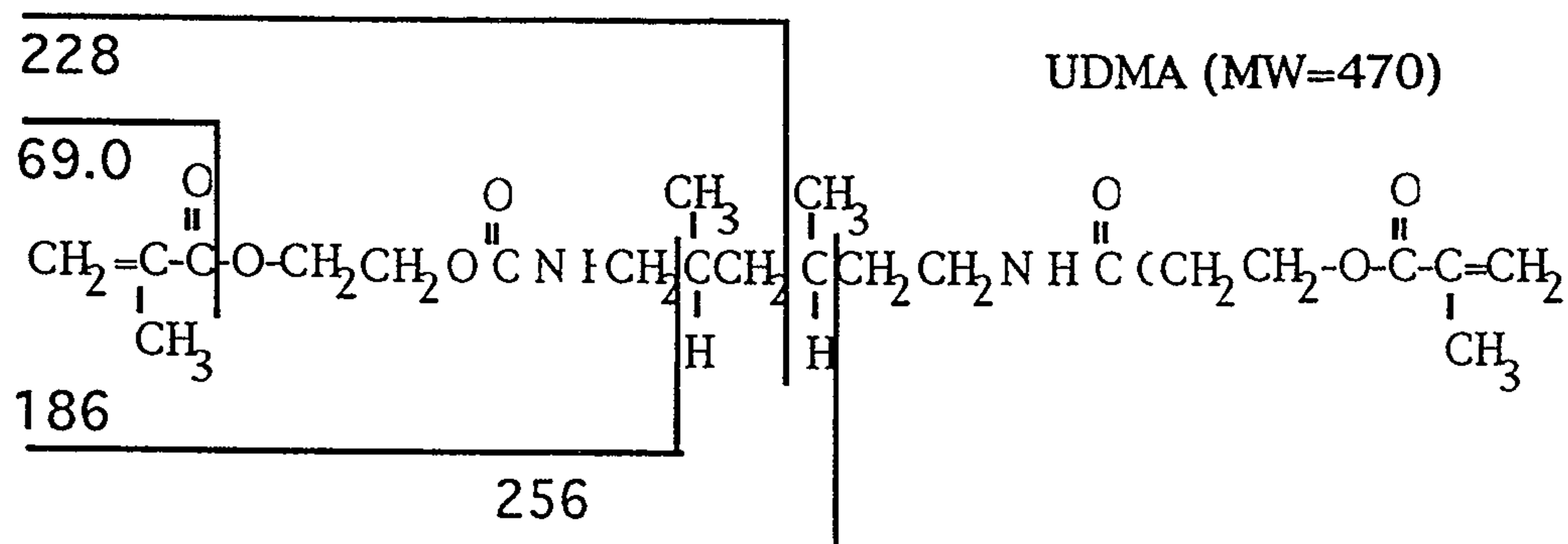


Figure 2.9 Mass spectrum for UDMA

Some common fragments occur in the four spectra presented. The peak at m/z 69.0 is the methacrylate ion, a reaction component for the synthesis of methacrylates and some portion probably persists as a contaminant in each of the monomers and oligomers. Ethyl methacrylate (MW=114) may be a degradation product of methacrylate monomers and may be similarly present as a contaminant. Peaks occurring elsewhere correspond to possible fragments: methacrylic acid (MW=86) and methyl methacrylate (MW=100) which corresponds well to NMR-analysis of composite resins (Asmussen, 1975a). By matching these unique spectra with those found for the sample the presence of corresponding peaks confirms identification.

2.5.5 HPLC and MS tandem analysis in current work

2.5.5.1 Experimental method of analysis

All equipment was routinely checked and appropriately equilibrated, prior to each day's analyses, until reproducible running of the equipment was established and chromatograms of injectionless assays under gradient conditions showed minimal baseline noise. Standards solutions and their dilutions were then assayed under isocratic HPLC conditions to establish calibration curves. Assays of control samples from each series of sample established that peaks of interest were real. The consistent appearance and symmetrical shape of the internal standard peak confirmed the reproducibility of the system.

Samples were assayed under gradient conditions and chromatograms from these analyses were compared to that of the mixed standard solution (containing all known resin components) to establish which components were present in the sample by retention time match. If the chromatogram showed obvious distortion at the retention time of the peak of interest, the sample was at first re-assayed to achieve uniform peak shape and retention time-match within $\pm 0.5\text{min}$ or another reason for the poor chromatographic appearance was investigated. Fractions were collected from the HPLC exiting at the retention times of peaks of interest and stored in individual polyethylene vials for preparation prior to mass spectrometric analysis. The fractions in vials thus consisted of approximately 1 ml of solvent containing an unknown amount of one eluted component. A slow stream of pure nitrogen was then continuously passed over each fraction to volatilize excess solvent and concentrate the contained sample which was

then sealed and submitted for analysis by CIMS. Samples of pure resin components were also submitted for CIMS standard preparations.

2.5.5.2 Quantitative analysis of experimental samples

Milne (1971) suggested that mass spectroscopy plays a crucial but supporting role to gas or liquid chromatography. The mass-spectrometric monitoring of the chromatogram is currently amongst the most accurate ways to identify compounds emerging from the chromatograph, but once this confirmation is achieved, HPLC retention time can be used to practically "identify" compounds.

In the current experimental work, CIMS data were used to confirm the identity of the peaks of interest in the collected fractions. This data confirmed the inferred identification completed by standard addition in HPLC assays. Peaks of interest in all unknown samples were then assayed under isocratic reversed-phase HPLC analysis and quantitated using the calibration curve established that day. HPLC assay series run beyond one day necessitated the preparation and assaying of a freshly prepared set of standard dilutions and the generation of a new calibration curve to allow valid quantitation. Similarity amongst different calibration curves for each component was checked and only when they were consistent were they used for quantitation. Chromatograms of standard dilutions and their associated calibration curves were stored to allow later recall during unknown quantitation. A typical calibration curve generated for TEGDMA is shown in Fig. 2.10.

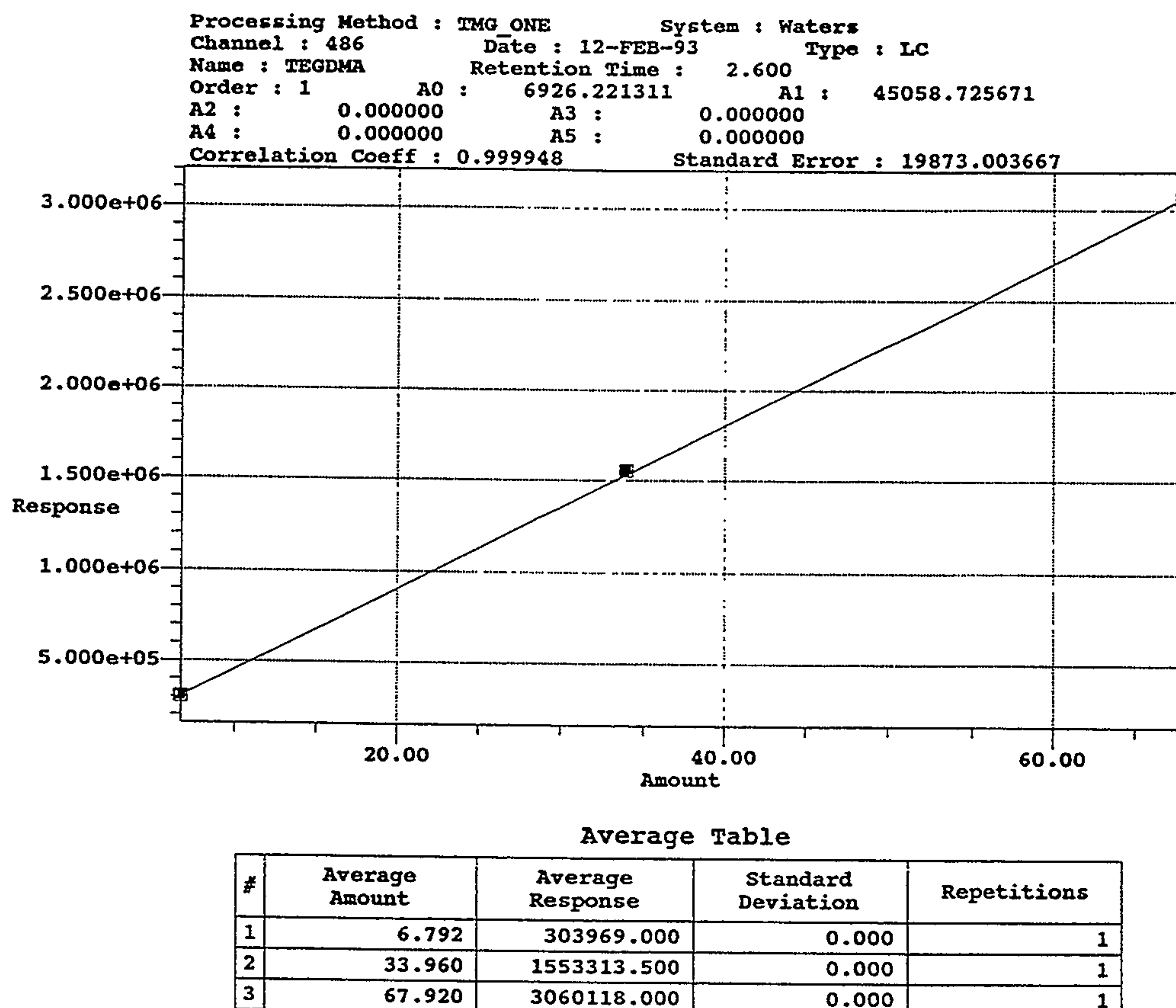


Figure 2.10 Calibration curve for the resin component TEGDMA

This calibration curve, generated from data for several different concentrations of the prepared standard solutions, can be applied to the quantitation of unknowns because it exhibits features which indicate its validity. These features include linearity, indicating a consistent response/concentration ratio of standards dilutions, a correlation coefficient close to a value of 1 and the curve approximately transects the origin. Calibration curves were, at first, generated for all components. Pilot study data, however, showed this not to be a routinely necessary procedure as not all the components were found in the unknown samples. Henceforth, calibration curves were generated for only the components which were identified in the unknown samples and for the internal standard.

Reproducibility of quantitation was validated using both retention time match and standard addition methods of validation. Valid chromatograms of

standard dilutions and for all unknowns and controls assayed under identical isocratic reversed-phase HPLC were stored as a file on the computer hard disk and integrated in the same way for that day's samples. All controls and standard chromatograms were processed first and validated, then all unknowns were similarly processed. Chromatograms for controls and standards for each day of analysis were stored on computer and matched to similar data for following days to monitor reproducibility of the analytical technique.

Each chromatogram from each series was imaged on the computer and initial peak detection by retention time was generated. Each peak of interest was then integrated manually on the computer screen by establishing the location of the baseline, setting peak starts and ends for the peaks of interest and disabling computer-driven integration for other areas of the chromatogram not of interest. These data were then used to determine the amount of the identified component (represented by the peak in the chromatogram) in the injected sample. Quantitative data generated were stored and retrieved as needed.

The following Chapter will describe the application of this analytical technique to the analysis of water elution samples from composite resins.

CHAPTER 3

Component Release from Composite Resin into Water

3.1 Introduction

3.2 Basic protocol

- 3.2.1 Materials
- 3.2.2 Experimental procedure
- 3.2.3 Analytical method
- 3.2.4 Data management
 - 3.2.4.1 Data conversion
 - 3.2.4.2 Quantification
 - 3.2.4.3 Statistical analysis
 - 3.2.4.4 Presentation of results
- 3.2.5 Results
 - 3.2.5.1 Component identification
 - 3.2.5.2 Component quantification
- 3.2.6 Discussion

3.3 Effects of variation in thickness on release

- 3.3.1 Materials and methods
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- 3.3.3 Discussion

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3.5 Effects of air-inhibition of the composite resin surface layer on release

- 3.5.1 Materials and methods
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3.6 Effects of variation in state of cure on release

3.6.1 Uncured

3.6.1.1 Materials and methods

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3.6.1.3 Discussion

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3.6.2.1 Materials and methods

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3.6.2.3 Discussion

3.7 Effects of variation in temperature on release

3.7.1 Materials and methods

3.7.2 Results

3.7.3 Discussion

3.8 Release of components from various composite resins

3.8.1 Materials and methods

3.8.2 Results

3.8.3 Discussion

3.9 Summary of TEGDMA release results from Foundation

3.10 General discussion of TEGDMA release from composite resin

3.1 Introduction

In order to examine the hypothesis that components of composite resin may interact with dentine, it was necessary to first investigate the potential of components to diffuse out of composite resin into aqueous solution under controlled conditions. The release of components, from one formulation of cured composite resin into water, was therefore examined by analysis of the water sample by high performance liquid chromatography/mass spectrometry (HPLC/MS). It was then relevant to examine the release with respect to the physical variables thickness and surface area of the same composite resin, ambient temperature during the experiment, air-inhibition of the surface during cure and state of cure. These variables were examined by varying the basic protocol. The release of components from other composite resin formulations was also examined. Data related to the components detected in the following experiments are described as **component release**. In a subsequent Chapter, the influence of dentine on this release, which will then be termed the **component diffusion**, will be described.

3.2 Basic protocol

3.2.1 Materials

The composite resin examined in the basic protocol was a Bis-GMA-type resin, Foundation, manufactured by Nulite Systems International (Hornsby, NSW, Australia). Syringes containing 3 g of shade A3 Foundation were used (Batch No. 1023). The components listed by the manufacturer and the acronyms used in the text are shown in Table 3.1.

Table 3.1 Components of composite resin material examined, Foundation

2,2-bis[4-(2-hydroxy-3-methacryloyloxy)phenyl]propane	Bis-GMA
triethylene glycol dimethacrylate	TEGDMA
di-2,3-di-keto-1,7,7-trimethylnorcamphane	CQ
2,6-di-tert-butyl-4-methylphenol	BHT
N,N,3,5-tetramethyl aniline	TMA
Sub-micron silica glass particles	
Precipitated particles	
Fibres	
γ -methacryloyloxypropyltrimethoxysilane	

3.2.2 Experimental procedure

A protocol was created which formed the basis for all subsequent release experiments, to be known as "basic protocol". The protocol consisted of experimental materials, procedure and the sampling and analytical methodology which allowed the monitoring of the release of components over time from a known quantity of composite resin into a known volume of water. Variations to this basic protocol are described in subsequent sections.

A cylindrical, stainless steel mold (Symes Metals, Newtown, NSW, Australia) into which composite resin could be placed was designed (Fig. 3.1, upper diagram). Internal dimensions of the mold were 6 mm diameter and 2 mm depth.

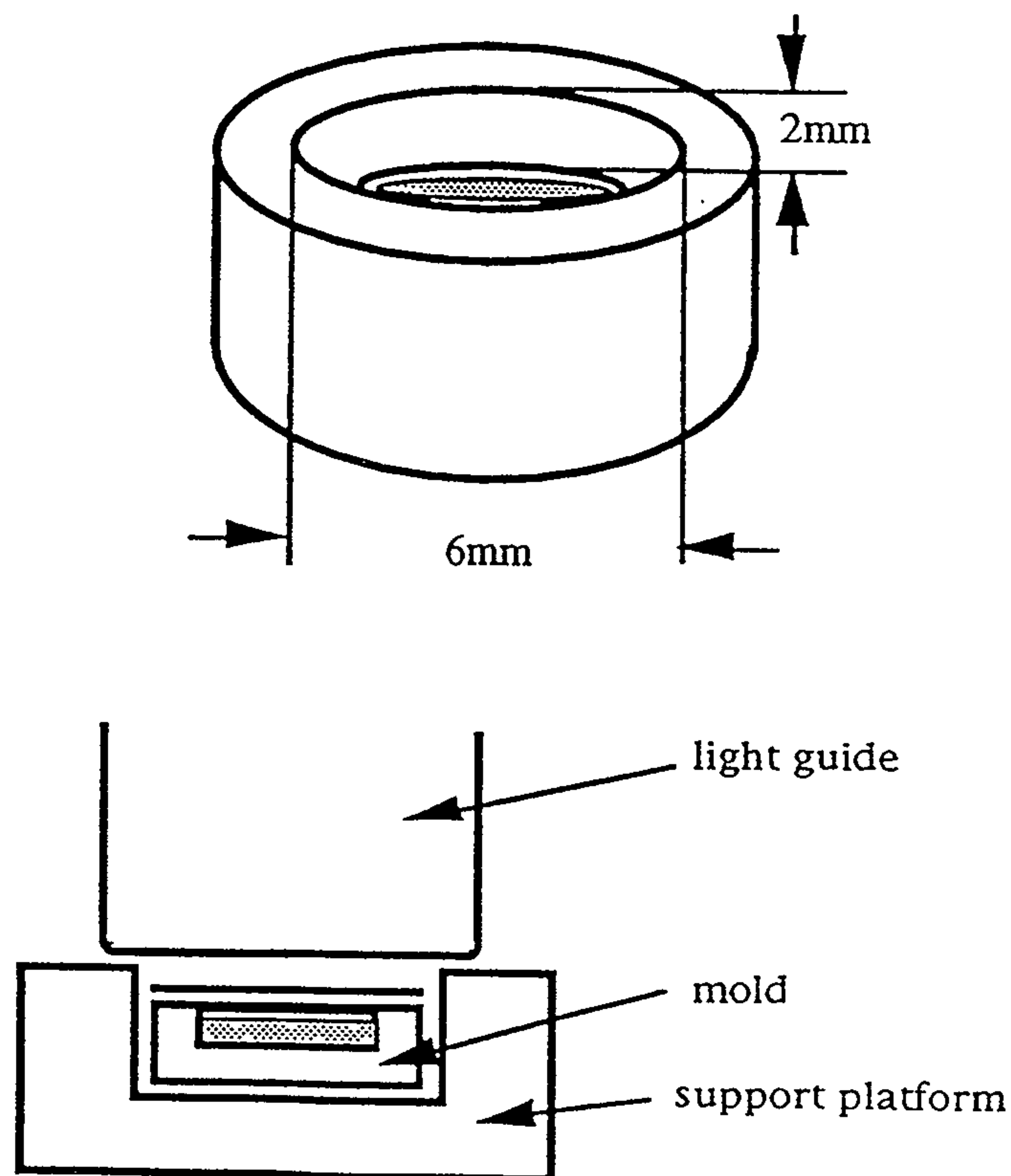


Figure 3.1 Mold and physical arrangement for light-activation of composite resin

Identical glass vials, with polyethylene lids, were used to contain the filled molds and water sample throughout the course of the experiment. These vials provided a convenient, reproducibly-sized container for the sample and mold and enabled easy mold transfer and sample retrieval. Double distilled type 1 water was used as the sample medium (MilliQ RO6 plus, MilliQ Plus, Millipore Corporation, Waters Chromatography Division, Milford, MA, USA). 4 ml of water was placed into each vial immediately before placement of the resin-filled mold and commencement of the experiment.

A strand of dental floss (Johnson and Johnson, New Jersey, USA) was attached to the circumference of each mold (n=49) with sticky wax (Associated Dental Products Ltd., Kemdent Works, Purton, Swindon, UK). The loose end of this floss was attached to a vial lid, through a small hole, using sticky wax. The floss length was adjusted to allow the mold to be suspended above the bottom of the internal floor of the vial. Following loading of uncured composite resin into the mold, a polyester matrix strip ("Melinex", De Trey, Surrey, England) was placed over the composite resin. The composite resin was then cured for 30 s, at $22\pm 1^{\circ}\text{C}$, using a dental activating visible light (Optilux 400, model 401, Demetron Research Corporation, Danbury, CN, USA). Standardizing of the distance from the light guide to the composite resin (3 mm) was achieved by using a recessed platform which held the mold for curing and supported the light guide for activation (Fig. 3.1, lower diagram). It is acknowledged that in the use of a stainless steel mold, no effective bonding occurred between the composite resin and the internal mold walls. It is therefore inevitable that shrinkage of the composite resin away from the mold walls, which would have occurred during polymerization, would create a gap surrounding the composite resin which would fill with water during the experiment. It can be suggested, therefore, that in addition to the top surfaces of the composite resin confined in molds, the vertical sides of the composite resin sample would also be associated with component elution. Any variation in the dimensions of the composite resin, as occurred when thickness and diameter were varied, would influence overall component elution. It is noted, however, that the relative contribution of these areas of the composite resin to the overall elution observed was not able to be discerned in the experiments.

After curing and removal of the matrix, the mold containing the composite resin was immediately lowered into a vial containing 4 ml of water and stored at 37°C until the end of each interval. The mold was then transferred

to a new vial containing fresh water and then returned to the 37°C incubator. The liquid sample from which the mold had been removed was then stored at 4°C until it was analysed. In this way, a total of 10 samples were obtained after the following intervals: 4.32, 14.4, 43.2, 144, 432 min and at 1, 3, 10, 30 and 100 days representing convenient logarithmic divisions of a day. Thus, for example, the sample obtained after the first interval contained all the components eluting from the material 4.32 min after the mold was placed into the water. The second sample contained all the components eluting over the next interval, that is, from 4.32 to 14.4 min. Experimental and chromatographic controls were used. Water samples from a series of the basic protocol without composite resin were used as experimental controls. Chromatographic controls included injectionless runs (to establish consistency of system disturbances) and runs of fresh water and standard solvents (to establish presence of impurities). A flow chart of the experimental sequence is presented in Fig. 3.2.

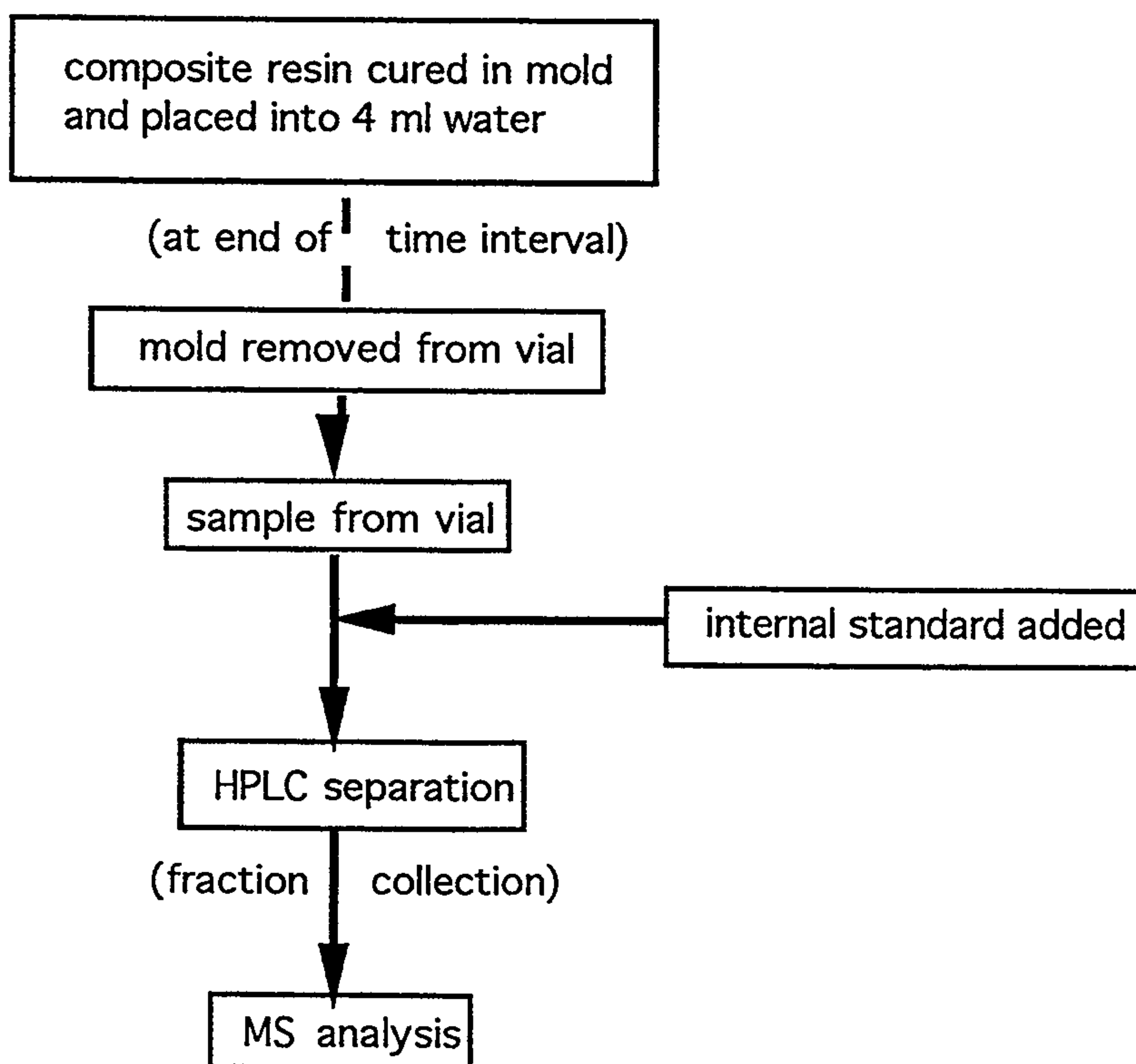


Figure 3.2 The basic procedure for the component release experiment

3.2.3 Analytical method

Each water sample was filtered through a 0.22 µm PVDF filter (Millex GV poly vanillidine difluoride, Millipore, Milford, MA, USA), and a known

amount of internal standard was added. Each filtered sample was then transferred to 1 ml or 200 μ l vials for placement in the autoinjector of the HPLC unit. An injection volume of 200 μ l was subjected to separation by reversed-phase HPLC. Both isocratic and gradient conditions were determined for HPLC analysis as described in Chapter 2 and were shown in Table 2.5.

Composite resin components of Foundation used to prepare chromatographic standards were all approximately 99% pure as supplied and reported by the manufacturer (see Table 2.2). Additional supplies of other components as required were obtained from Sigma Chemical Company (Sydney, NSW, Australia) and ICN Biomedicals (San Francisco, CA, USA).

Validation tests of reproducible operation of the HPLC equipment were carried out. System chromatograms, and those of injectionless assays, confirmed the absence of peaks which could confuse chromatogram interpretation. Following separation of the unknown sample into its components, the individual components exited the detector of the HPLC at their characteristic retention times. In order to obtain each of the separated components for subsequent CIMS analysis, a small volume of solvent (approximately 1 ml) was collected from the detector at the retention time of each component. These volumes or "fractions" contained only the separated component dissolved in mobile phase. Each fraction was collected into a clean, polyethylene centrifuge tube, concentrated under a nitrogen stream to reduce the volume to approximately 500 μ l and then immediately sealed and stored at 4°C until analysed. Confirmation of component identification by HPLC was provided by CIMS analysis performed at the Mass Spectrometry facility at the University of California, San Francisco USA. Similar samples were collected in clean glass vials, identically treated and submitted for MS analysis. Analysis of samples submitted in polyethylene vials were not different from these glass vial samples and so all further sampling was completed using the polyethylene vials.

3.2.4 Data management

Quantities of identified component present in each unknown were calculated using peak height-amount ratios obtained from a series of individual standards used to generate a calibration curve. Heights of peaks

of identified components in the unknown were fitted to the calibration curve to derive amounts. A calibration curve was also generated for the internal standard to establish the consistency of chromatographic performance.

Data processing and calculation from chromatograms were completed using Millenium 2010 software Version 1.10 provided as part of the Millenium Chromatography Manager Processing package (Millipore Corporation, Waters Chromatography Division, Milford, MA, USA). This processing provided the quantity of each component found in the injection volume used for each unknown. From this, the quantity of components in the entire sample volume was determined. These data were then entered into a spreadsheet program (Microsoft Excel version 4.0, Soft-Art, Inc. Berkeley, CA, USA) and imported into a statistical program (StatViewII 512+, Abacus Concepts Inc, Berkeley, CA, USA).

3.2.4.1 Data conversion

Chromatogram integration and processing provided the quantity, in μg , of each component present in each injection volume introduced onto the HPLC column. By simple calculation this quantity was converted into the amount present in the entire sample volume and then converted into a concentration in $\mu\text{g/ml}$. Concentrations were then converted into moles released.

3.2.4.2 Quantification

The accumulation of components over the time course was calculated by addition of the amounts (nmol) detected in each interval. Thus, the cumulative amount detected at 1 day equalled the addition of all amounts up to 1 day for that component.

Rate of release was calculated per collection interval, so that the rate recorded at 1 day was the average rate of release over the interval 432 min to 1 day after commencement of the experiment. An assumption that release was constant over the time interval has been made when determining the rate of release.

3.2.4.3 Statistical analysis

Means plus or minus standard deviations about the means for cumulative amounts and rates of release were derived. Assumptions of normality of the

data were checked and only outliers due to experimental error, such as vial breakage, were excluded. Comparisons among data at different time points for each group were made using one factor ANOVA-repeated measures and Scheffé statistical tests at $p < 0.05$.

3.2.4.4 Presentation of results

Graphic presentations of cumulative release and release rate data are presented for each of the experiments described. Time course, as the axis, is presented as $\log_{10}(\text{day})$. For cumulative release data, the abscissa is plotted in nmol and for the release rate data a logarithmic transformation is presented, $\log_{10}(\text{nmol/min})$. Results for each variation of this basic protocol considered are presented in subsequent sections, as each variable is described.

3.2.5 Results

3.2.5.1 Component identification

An examination of the control chromatograms confirmed that there were no significant peaks at the retention times of known standard components that would interfere with interpretation of the chromatograms of the unknowns. A typical chromatogram of an unknown sample removed after 1 day is shown in Fig. 3.3. Comparisons of chromatograms of unknowns with that of the mixed standard (Fig. 2.2) shows that only one component of the composite resin was consistently detectable in the samples, the peak at 20.117 min. This component corresponded to the peak retention time of the diluent monomer, TEGDMA. This was corroborated by both retention time match over duplicate separations and by "spiking" selected unknowns with pure component as described in Chapter 2. In some unknowns, other peaks were present but these were small, inconsistently present and were not able to be reliably integrated. All other components were below the valid detection level of the HPLC. The consistent appearance of the internal standard confirmed that conditions of the experiment and of analysis were reproducible.

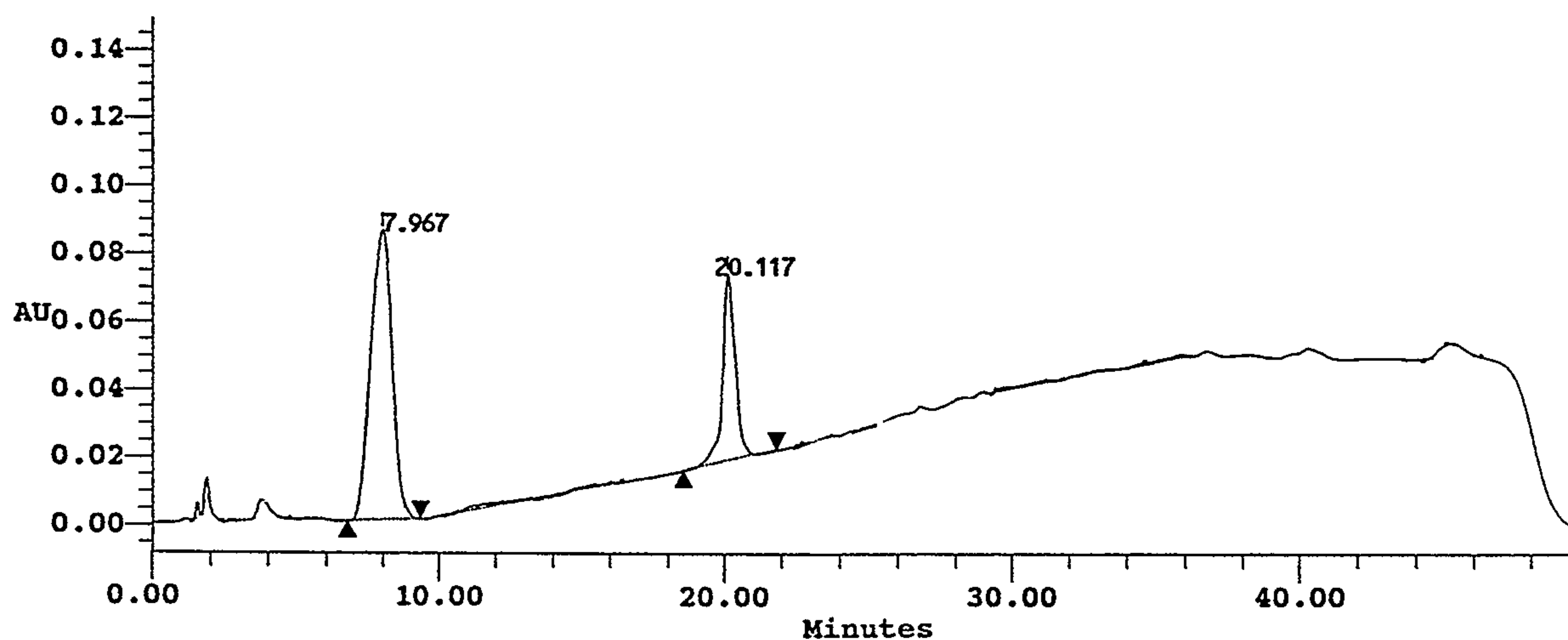


Figure 3.3 Chromatogram of a typical unknown (taken at 1day)

The HPLC chromatogram of an unknown sample showed no other notable peaks apart from those which matched the retention times of TEGDMA and the internal standard. A fraction was therefore collected exiting the HPLC detector at the peak retention time of TEGDMA and submitted for MS analysis. A typical mass spectrometric scan of a fraction collected corresponding to an unknown is shown in Fig. 3.4. The spectrum features several abundant ions, with molecular weights identical to those noted in the MS spectrum of "pure" TEGDMA that was shown in Fig. 2.6. The protonated TEGDMA molecule is seen at 287.2, the quasi-molecular ion of TEGDMA with ammonia is seen at 304.2 and the contaminant is again seen at 113.1. An additional contaminant is seen at 61.0. The two spectra are sufficiently similar to confirm that TEGDMA was the principal component present in the unknown.

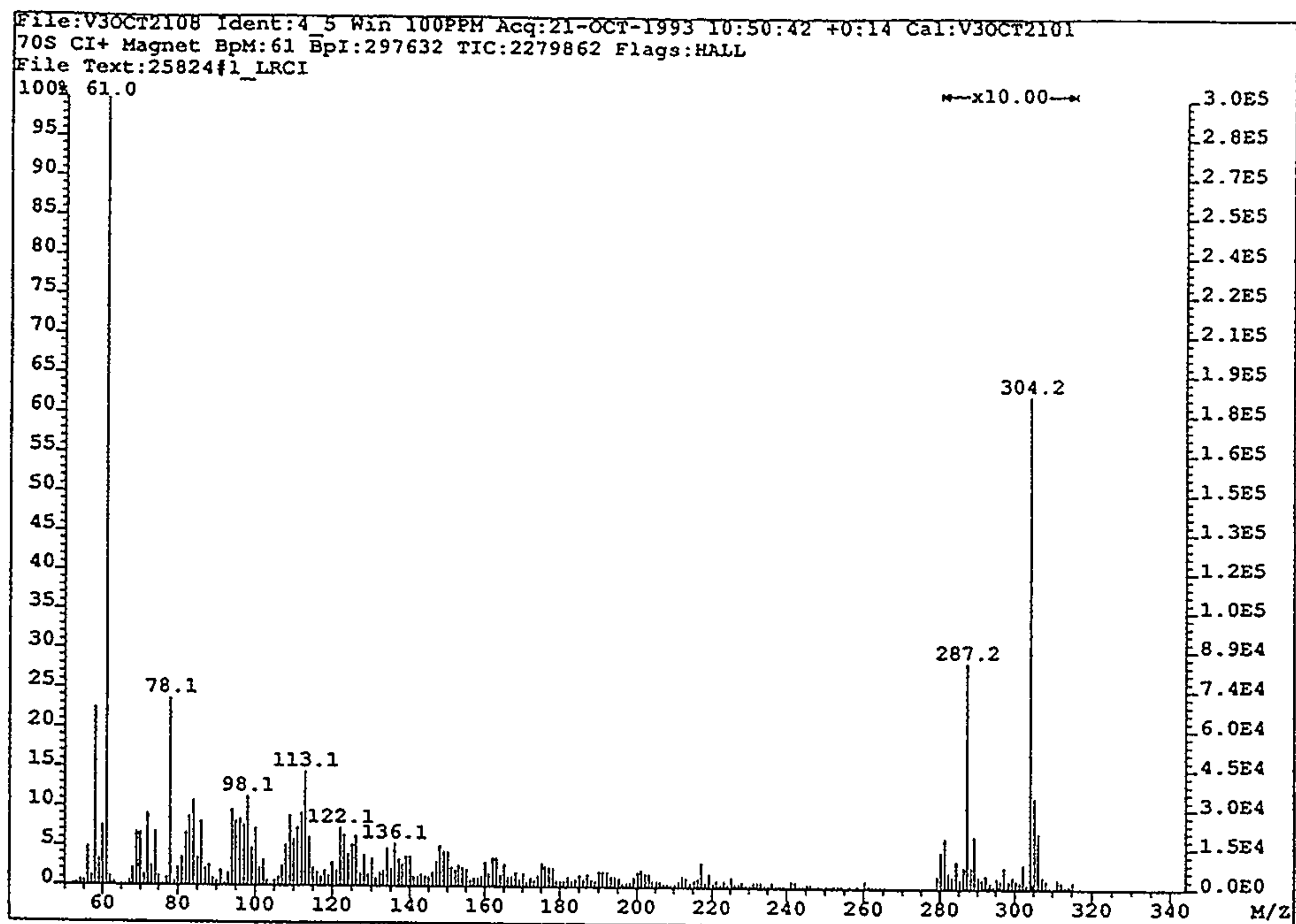


Figure 3.4 Mass spectrometric scan of a typical unknown
 (taken at 1 day)

3.2.5.2 Component quantification

The cumulative release of TEGDMA from Foundation is shown in Fig. 3.5. The rates of release of TEGDMA from Foundation over the time course is presented in Fig. 3.6. A common logarithmic time scale is used on the abscissa so that " $\log_{10}(-2.5)$ day" is equivalent to 4.32 min, " $\log_{10}(-2)$ day" is equivalent to 14.4 min, " $\log_{10}(-1.5)$ day" is equivalent to 43.2 min, " $\log_{10}(-1)$ day" is equivalent to 144 min, " $\log_{10}(-0.5)$ day" is equivalent to 432 min, " $\log_{10}(0)$ day" is equivalent to 1 day, " $\log_{10}(0.5)$ day" is equivalent to 3 days, " $\log_{10}(1)$ day" is equivalent to 10 days, " $\log_{10}(1.5)$ day" is equivalent to 30 days and " $\log_{10}(2)$ day" is equivalent to 100 days. TEGDMA was detected from the earliest time interval and release rate data showed that the highest rate was in the first sampling period. After 3 days, little further release of TEGDMA was detected.

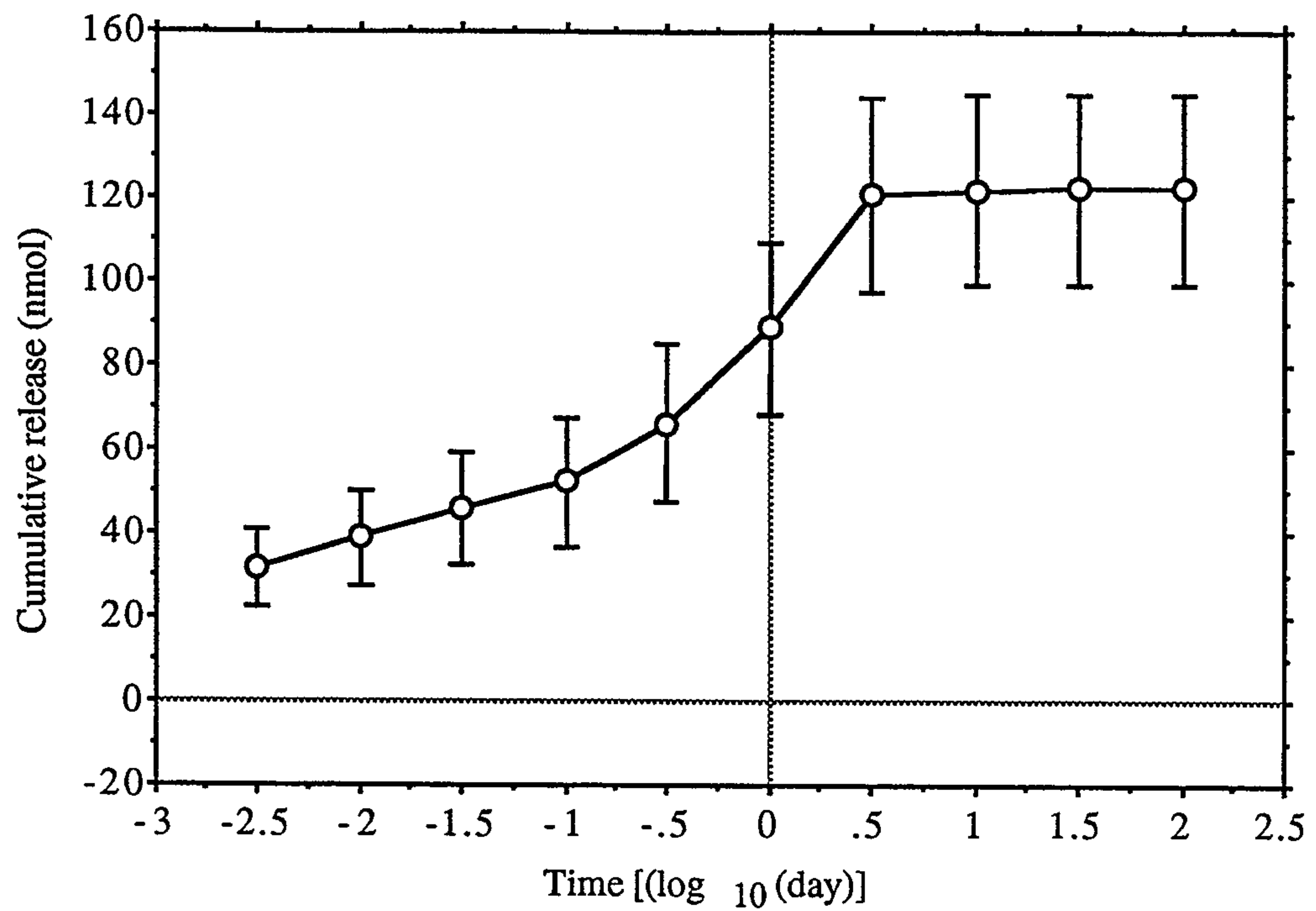


Figure 3.5 Cumulative release of TEGDMA from Foundation: basic protocol

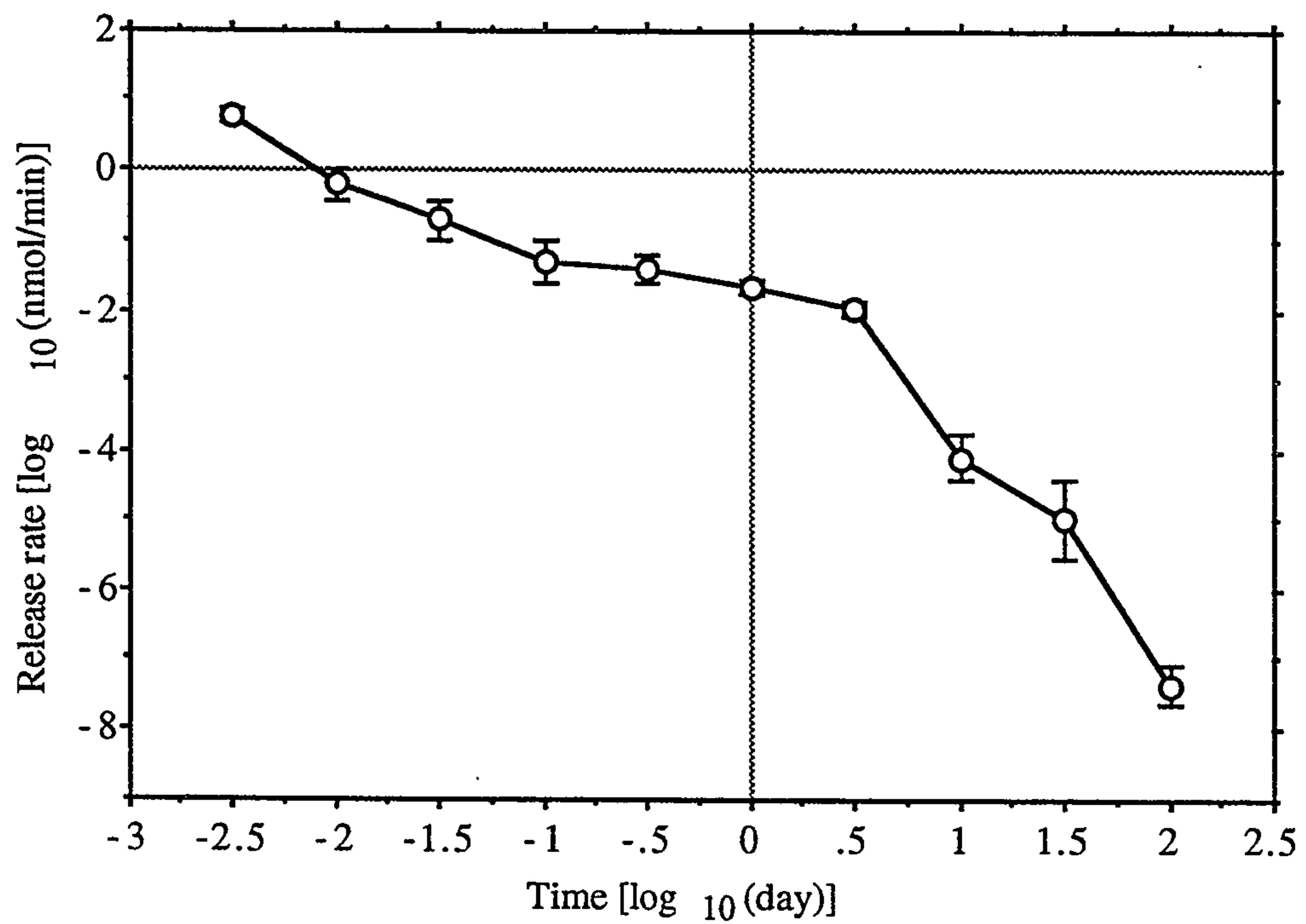


Figure 3.6 Rate of release of TEGDMA from Foundation: basic protocol

Highest release rate of 7.376 ± 2.116 nmol/min occurred at the earliest time interval, up to 4.32 min after the start of the experiment. Rates dropped substantially thereafter.

3.2.6 Discussion

It was notable that only one of the known components, TEGDMA, was detected in the samples. Aspects which may have contributed to this include limited solubility in water of the other components and limited content of the components in the composite resin. TEGDMA functions as the diluent monomer in the composite resin and exhibits moderate solubility in water. Since this component is often present in amounts in excess of that which participates in complete free-radical addition polymerization of the composite resin, it is unlikely that TEGDMA is completely converted during polymerization. In fact, double-bond conversion during polymerization in composite resins has been determined by Ruyter and Øysaet (1982) to only be between 55 and 73%.

The early release of TEGDMA from the cured material and the high rate of release at that time suggested that a major source might be the surface layer of the material. Continued low release for the duration of the experiment, however, suggested that "leaching" from the body and the sides of the material also occurred but that this was a smaller contributor to the overall release. Most TEGDMA release was completed early, which supports the findings of Ferracane (1994).

Component release, therefore, could be considered the result of both a surface phenomenon and a "bulk" phenomenon. It was of interest, then, to examine this release pattern as a function of variations to the bulk, namely thickness and surface area, and to variations in the surface condition of the composite resin. Data from the physical variables are reported in the following sections. In each case the basic protocol was varied only by a change in the variable of interest, all other physical aspects remained identical to that in the basic protocol. Results from the basic protocol for cumulative release and rate of release, where relevant for comparison, are plotted in each case.

3.3 Effects of variation in thickness on release

The first variable examined which varies *material bulk* is the thickness of composite resin.

3.3.1 Materials and methods

In this set of experiments, molds of different internal depths were used: 4.00, 1.00, 0.50 and 0.25 mm (each group, $n=7$). Mold diameter was the same in all cases, *viz* 6 mm. All other aspects of the experimental procedure were carried out as described previously for Foundation (section 3.2.2), that is, a polyester matrix was applied to the composite resin during light activation, cure time was 30 s and the time of sampling was as previously described.

3.3.2 Results

Cumulative release and rate of release of TEGDMA are shown in Figs. 3.7 and 3.8 respectively. Data for the basic protocol are plotted for comparison (2 mm thickness). A general direct relationship between component release and composite resin thickness was seen although release from material at depths below 1.00 mm was not significantly different (at $p<0.05$). Significantly more TEGDMA was released from the 4.00 mm thickness of material after 144 min, and for the duration of the experiment, than for any other thickness.

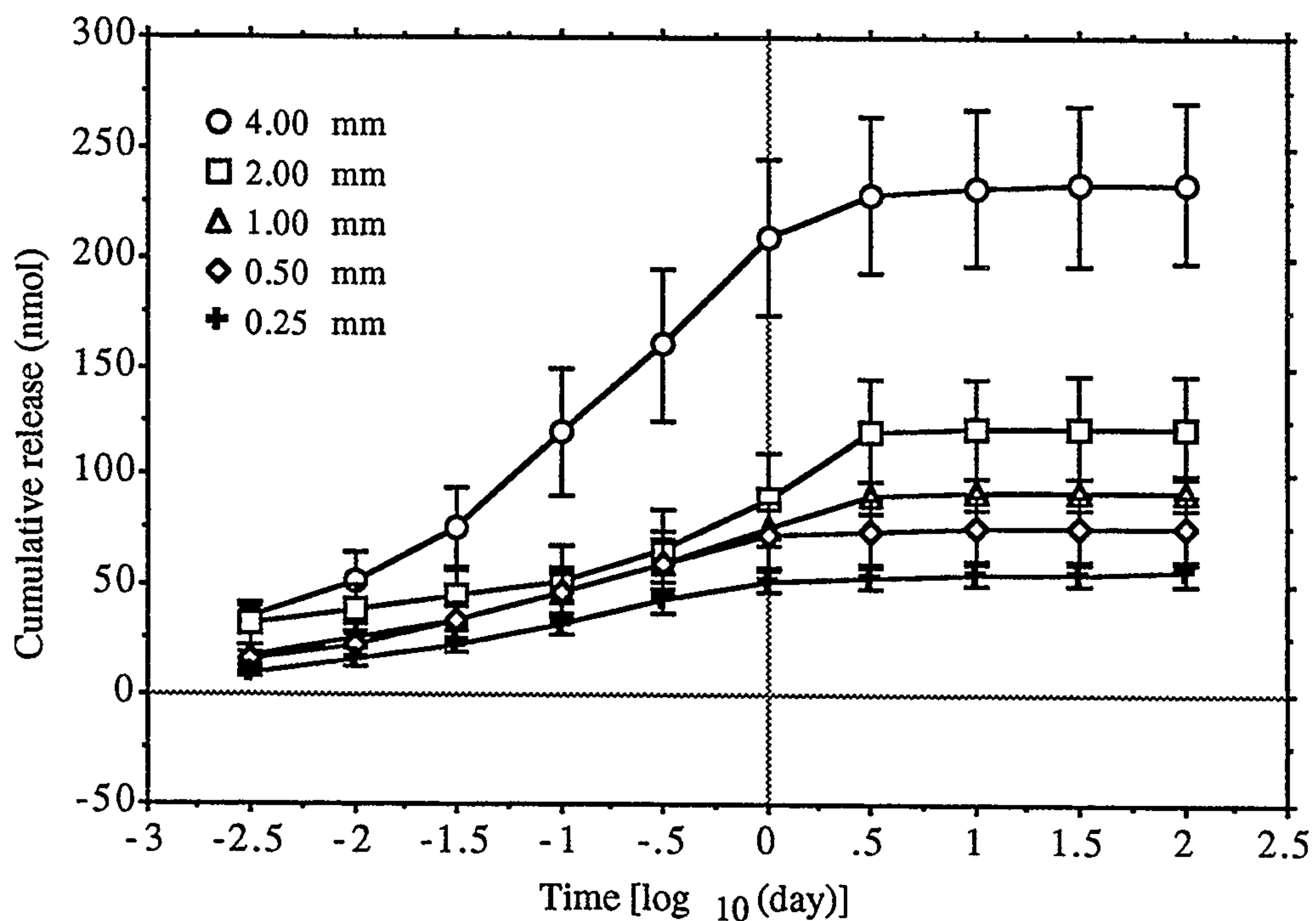


Figure 3.7 Cumulative release of TEGDMA relative to thickness

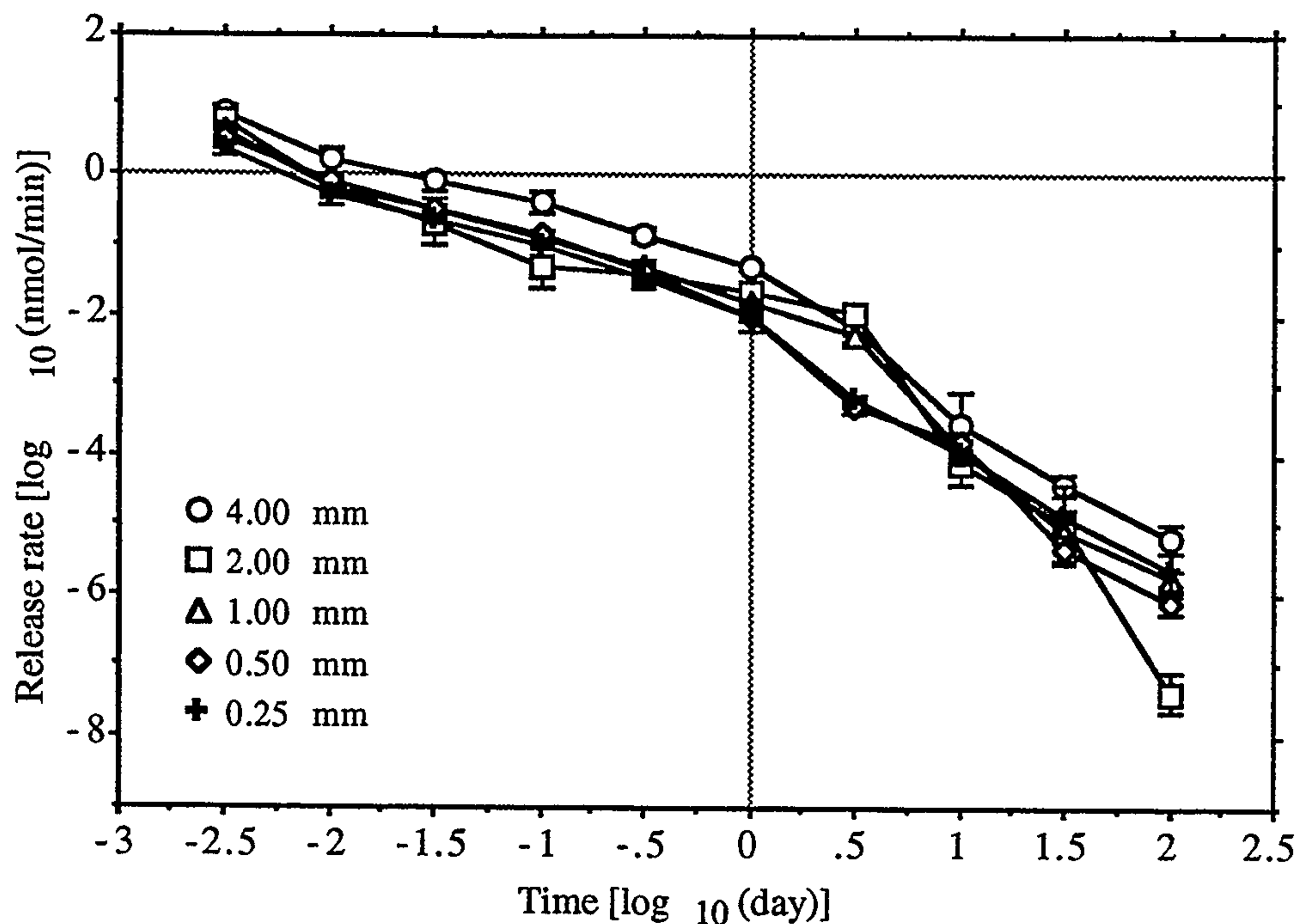


Figure 3.8 Rate of release of TEGDMA relative to thickness

3.3.3 Discussion

The amounts of TEGDMA released and the release rates from samples of different thicknesses in the early sampling periods (up to 14.4 min) were similar, indicating strongly that the predominant early release was from the surface layer of the composite resin, that layer being a maximum of 0.25 mm thick. This finding can be correlated to those of Hood and Hume (1990) who, in examining cytotoxicity of eluates from different depths of cured composite resins, found no significant difference between IC_{50} values of the respective eluates. This is very relevant to considerations of potential adverse effects in clinical use; it would appear that thickness of a resin composite filling would have little or no effect on component release in the early period after placement. However, as pointed out in section 3.2.2, the gap which developed around the composite resin within the molds enabled the sides of the composite resin to be bathed in water and these surfaces would have contributed, in some measure, to the overall component elution observed. The different thicknesses of composite resin used effectively varied the height of the sides of the composite resin sample which meant that the surface area contributing to component elution was directly proportional to height.

The apparent correlation between mean cumulative release and sample thickness from 144 min and thereafter suggests that release is also a bulk phenomenon over the later intervals examined with available component able to diffuse through the material. However, it is also likely that the deeper layers of the 4 mm thick samples were less well cured, since degree of conversion may have been less at greater distances from the activating light, as suggested in Chapter 1. This may contribute to the significantly higher amount of TEGDMA detected (at $p < 0.05$) and supports the desirability of restricting the thickness of composite resin increments placed in a cavity.

3.4 Effects of variation in diameter on release

Another physical variable which would vary the *bulk* of the material exposed to water in the current experiment, is the variation of surface area, for example, by varying diameter of the composite resin.

3.4.1 Materials and methods

Three different diameters of composite resin were used: 6 mm (as basic protocol), 8 mm and 12 mm to examine component release representing surface areas of approximately 29, 51 and 115 mm² respectively. The light guide used with the activating light accommodated all the mold diameters so that the same activation time, 30 s, was used for all molds. All other aspects of the experiments were as described for the basic protocol. It may be said that, similar to the comment made in section 3.3.1, a gap developed around the composite resin as a consequence of polymerization shrinkage away from the steel mold. For the different diameter molds it is likely that this gap would differ in size proportionally with the circumference.

3.4.2 Results

Cumulative release and release rate of TEGDMA relative to composite resin surface diameter in the mold are shown in Figs. 3.9 and 3.10 respectively. Significantly more TEGDMA was released from the 12 mm diameter mold (at $p < 0.05$) than for the 6 mm diameter mold. There was no significant difference between cumulative release for the 6mm and 8mm diameter molds (at $p < 0.05$).

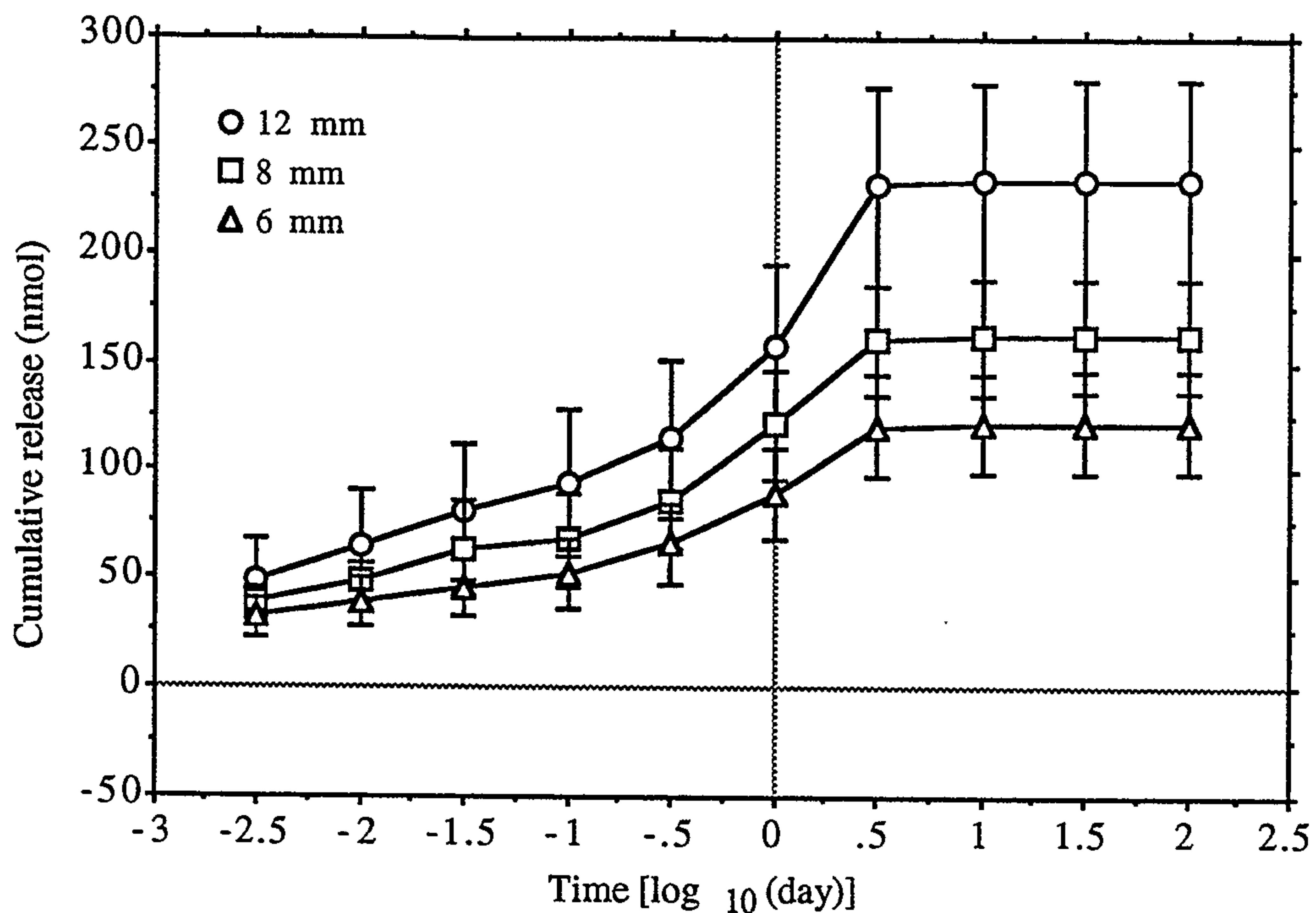


Figure 3.9 Cumulative release of TEGDMA relative to sample diameter

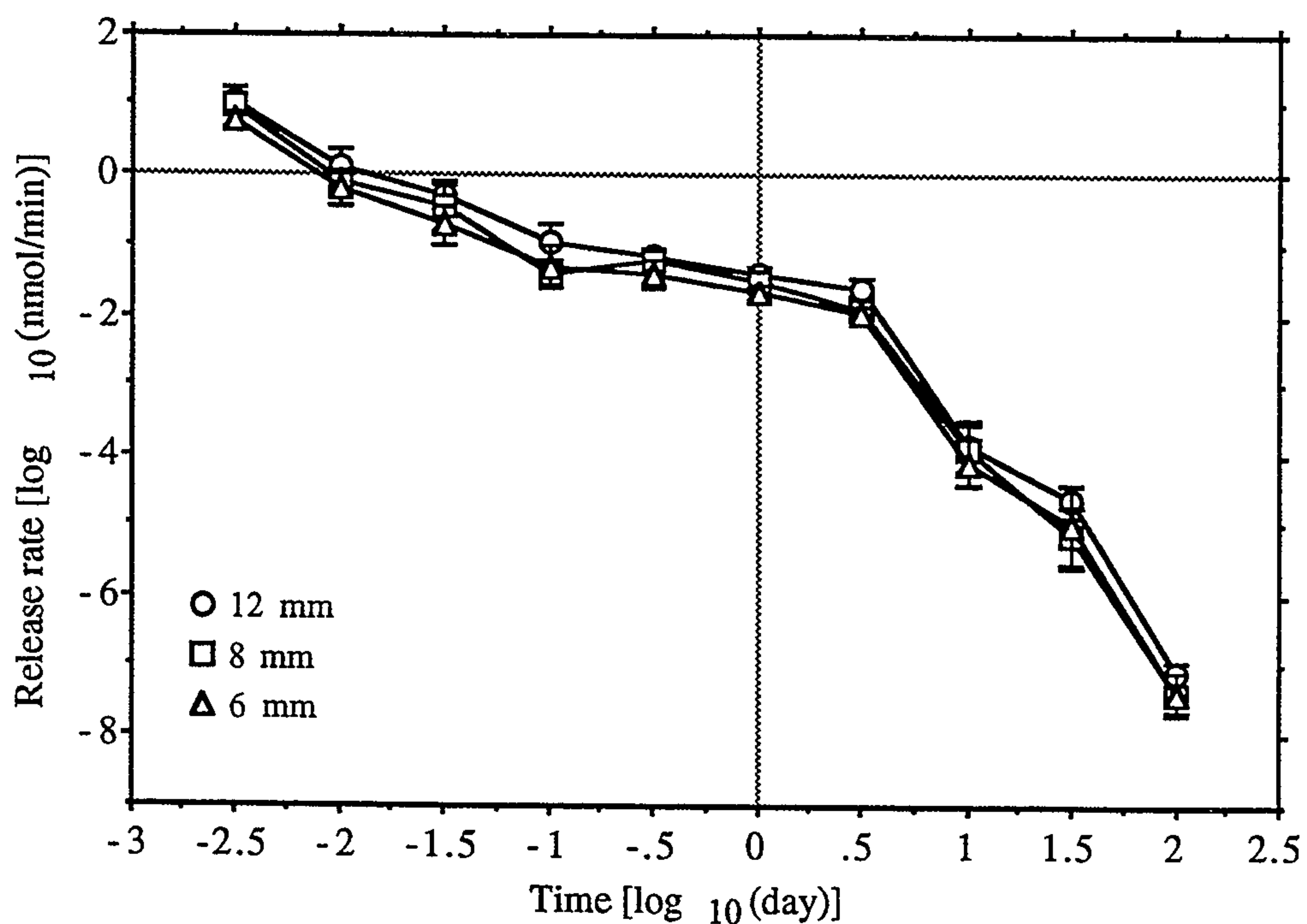


Figure 3.10 Rate of release of TEGDMA relative to sample diameter

3.4.3 Discussion

From the data it can be seen that, by 3 days, TEGDMA release appeared to be proportional to the diameter of the composite resin confined in the molds. Clinically, the data suggest, not surprisingly, that restricting the diameter

of composite resin applied to the cavity floor would restrict the potential for TEGDMA release. Again, as pointed out in section 3.2.2, the vertical sides of the composite resin also contributed to component elution. When diameter was varied, the area of the vertical sides of the composite resin sample also varied so that the effective surface area of the composite resin contributing to component elution was directly proportional to the diameter of the composite resin sample. This means that the contribution of the side surfaces of the composite resin sample to overall component elution was greater in the molds of larger diameter.

3.5 Effect of air-inhibition of the composite resin surface layer on release

The early stage of polymerization can be inhibited for a time by oxygen (Finger and Jørgensen, 1976) which successfully competes with monomer for reaction with activated radicals. This results in the development of an "air-inhibited" layer which is possibly monomer-rich. Thus, an important physical variation which is likely to influence the *condition of the surface* of the composite resin is air-inhibition of the surface during light activation.

3.5.1 Materials and methods

Air-inhibition of the surface of the composite resin, whilst polymerization was occurring, was examined in these experiments. No matrix was used during light activation of the composite resin placed in these molds, $n=7$. This group of molds is referred to as the **air-inhibited** group. The group constituting the basic protocol, where a polyester matrix was applied to the uncured material and left in position for the duration of light activation, is referred to as the **air-excluded** group, $n=49$.

3.5.2 Results

Figs. 3.11 and 3.12 present the cumulative release and rate of release of TEGDMA in the two groups examined respectively. Significantly more TEGDMA was detected throughout the time course in the air-inhibited group (at $p<0.05$). In the air-inhibited group the amount released was high from the earliest time interval, as illustrated in the high rate. The degree of difference between the two groups was less after 1 day.

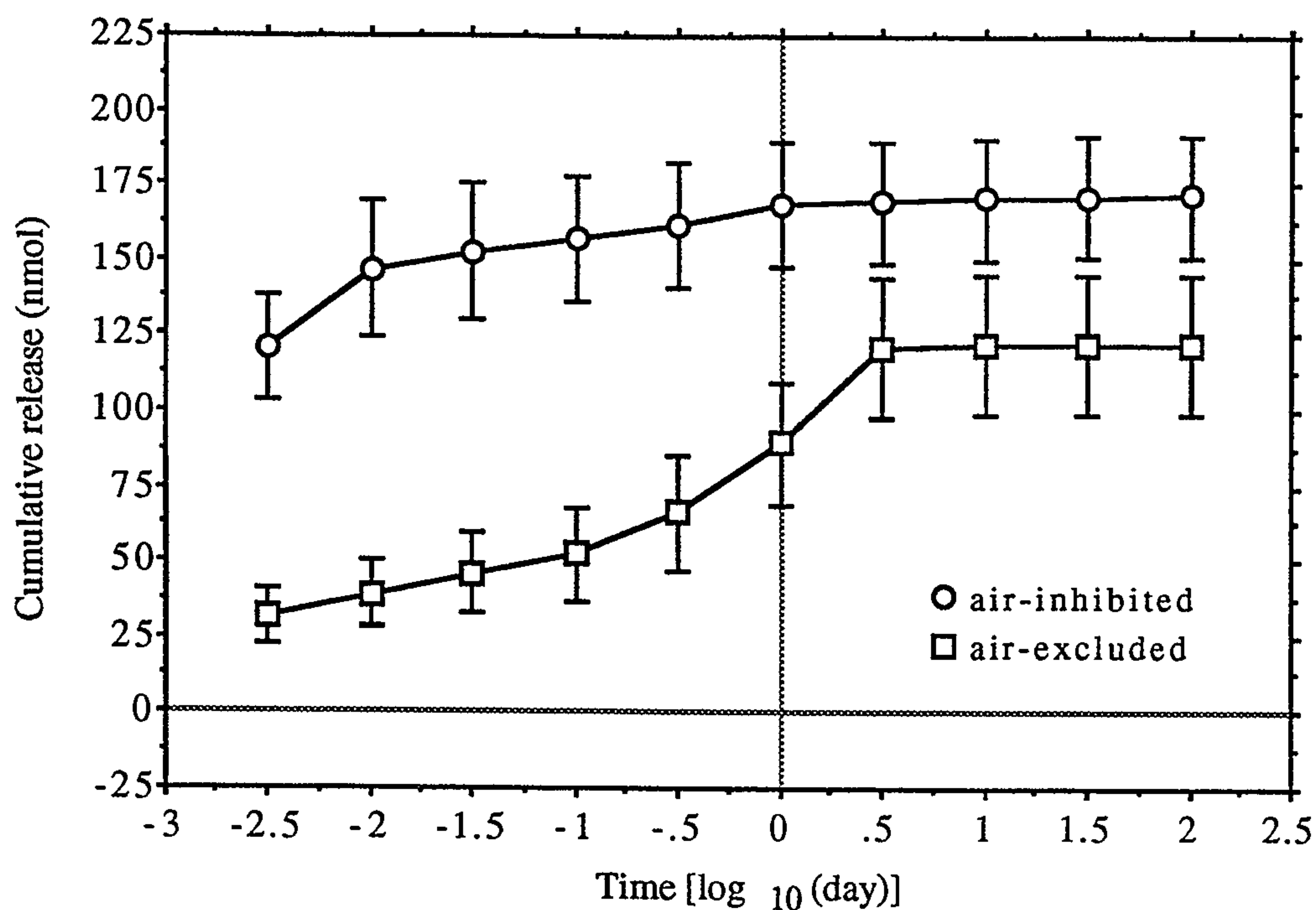


Figure 3.11 Cumulative release of TEGDMA relative to surface protection during cure

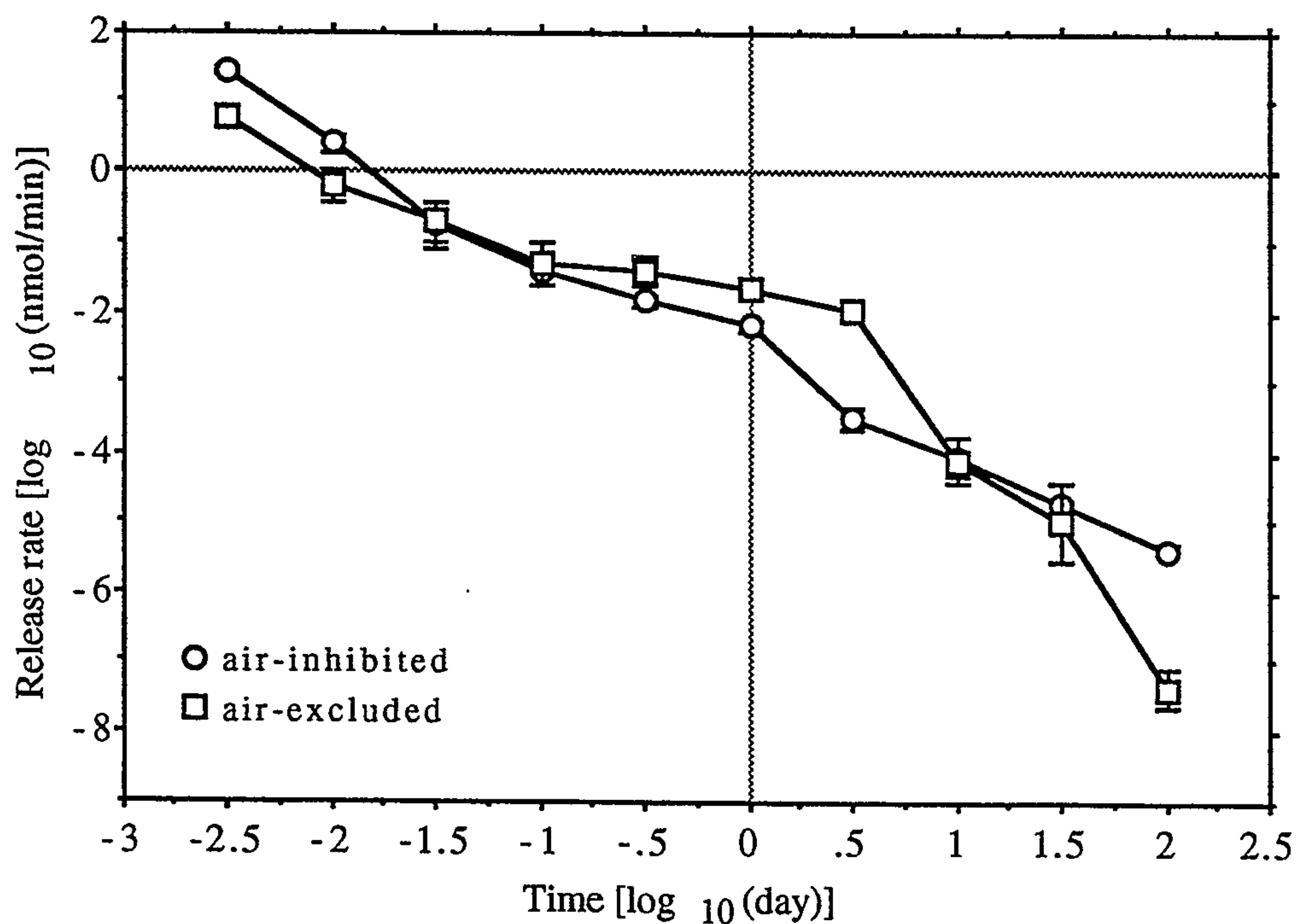


Figure 3.12 Rate of release of TEGDMA relative to surface protection during cure

3.5.3 Discussion

The marked difference in accumulated amounts of TEGDMA seen in the air-inhibited and air-excluded groups occurred despite the same thickness and diameter of composite resin being used in the two groups, and the cure time being identical. The marked difference in the early release strongly suggests that the *condition of the composite resin surface* has a major effect on release.

Oxygen acts as an inhibitor of polymerization in resin materials (Ruyter, 1981) resulting in the formation of an inhibited layer on the surface of the material which is similar in composition to the uncured resin except that the initiator-activator complex has been consumed. In unfilled resins this layer is a porous, tacky film on the surface, is reported to be $37 \pm 16 \mu\text{m}$ thick and exhibits a degree of conversion one quarter that of air-excluded resin (Rueggeberg and Margeson, 1990). The present data indicate that the air-inhibited layer offered little resistance to the diffusion of TEGDMA from deeper, better converted layers of composite resin and was itself very probably a source of TEGDMA. Thus, release in the air-inhibited group is likely to be derived from unconverted TEGDMA from the body of the composite resin (as seen in the air-excluded group) plus that arising from the air-inhibited layer itself.

3.6 Effects of variation in state of cure on release

A fundamental requirement for adequate polymerization is optimal initiation of polymerization, which in light-activated composite resins, is achieved by an appropriate light source. During curing, growth of the resin network occurs as the mobile monomer molecules establish cross-links. It is therefore important to examine component release when polymerization activation of the composite resin is varied, such as when no light activation is used, which here is termed *uncured*, or when activation time is shortened or increased.

3.6.1 Uncured

3.6.1.1 Materials and methods

In the uncured group in this experiment, placement of composite resin into molds, $n=7$, and placement of molds into the vials was conducted within a light-excluded photographic dark room. All vials were then sealed in light-safe containers and then placed into the 37°C incubator. Sample retrieval at

each time interval was also carried out under light-excluded conditions, in the photographic dark room. These data are compared to those from the basic protocol, which here is termed **cured** where an activation time of 30 s was used. In other respects mold dimensions and experimental procedures were identical to those used in the basic protocol. At the end of the entire time course, composite resin in the molds was retrieved and examined for evidence of hardening which would have suggested some degree of cure had occurred. All composite resin retrieved was in the uncured state.

3.6.1.2 Results

Only one component was detected in all the samples for both groups, TEGDMA. In some uncured samples, however, small amounts of Bis-GMA were detected but this was not a consistent finding for samples in a given series. Quantitation of these irregular amounts of Bis-GMA was not completed. Figs. 3.13 and 3.14 present the cumulative release and rate of release for TEGDMA found in the two groups examined. Significantly more TEGDMA was detected in the uncured group than in the cured group (basic protocol) as illustrated in the cumulative release by 3 days of 4000 nmol and 120 nmol respectively. The high release rate in the uncured group was sustained throughout the time course and exhibited reasonably good linearity for the entire time course.

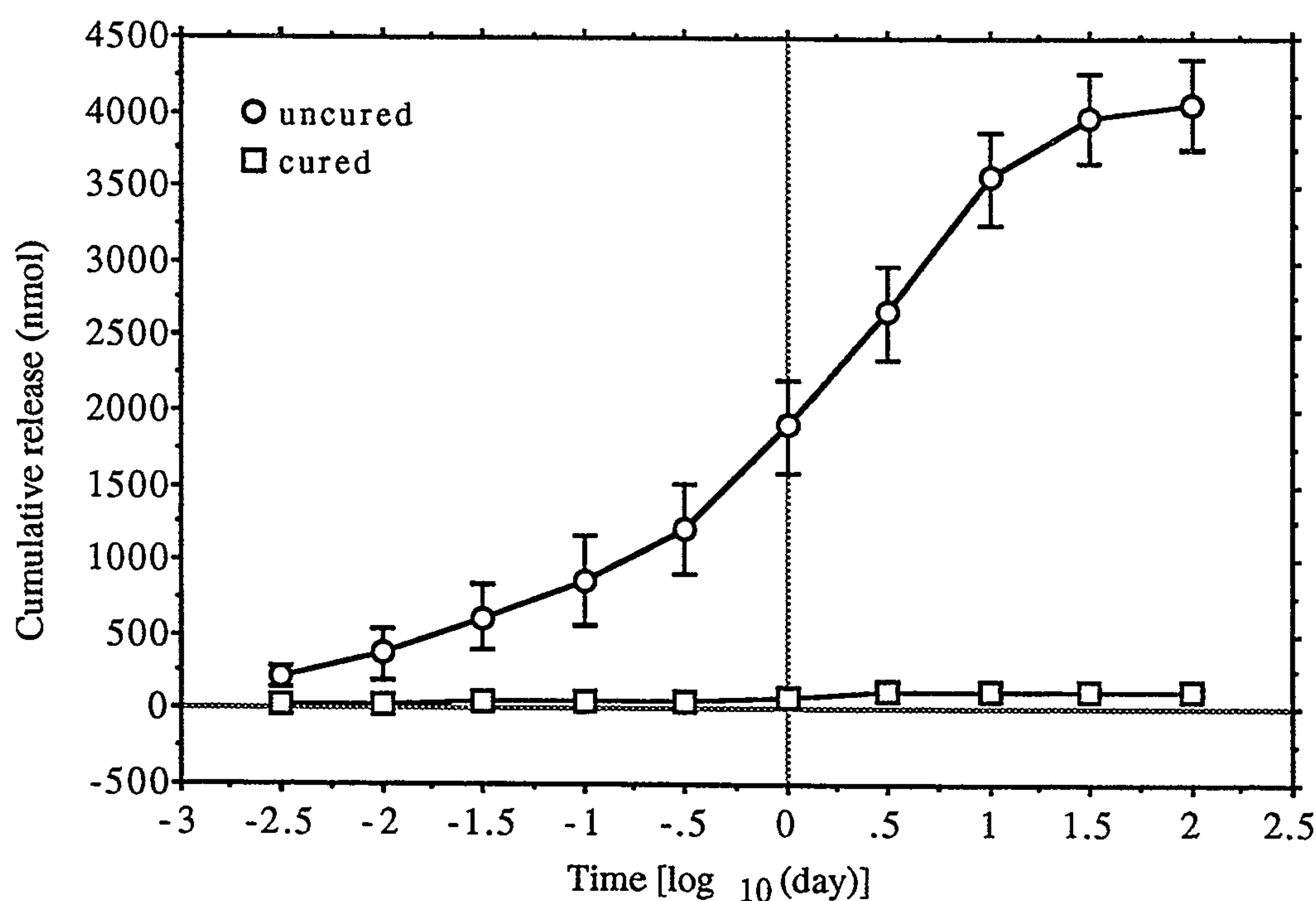


Figure 3.13 Cumulative release of TEGDMA relative to state of cure

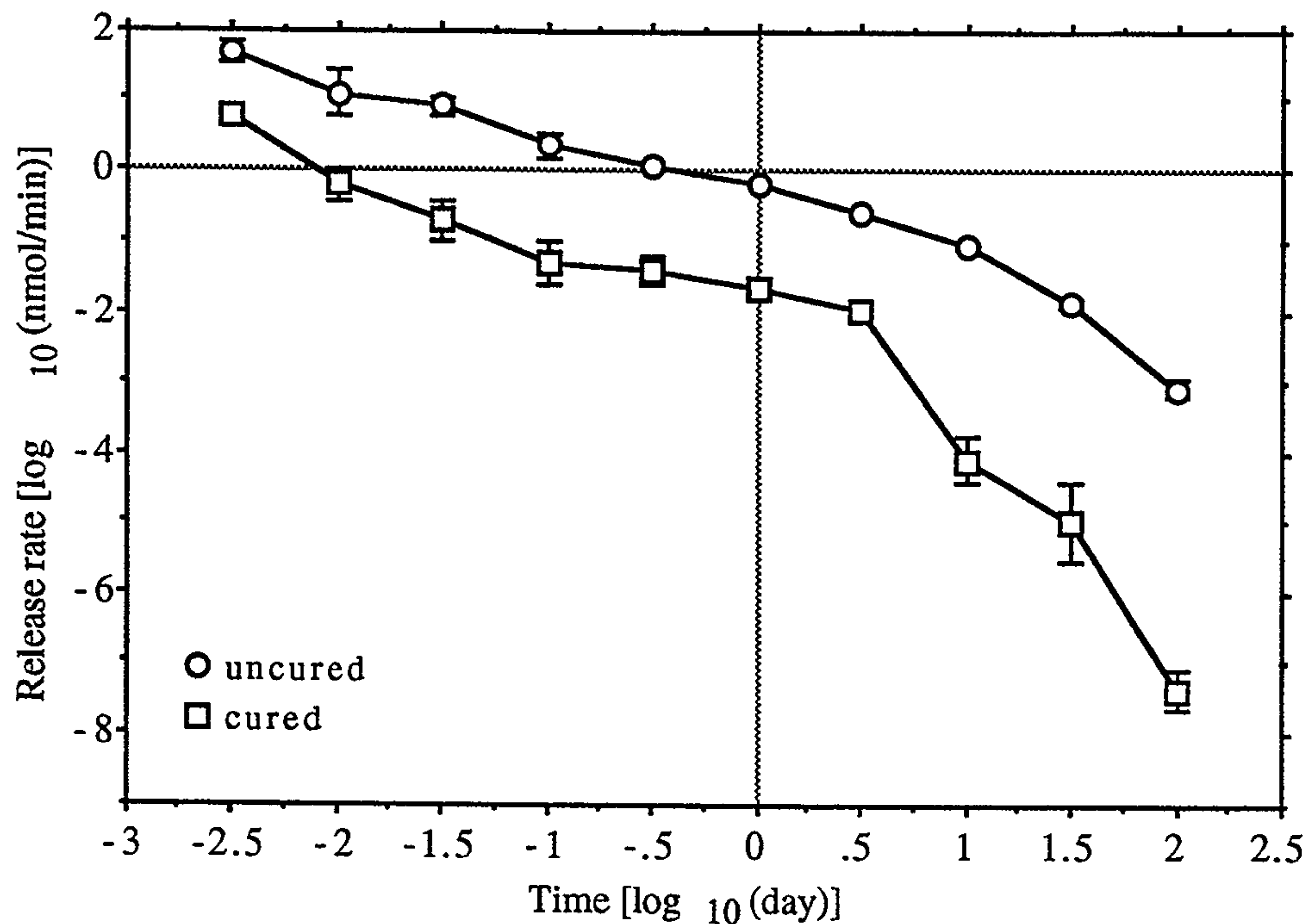


Figure 3.14 Rate of release of TEGDMA relative to state of cure

3.6.1.3 Discussion

It was of interest that of the components present in composite resin, only TEGDMA was detected reliably and consistently in the water samples, although Bis-GMA was also detected in some samples. It can be suggested that the more viscous nature and the larger molecular weight of the Bis-GMA molecule probably prevented its sustained release from the composite resin. Further comment about this will be made in section 3.10.

A higher amount of TEGDMA was released when light was excluded and the composite resin remained uncured. By 3 days, the amount of TEGDMA accumulated in the uncured group was approximately 30X that seen in the basic protocol group cured. Assuming that no polymerization took place in the uncured group, all of the TEGDMA content of the composite resin was potentially able to diffuse through the material. In this case, the only hindrance to diffusion is that of the resin matrix itself and the molecules of the other components. The limiting factor influencing TEGDMA release would therefore be the ability of the water to enter the uncured material and facilitate TEGDMA release. It would be important to accurately measure

the degree of conversion in the uncured composite resin to enable correlation of this with TEGDMA release.

These findings suggest a possible reason for the higher toxicity found for uncured composite resins compared with that found for cured composite resins (Hume and Hood, 1990). This strongly highlights the necessity to cure composite resin adequately, thereby limiting component release.

3.6.2 Variation in light activation time

3.6.2.1 Materials and methods

Variation of the duration of light activation (cure time) of the composite resin was examined in this study. Two duration times for light activation of the composite resin were used, 15 s and 60 s. These results are compared to those results for the basic protocol, where light activation was 30 s. In all other respects the experimental procedures were as those described in section 3.2.2.

3.6.2.2 Results

Cumulative release and rate of release of TEGDMA are shown in Figs. 3.15 and 3.16. respectively. No significant difference (at $p < 0.05$) was seen among the cumulative release amounts in the different groups although there was a tendency to more release in the 15 s group. Release rates for the groups were also comparable.

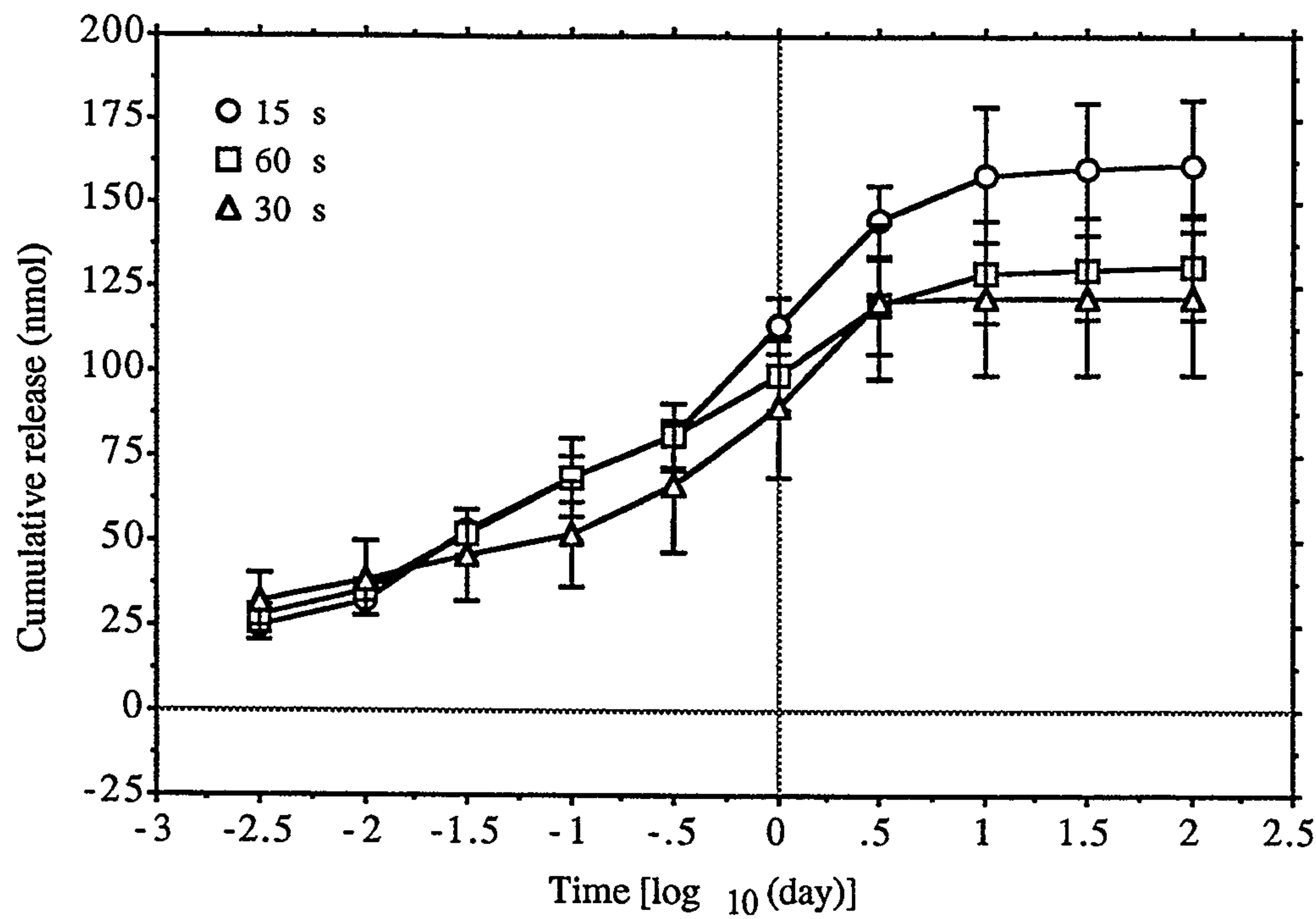


Figure 3.15 Cumulative release of TEGDMA relative to variation in light activation time

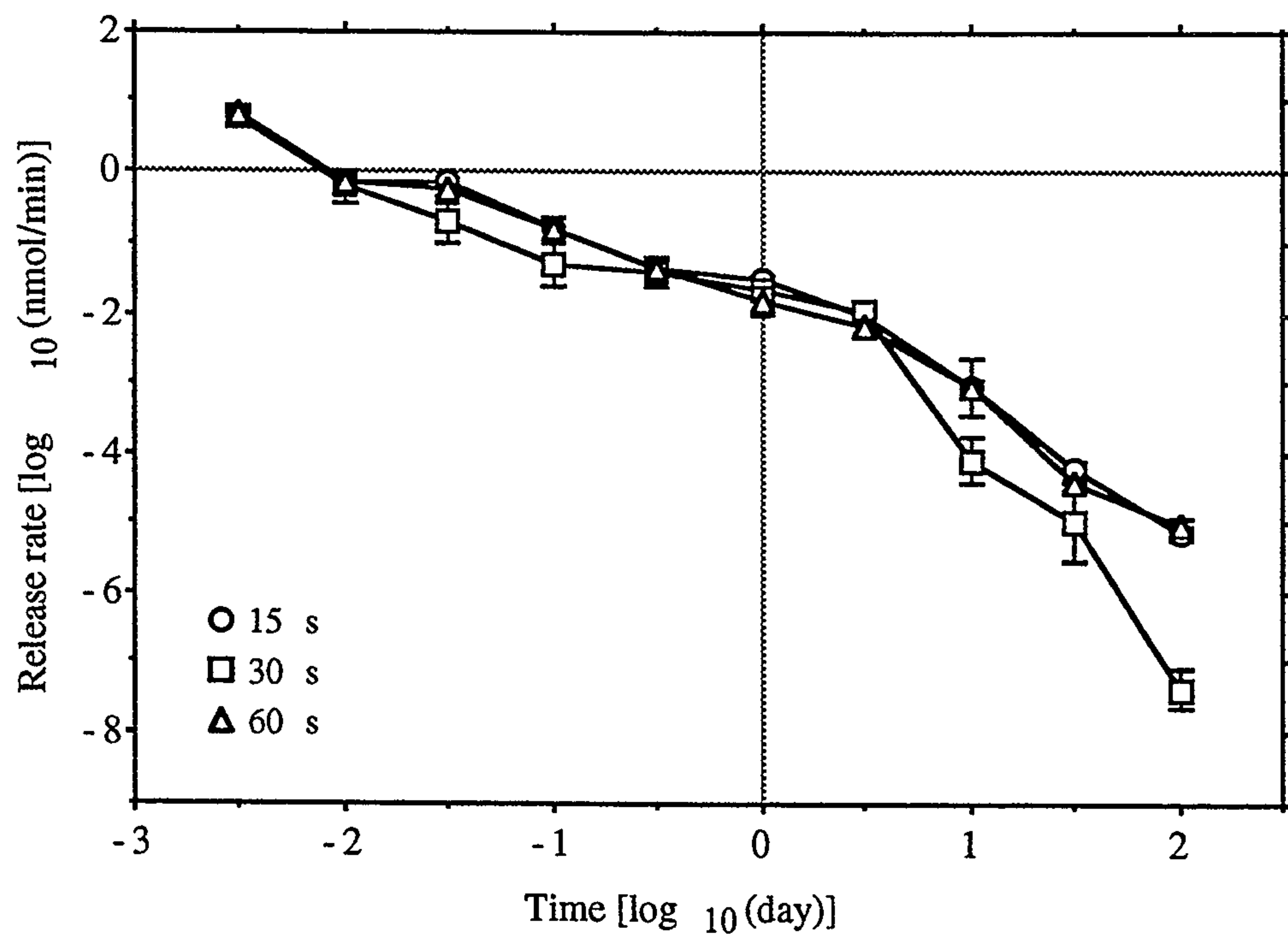


Figure 3.16 Rate of release of TEGDMA relative to variation in light activation time

3.6.2.3 Discussion

The data indicate that a cure time less than 30 s for the 2 mm thickness specimen was possibly associated with slightly greater TEGDMA release, probably due to a poorer degree of conversion. However, doubling activation time from 30 s to 60 s did not reduce TEGDMA release. It may be inferred from these data that 30 s was an adequate light activation time for conversion under the experimental conditions, that is, there appeared to be no reduction in TEGDMA release when activation times were increased.

3.7 Effect of variation in temperature on release

Unconverted monomer molecules within the resin matrix may move within the network and this movement is probably enhanced if the energy of the system rises. The following experiment aimed to examine whether clinically relevant temperatures had any influence on component release from the composite resin.

3.7.1 Materials and methods

For this experiment, initial composite resin placement into molds and polymerization activation was conducted at room temperature. Molds were divided into two groups which differed in the temperature of storage of the filled molds in their individual vials during the time course. In the 4°C group, after activation, the mold was placed into a vial containing 4 ml of water and held at 4°C for the duration of the time course. In the 60°C group, all molds were stored in vials containing 4 ml of water held at 60°C. Fresh water samples for each time interval were similarly kept at these temperatures and only during the very brief periods of sample retrieval were the molds able to equalize to room temperature. Following sample retrieval and fresh water replacement, samples were promptly returned to the temperature for that experiment.

3.7.2 Results

Results were compared with those for release at 37°C, that is, the basic protocol. Cumulative release of TEGDMA found over the time course in the three groups is shown in Fig. 3.17. Rate of release is shown in Fig. 3.18. After 1 day, there was a significant difference (at $p < 0.05$) between the amounts of TEGDMA accumulated at 4°C and 60°C, but not between each of these groups of data and those for the basic protocol, that is at 37°C. No

significant difference was seen between the rates of release for the groups (at $p < 0.05$).

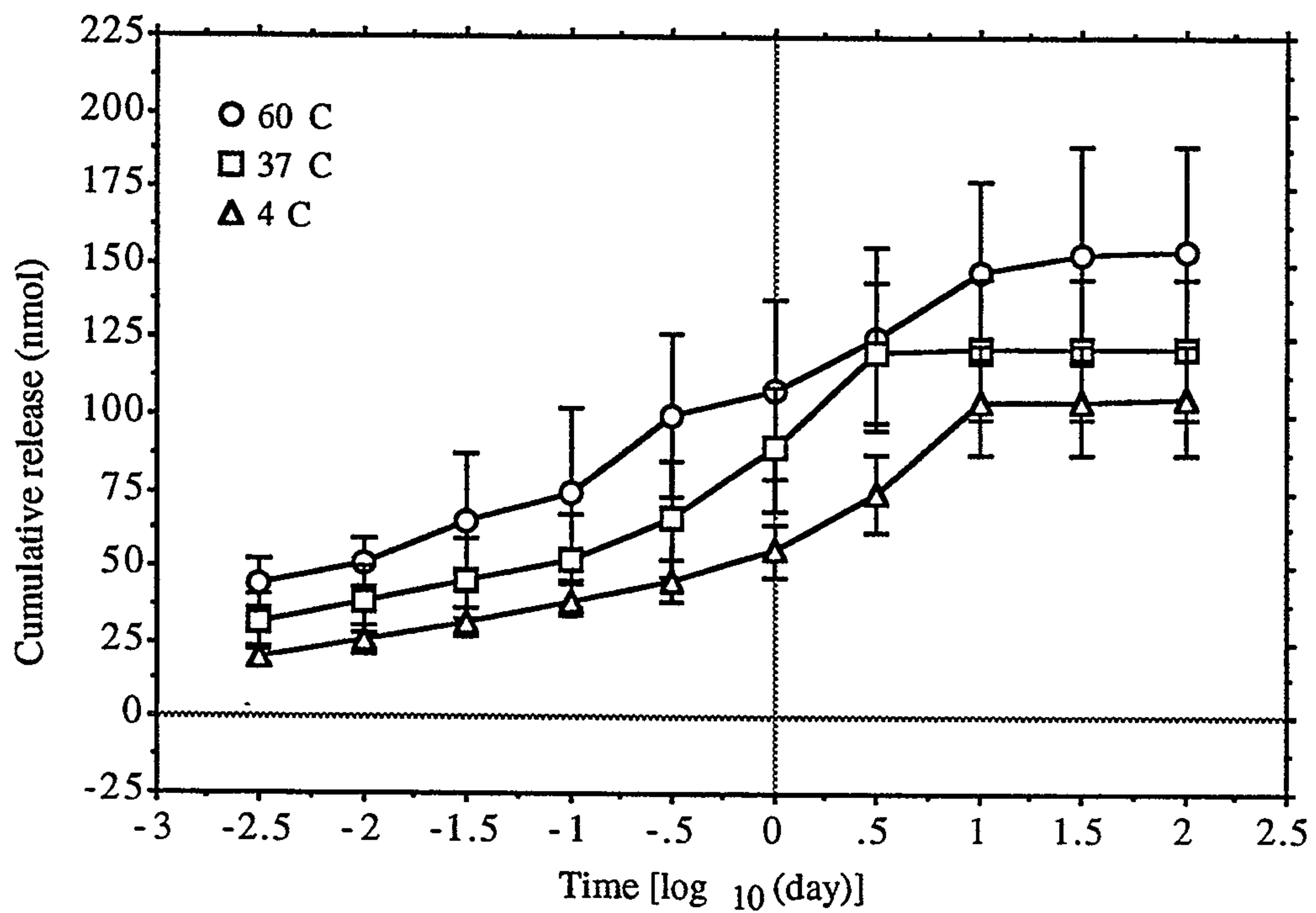


Figure 3.17 Cumulative release of TEGDMA relative to temperature

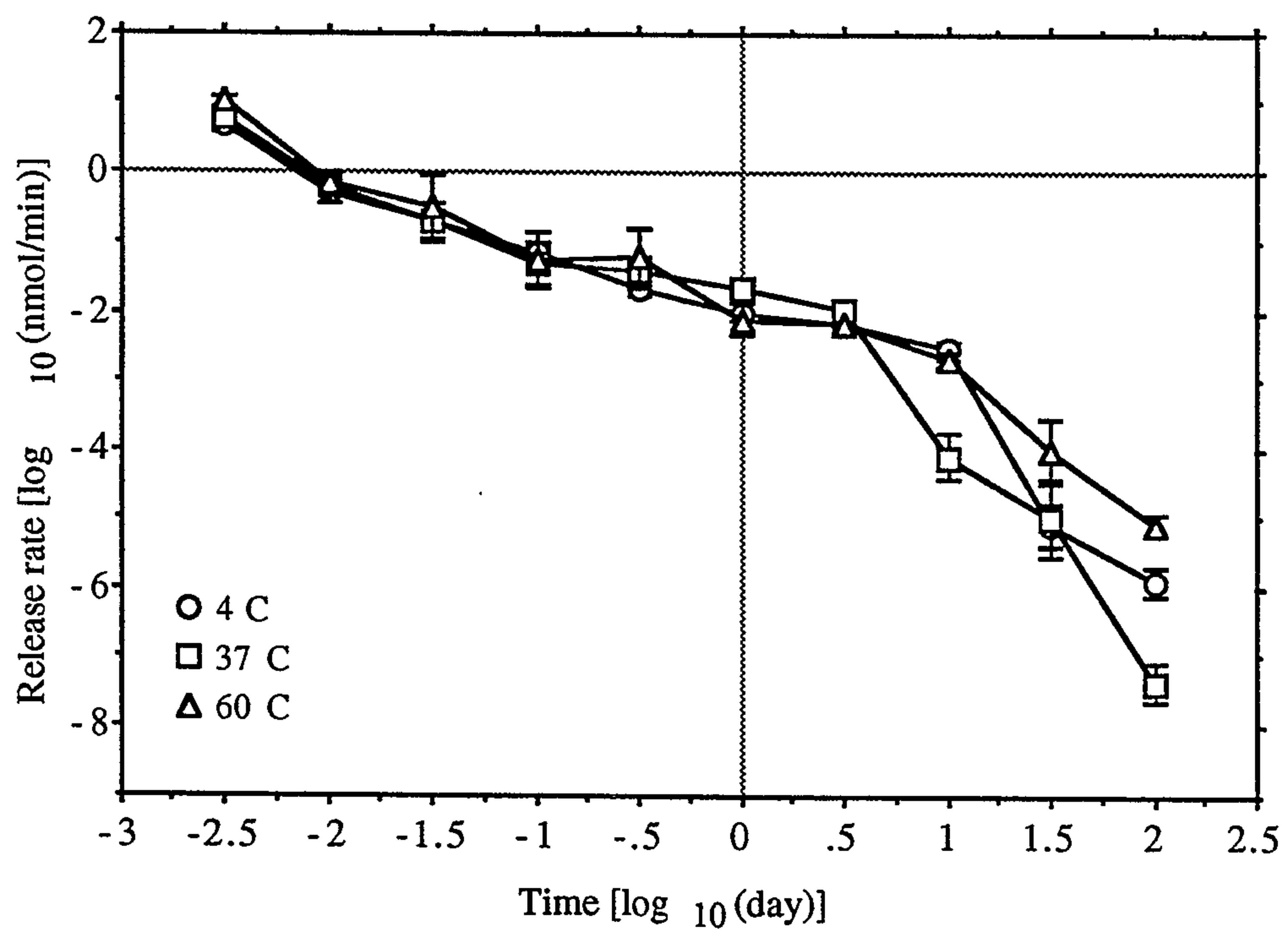


Figure 3.18 Rate of release of TEGDMA relative to temperature

3.7.3 Discussion

These data suggest that release is probably slightly inhibited by lowered temperature, very probably because decrease in temperature increases the energy required by molecules to diffuse through the material. The increased release noted in the 60°C group may have been due to the higher energy acquired discriminantly by unconverted monomer molecules which therefore experience facilitated diffusion. Thermal degradation of the resin matrix is unlikely to have occurred at even the higher temperature (Nicholson, 1991a).

Clinically, composite resin restorations are often exposed to mouth temperatures in the ranges examined here. These temperatures are sustained only briefly and quickly stabilize to body temperature. During curing of composite resin into a cavity, however, thermal energy in excess of the common range recorded intra-orally may be released via some light activating units to the curing composite resin. This, however, would probably only be significant if activation was sustained well in excess of the recommended curing time.

3.8 Release of components from various composite resins

3.8.1 Materials and methods

Component release from four other composite resins (n=7 samples for each composite resin) was determined using the basic experimental protocol described in section 3.2. Briefly, stainless steel molds, 6 mm internal diameter and 2 mm depth, were filled with the composite resin and light activated for 30 s. A polyester matrix was used during light activation to exclude air during activation. The molds were then dropped into 4 ml of fresh water and this sample removed at the time intervals previously listed.

These composite resins and their classification, as suggested by Willems and co-workers (1992), are listed in Table 3.2. The classification listed has used the codes shown in Table 1.1 where 'f/U' is Fibre-filled, Ultrafine; 'F C-F' denotes Fine Compact-Filled, 'MFspf' is Midway-filled with splintered prepolymerized filler, 'U M-F' is Ultrafine Midway-filled and 'U C-F' is Ultrafine Compact-filled. Also shown in Table 3.2 are the TEGDMA content, where known, and the inorganic filler volume percentage as listed by Willems and co-workers (1992). From manufacturer data, all the composite resins are known to contain TEGDMA and, apart from Occlusin, Bis-GMA.

Occlusin contains a mixture of di-, tetra- and oligo-UDMA (Ruyter and Øysaed, 1987).

Table 3.2 Additional composite resins examined

Resin (manufacturer)	Class ¹	TEGDMA (% Vol)	Filler (% Vol)
Foundation (Nulite Systems International, Hornsby NSW, Australia)	f/U	63	72.0 ²
Occlusin (GC, Tokyo, Japan)	FC-F	55	69.0 ³
Perfection (Den-Mat Corporation, Santa Maria, CA, USA)	MFspf	present	67.5
Herculite XR (Kerr Manufacturing, Romulus, MI, USA)	U M-F	present	57.0 ³
Conquest (Jeneric/Pentron, Wallingford, CT, USA)	U M-F	present	68.5 ³
Z100 (3M Dental Products, St. Paul, MN USA)	U C-F	52	71.0 ³

1 Classes adapted from Willems and co-workers (1992)

2 Manufacturer's data (Nulite Systems International, Australia)

3 Inorganic filler volume, manufacturer data adapted from Willems and co-workers (1992)

3.8.2 Results

TEGDMA was detected in all eluate samples. Trace amounts of unidentified components were also found in Perfection and Conquest. Data for the cumulative release of TEGDMA from the composite resins examined are presented in Fig. 3.19 and release rates over the course of the experiments is shown in Fig. 3.20. Similar release patterns for TEGDMA were seen for all the composite resins. If the cumulative amounts are compared for all the composite resins, Perfection and Conquest each eluted more TEGDMA than any other composite resin over the period of testing and the amounts were similar. Amounts of TEGDMA released from Occlusin, Herculite XR and Foundation were significantly different from each other (at $p < 0.05$).

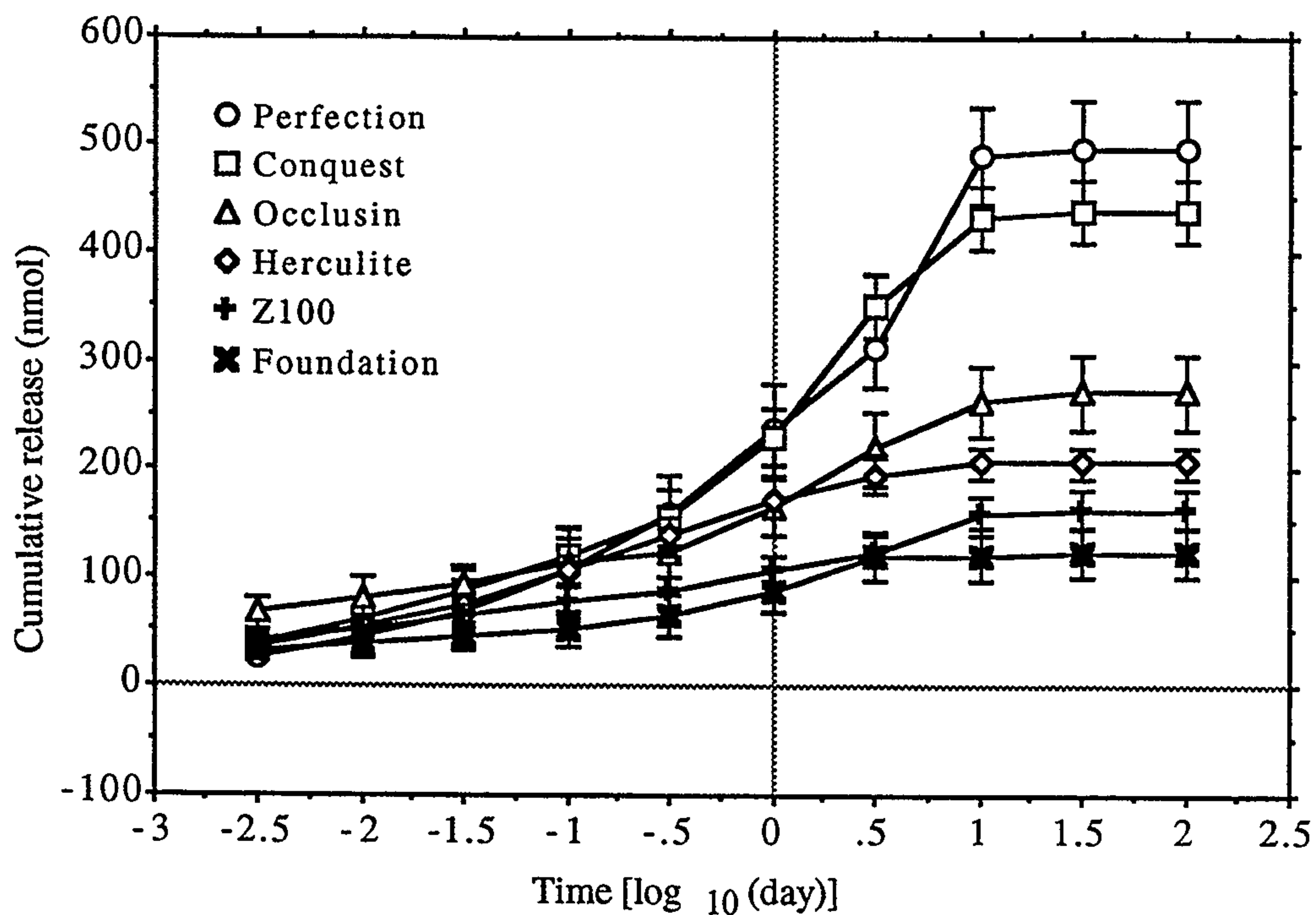


Figure 3.19 Cumulative release of TEGDMA from six composite resins

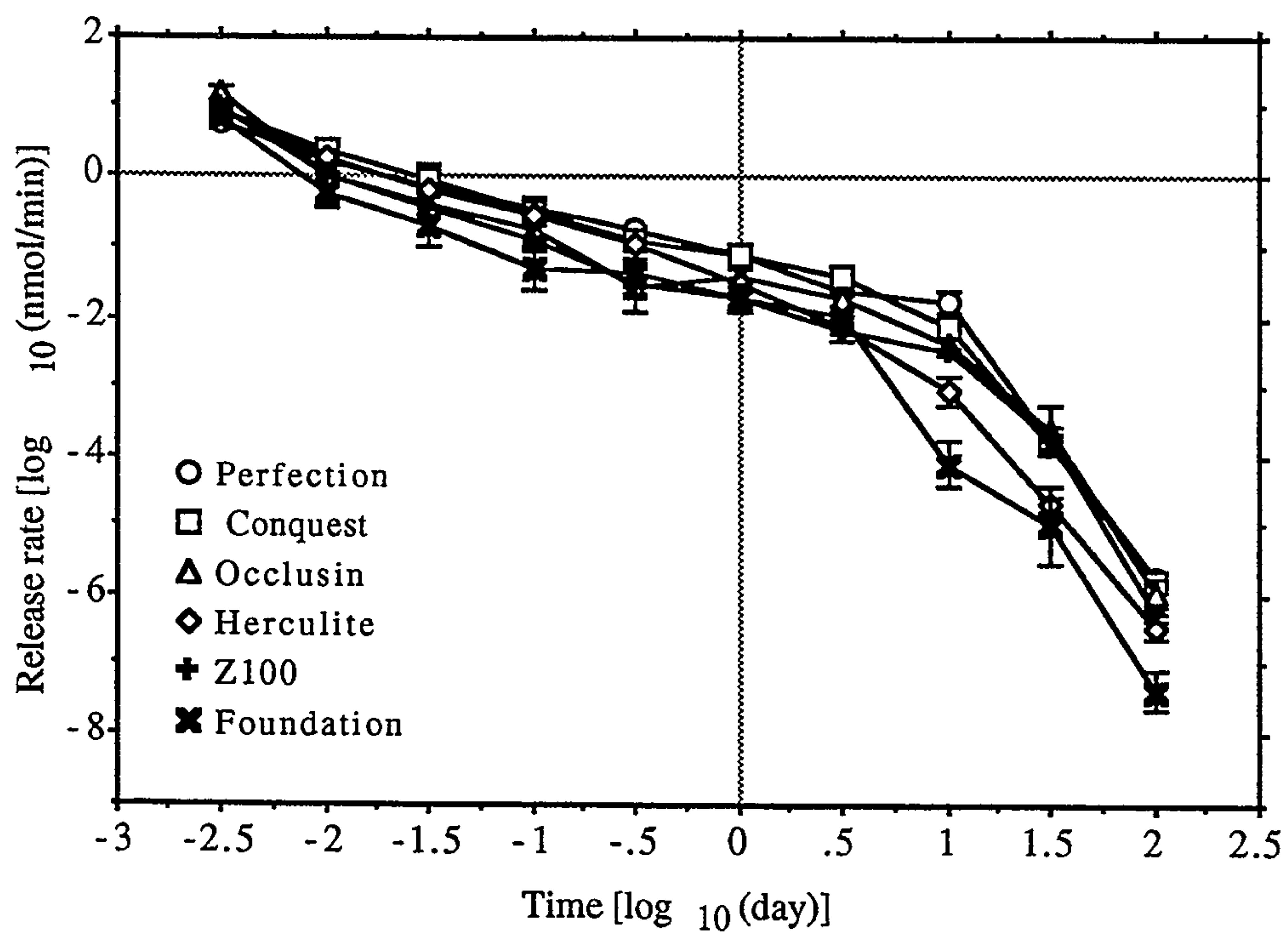


Figure 3.20 Rate of release of TEGDMA from six composite resins

To enable a convenient comparison between these composite resins, for cumulative amounts released and release rates of TEGDMA, data for the interval from time zero to 4.32 min is presented in Table 3.3. Table 3.4 presents the cumulative amounts of TEGDMA released up to 3 days from the five additional composite resins and for Foundation. Vertical lines unite means which are not significantly different (at $p < 0.05$).

Table 3.3 Comparison of cumulative amounts released at, and release rate to, 4.32 min for six composite resins

Composite Resin	Cumulative amount of release (nmol)	Release rate (nmol/min)
Occlusin	66.96 ± 14.64	15.50 ± 3.39
Z100	42.99 ± 8.45	9.32 ± 1.92
Conquest	37.96 ± 7.10	8.79 ± 1.64
Herculite XR	37.09 ± 6.07	8.59 ± 1.41
Foundation	31.87 ± 9.14	7.38 ± 2.12
Perfection	27.82 ± 5.67	6.44 ± 1.31

Table 3.4 Cumulative amount of TEGDMA released by 3 days for six composite resins

Composite Resin	Cumulative amount of release (nmol)
Conquest	351.1 ± 28.19
Perfection	313.1 ± 35.91
Occlusin	220.8 ± 34.08
Herculite XR	196.3 ± 16.80
Z100	125.0 ± 14.92
Foundation	120.7 ± 23.13

Several observations can be made from Tables 3.3 and 3.4. Initial release rate (to 4.32 min) of TEGDMA for Occlusin was significantly higher (at $p < 0.05$) than that for the other composite resins, which were comparable, but by 3 days Perfection and Conquest had released more TEGDMA. This finding suggests that the condition of the surface layer is relatively characteristic in different composite resins and early release has little

predictive value with respect to longer term release. Other factors peculiar to the composite resins themselves may influence release, for example, the nature of the base resin oligomer, which in Occlusin is UDMA, or the physical structure and distribution of the filler. By 3 days, accumulated amounts of TEGDMA released were similar for Z100 and Foundation, and for Occlusin and Herculite XR, but was significantly higher for Conquest than for all other resins except Perfection.

3.8.3 Discussion

Composite resin materials differ greatly in basic formulation and this is most probably reflected in the different patterns of TEGDMA release seen for the range of composite resins examined. However, the correlation between formulation and release is not a close one, as indicated by the significantly lower TEGDMA release in Foundation compared to Occlusin, despite Occlusin having a similar TEGDMA content. Other physical characteristics very probably enhance or inhibit release. Another important factor which may have contributed to the variation seen for the different composite resins may have been the difference in degree of cure. It would be of interest to determine degree of cure for the different composite resins and relate this to the component release seen.

Filler volume has been found to correlate well to several mechanical properties (Braem *et al.*, 1989) and is inversely proportional to water sorption. Increased filler volume may also contribute to improved depth of cure (Li *et al.*, 1985). Foundation exhibited the lowest TEGDMA release among the materials examined; its fibre content may influence TEGDMA diffusion through the material.

3.9 Summary of TEGDMA release data for Foundation

Several variables influencing TEGDMA release from Foundation were examined. For all variables, rate of release was highest in the first time interval, that is, from time zero to 4.32 min, and only small additional amounts were detected after 3 days. The following tables (Tables 3.5 and 3.6) compare TEGDMA release data for these variables and the vertical lines in the tables unite those means within groups which were not significantly different (at $p < 0.05$).

Table 3.5 presents the cumulative amount of release and the release rate of TEGDMA from Foundation for all the conditions examined at the time of highest rate (time zero to 4.32 min); means and standard deviations about the mean are shown.

Table 3.5 Comparison of cumulative amount of TEGDMA release to, and release rate at, 4.32 min for Foundation

Category	Variable	Cumulative amount of release (nmol)	Release rate (nmol/min)
Thickness	0.25 mm	10.59 ± 2.40	2.45 ± 0.15
	0.50 mm	15.63 ± 4.42	3.62 ± 1.02
	1.00 mm	18.22 ± 4.60	4.22 ± 1.06
	2.00 mm	31.87 ± 9.14	7.38 ± 2.12
	4.00 mm	35.38 ± 7.42	8.19 ± 1.72
Diameter	6 mm	31.87 ± 9.14	7.38 ± 2.12
	8 mm	39.73 ± 6.53	9.20 ± 1.51
	12 mm	49.53 ± 18.67	11.46 ± 4.18
Surface condition	Air-inhibited	120.5 ± 17.22	27.89 ± 3.98
	Air-excluded	31.87 ± 9.14	7.38 ± 2.12
Temperature	4 °C	20.58 ± 3.59	4.76 ± 0.83
	37°C	31.87 ± 9.14	7.38 ± 2.12
	60°C	44.81 ± 8.27	10.37 ± 1.91
Curing time	15 s	25.51 ± 4.26	5.91 ± 0.99
	30 s	31.87 ± 9.14	7.38 ± 2.12
	60 s	28.52 ± 2.98	6.60 ± 0.69
	Uncured	214.9 ± 69.08	49.75 ± 15.99

Two conditions, air inhibition of the surface during cure and non-light activating the material, were associated with much higher release than any other variable. Therefore, early release was greatly enhanced by air inhibition of the surface during cure or if the resin was uncured, 4X and 7X that of the air-excluded and 30 s cure (that is, basic protocol) respectively. Early release was not directly related to amount of composite resin present, since doubling the thickness or diameter did not double the release but thicknesses below 1 mm showed a trend toward a direct relationship. The geometric arrangement of composite resin also had little influence on early release since molds of similar volume but of different linear dimensions (*viz.* 4 mm thickness and 8 mm diameter) exhibited comparable release.

Table 3.6 presents the findings for the cumulative release of TEGDMA from Foundation by 3 days for each variable examined. Means and standard deviations about these means are listed. The data presented in Tables 3.5 and 3.6 provide a convenient comparison between total release and early release respectively for all the composite resin examined.

Table 3.6 Comparison of cumulative amount by 3 days for Foundation

Category	Variable	Cumulative amount of release (nmol)
Thickness	0.25 mm	54.42 ± 5.482
	0.50 mm	74.19 ± 15.38
	1.00 mm	90.51 ± 7.39
	2.00 mm	120.7 ± 23.13
	4.00 mm	228.2 ± 35.68
Diameter	6mm	120.7 ± 23.13
	8mm	97.07 ± 7.09
	12 mm	164.0 ± 7.56
Surface condition	Air-inhibited	169.2 ± 20.32
	Air-excluded	120.7 ± 23.13
Temperature	4 °C	74.34 ± 12.5
	37 °C	120.7 ± 23.13
	60°C	125.4 ± 30.18
Curing time	15 s	144.6 ± 11.06
	30 s	120.7 ± 23.13
	60 s	119.9 ± 14.55
	Uncured	2662 ± 312.4

By 3 days, the highest accumulated amounts of TEGDMA occurred when the composite resin was uncured and the smallest release occurred with the least thickness. Not surprisingly, greater surface area of composite resin exposed to water was associated with an increased total TEGDMA release and it appeared that increased bulk (thickness) of material also had this effect. Air-inhibition during curing increased TEGDMA release as did halving the curing time.

3.10 General discussion

In the current work, the release of TEGDMA from composite resin into water was examined by analysis of the water sample (release medium) by HPLC and MS. In order to discuss the findings, it is pertinent to first briefly address the merits and shortcomings of the experimental apparatus used.

It was intended that the dimensions of the physical experimental apparatus used would duplicate a typical order of magnitude of the surface area and composite resin thickness obtained clinically. The stainless steel molds standardized these physical parameters whilst enabling easy manipulation of the physical variable of interest, such as thickness. The composite resin remained confined in the mold throughout the experiment. This experimental arrangement differed from that used by other workers, for example, where composite resin was removed from the mold and immersed (Øysaet *et al.*, 1988; Ferracane and Condon, 1990; Ferracane 1994) or where composite resin was shaped into spherical units and immersed (Inoue and Hayashi, 1982). The surface of the composite resin was not treated in any way prior to immersion in water so release was neither enhanced nor diminished by these factors. This contrasts to the experimental design of other workers who either removed the inhibited surface layer by surface grinding with carbide paper prior to immersion (Øysaet *et al.*, 1988) or heated the resin prior to immersion (Caughman *et al.*, 1991). For these reasons, only limited quantitative comparisons between different studies and the current data can be made.

The choice of type 1 water as the release medium was important from both clinical and experimental points of view. The release of components into water more likely represents the release that may occur clinically (into saliva) although other release media have been used by other workers. Alcohol, as the release medium, has been used by several workers. The use of artificial saliva or artificial extracellular fluid, although more clinically appropriate, would have severely compromised HPLC analysis of released components due to matrix interferences. The water used was free of detectable contaminants, maintained a small pH range (7.3-6.9) and was freshly obtained for each time interval. During longer time intervals of the experiment, when water replacement was not frequent, the pH range decreased to approximately 6.2-6.8.

The diluent monomer, TEGDMA, was released from composite resin into water in readily detectable amounts throughout the time course. Other components, such as Bis-GMA, may also have been released, but in amounts below the level of detection of the analytical technique used. This observation is consistent with the findings of other workers using HPLC (Fujisawa and Masuhara, 1979; Inoue and Hayashi, 1982; Fan, 1985) and other analytical technologies (Thompson *et al.*, 1982; Munteanu *et al.*, 1987a; Ferracane and Condon, 1990) although Inoue and Hayashi (1982) also found Bis-GMA. The minimal (trace amount) release of other components is probably attributable to either their small proportion in the material, their limited solubility in water (Table 2.3) or their virtually complete reaction during polymerization, for example, the component BHT (Cook, 1985).

The TEGDMA release seen for Foundation suggests that this composite resin may not be typical of Bis-GMA composite resins. The data showed that cumulative TEGDMA release from Foundation by 3 days was approximately 4X that for the interval time zero to 4.32 min, using the basic protocol. This relationship was similar for the variables relating to material bulk (that is, thickness and diameter). For those variables relating to the surface condition (that is, air-inhibition during light activation) the relationship differed markedly (1.4 X and 12.4 X data for the basic protocol respectively). This suggests that release was highly dependent on the conversion achieved in the composite resin, which supports the comments of Ferracane (1994).

Component release has been examined by other workers in different contexts. Component elution has been examined as an index of degree of conversion (Ruyter and Svendsen, 1977; Ruyter and Øysæd, 1982; Venhoven *et al.*, 1993), for analysis of monomer content (Asmussen, 1975a; Asmussen, 1980a; Ruyter and Sjøvik, 1981; Vankerckhoven *et al.*, 1981; Ban *et al.*, 1982; Vankerckhoven *et al.*, 1982; Munteanu *et al.*, 1987a; Ruyter and Øysæd, 1987; Rueggeberg and Craig, 1988; Yoshida *et al.*, 1992), as a function of change in mechanical properties (Ferracane and Marker, 1992) and correlated with degree of cure (Ferracane, 1994). Monomer release can also vary according to factors likely to have effected conversion, for example, post-cure heat treatment of the composite resin. Monomers are also released from the composite resin when under the action of alcohol solvents. Inoue and Hayashi (1982) found that only Bis-GMA was eluted from composite resin, but sample treatment prior to analysis probably caused premature loss of lower molecular weight monomers by volatilization. The early high release

rate of TEGDMA observed for the composite resins examined in the present study did, however, confirm the observations of Ferracane and Condon (1990) who found 75% of "elutable species" were extracted from composite resin within several hours.

Having discussed some limitations of the experimental method, it is now relevant to consider composite resin as a material capable of component release to the biophase and to consider which characteristics of the material are responsible for this phenomenon. Composite resin is a heterogenous, highly cross-linked polymer dependent on intermolecular secondary bonds to maintain structure. Ideally, the polymer contains minimum residual monomer after polymerization (Brauer and Argentar, 1983). It is susceptible to both disintegration of its network and chemical reaction of its component units when subjected to adverse conditions (Guy, 1972). It may be suggested that component release from dental composite resin can be considered the consequence of several physical factors: *incomplete conversion* and *residual unsaturation* of a polymer allowing monomer release, *solubility of a solid in a solvent* and, possibly, *polymer degradation*. These factors are discussed below.

Residual unsaturation in composite resin materials corresponds to degree of conversion and is a complex physical phenomenon in composite resin materials. A proportion of the methacrylate groups remain unreacted even under conditions of optimal cure and this degree of unsaturation makes available variable amounts of methacrylate-containing species or unpolymerized dimethacrylate molecules which can be retained unreacted or diffuse out of the material. A degree of residual unsaturation is exploited clinically in optimizing adherence between layers of composite resin by the reaction of unreacted species within the new layer with available species in the deeper layer (Vankerckhoven *et al.*, 1982). Component release can be considered a consequence of imperfect conversion occurring within the material, or on the surface, during polymerization. In fact, an indirect correlation between the proportion of "leaching" monomers and the degree of conversion has been noted (Rueggeberg and Craig, 1988; Pham and Ferracane, 1989). Many physical aspects determine the degree of conversion in composite resin materials. Some of those are discussed below in light of the present study, namely, diluent monomer content, resin thickness and curing depth, polymerization kinetics, filler characteristics and oxygen inhibition of cure.

The *nature of the diluent monomer* can effectively lower conversion by hastening "vitrification" of the polymer in contributing to relatively heavy cross-linking (Venhoven *et al.*, 1993). The quantity of double bonds also varies inversely with the concentration of the diluting monomer (Ruyter, 1985). In the present study, the composite resin, Foundation, eluted significant amounts of TEGDMA as did the other TEGDMA-containing resins tested. It could be predicted, therefore, that smaller component release would be observed from a composite resin containing a higher order diluent methacrylate due to better conversion in these resins. It may be relevant to examine component release from experimental resins containing varying blends of TEGDMA/Bis-GMA or indirect composite resin curing technology employing post-cure treatments such as heat or Argon perfusion.

Conversion is *thickness* dependent, such that thicker layers of composite resin receive less reliable light activation in their depths and so are less reliably cured (Ruyter and Øysæd, 1982). Although it can be reasoned that greater thicknesses of material must contain more of each component, the question of degree of conversion in composite resins influences the availability of these components. Material below the critical depth for efficient curing by the light source, in fact, is likely to remain uncured and therefore poorly converted. Clinically, this depth is considered to be 2-3 mm. Below this depth the quantity of remaining methacrylate groups increases rapidly (Ruyter and Øysæd, 1982). The concentration of available unreacted methacrylate groups is presumably higher in these deeper regions and, it may be suggested, in sufficient time these species may diffuse through the matrix and be released from the surface. Data consistent with this scenario were observed in composite resin of 4 mm thickness where a significantly higher amount of TEGDMA was released from 144 min after cure. Up to this time, TEGDMA release was comparable for all thicknesses, indicating the surface had been similarly cured in all the molds and was a consistent source of components. Diluent monomers like TEGDMA are present in the resin matrix in part because they readily diffuse through the polymer matrix to participate in chain growth (Venhoven *et al.*, 1993).

Polymerization kinetics as presented in Chapter 1, describes the physical changes which take place in composite resin following activation. From equation 1.2 it can be seen that early in polymerization of common

polymers, the rate of polymerization is directly related to monomer concentration, especially when poorly efficient initiators are used (Nicholson, 1991b). For light-activated polymers, polymerization kinetics are similar but other factors exist. Polymerization shrinkage, in occurring before and after the gel point is reached, is accompanied by an abrupt rise in viscosity which limits monomer diffusion through the polymer matrix (Cook, 1985) and reduces the rate constant for termination. TEGDMA was observed to be freely released from uncured composite resin in relatively large amounts. In cured material, TEGDMA release is very much lower. Although this effect is most likely due to the reaction of the greater proportion of the TEGDMA content, the rigidity developing during polymerization may also limit TEGDMA diffusion through the composite resin.

Related to polymerization kinetics in composite resin is an interesting finding of Ruyter and Øysæd (1982) who identified a region within composite resin exhibiting an almost constant increase in the quantity of remaining methacrylate groups with increasing depth, terming this region an area of "iso-conversion". These authors found a "moderate increase in the region of iso-conversion with increasing exposure time" for visible light-activated composite resins. Although the current methodology did not employ a method of determining conversion, a trend toward increased component release with lower exposure time was noted in the current study, suggesting a possible decrease in the region of iso-conversion. It would be of interest to examine this concept of the iso-conversion region and the possibility of this being an index of the degree of conversion for composite resin.

Component release rate was highest in the earliest time interval, (under 5 min), suggesting that the surface was a rich source of components. This surface probably exhibits a characteristic energy which is lowered (to reach equilibrium) by segregation of components entering and leaving from the surface (adsorption) (Brophy *et al.*, 1970). Before equilibrium is established, diffusion from the surface creates a concentration gradient. In the current study, physical factors which increased diffusion include increased surface area and porosity but even greater component release was seen when the surface was uncured or air-inhibited during cure.

Filler particle characteristics such as particle size and refractive index also influence conversion. A match of the refractive indices of the resin matrix and filler promotes the passage of light into the material. Microfilled resins exhibit smaller depths of cures and higher amounts of unconverted bonds than that seen in the conventionally-filled resins. Larger particles admit deeper penetration of activating light and so exhibit better conversion (Ruyter and Øysæd, 1982). Fillers also reduce permeation in polymers, except when the filler is not bonded or uniformly wetted by resin so that the filler or the interface absorbs penetrant. Increased diffusion can be attributed to cracking or grazing of the material, characteristics which are intrinsic to filler nature (Sheldon, 1972) although silane-coupling of filler reduces the potential for this to occur. The filler itself may "leach" out of the composite resin. Söderholm (1990), for example, found that leaching of filler was linear with time over a six month observation period. Whilst the current work did not examine filler characteristics, it is reasonable to suggest that the interface disturbance, which must accompany this leaching pattern, has implications for release of resin components.

Oxygen inhibition during curing reduces the degree of conversion by successfully competing with monomer for reaction with radicals (Rueggeberg and Margeson, 1990) although inhibitor content is also relevant (Asmussen, 1981). This effect causes the formation of a layer which is monomer-rich and similar in basic chemistry to an unfilled resin. The layer is reported to be 5-100 μm thick (Ruyter, 1981) but varies with material viscosity and is probably responsible for the high component release seen in the air-inhibited group.

The release of components can also be considered to be an example of the solubility of a solid in a liquid. Factors that determine the solubility of a solid in a liquid are well established in physical chemistry. These include the area of contact between the solid and the liquid and the potential energy of the molecules to overcome attractive forces within the solid, which determines the rate at which the molecules leave the surface, and the rate of return. In general, an equilibrium is attained when the rate of molecules leaving a solid is proportional to the rate of return (Parry *et al.*, 1970). This, however, assumes that the elution volume is constant, which is not the case when composite resin is placed clinically and components are released into either the oral fluids or (potentially) through dentine to the pulpal space. In both cases, the elution volume is constantly renewed. Component release

from composite resin under the present experimental conditions can be considered to demonstrate solubility of some components of a multi-phase in water where increased component release was observed with increased surface area. It is unlikely that a characteristic solubility equilibrium was attained in the current experiment since composite resins demonstrate a low diffusion coefficient of 1.1 to $3.1 \times 10^{-9} \text{ cm}^2/\text{s}$ (Craig, 1981) and the elution volume was changed regularly.

Degradation of polymers is a complex phenomenon and accounts for component release from materials in service (Nicholson, 1991a). In the current experiment, both the time course and the conditions were clinically relevant, but were probably insufficient to have caused detectable degradation of the composite resin. What was probably of more importance was the influence of water immersion on the material.

The effect of water on composite resin is influenced by temperature, stress created by the time of contact, nature and pre-treatment of the filler. The initial effect of water immersion of composite resin is simple void replacement (Kalachandra, 1989). Subsequently, water sorption and loss of components due to solubility and hydrolysis occur. The absorbed water exists in the form of hydrogen-bonded molecules and causes swelling of the resin matrix thereby facilitating diffusion along filler/resin interfaces (Delasi and Whiteside, 1978). The physical integrity of the network structure is thus disturbed. Craig (1981) cited the water sorption of composite resin as 0.6 mg/cm^2 . The hydrophilic nature of TEGDMA tends to promote water sorption, but this is at least partially resisted by the common practice, in the manufacture of dental composite resins, of improved cross-linking. Incomplete polymerization is characterised by higher water sorption and therefore a greater potential for release of components. Foundation composite resin is heavily cross-linked and hydrophilic, and in its cured state showed only low-grade component release in comparison to the uncured state. In the uncured state, the effects of water sorption would have contributed to component release, by facilitating diffusion. Water is a weak solvent of cured composite resin and so is likely to be responsible for only very slow component release by dissolution. Initial component release appears to be a superficial phenomenon independent of changes in deeper layers. Hydrolytic degradation accounts for component release over a longer time period although the action of enzymes dissolved in saliva is more marked in this effect (Munksgaard and Freund, 1990). TEGDMA, for

example, is most susceptible, regressing by hydrolysis, to EGDMA and methacrylic acid.

In summary, the release of a component of composite resin into water is important for several reasons. A diluent monomer component is a consistent and major component of all dental composite resins, vital to the basic physical state of the material, and although this component participates in polymerization, it is rarely completely expended in the process. Release may be enhanced in clinical use by such practices as variation in the curing process, and particularly if the material is incompletely uncured or the surface is exposed to air during activation.

It is important now to examine how the observed patterns of release are modified by dentine. This will be examined in subsequent Chapters. The following Chapter presents a brief review of dentine structure and characteristics.