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Summary

From the extensive literature review presented in the first chapter, it could be concluded that food safety and quality can be influenced by the chemical interactions between packaging materials and foodstuffs. Especially for plastics a material transfer, defined as migration, from the contact material to the food has been intensively studied within this respect. Based on the fundamental aspects of migration, important parameters controlling the phenomenon were identified. In addition, two legislative approaches, issued to protect public health by reducing the exposure to migrants up to tolerable levels, were discussed in detail. Finally the current analytical methodology to assess overall and specific migration was reviewed.

In the presented study, an additional contribution to this particular field of food science was made. Basically research concentrated on the following three main topics:

- chemical characterization of polyglycerol fatty acid esters (Chapter 2)
- the development of an immunochemical method for bisphenol A analysis in foodstuffs (Chapters 3 and 4)
- evaluation of the chemical interactions between food and active and intelligent packaging materials (Chapter 5)

In each of these sub-studies, innovative analytical approaches in the particular field of chemical interactions between packaging materials and foodstuffs are introduced.

Polyglycerol fatty acid esters consist of a complex group of compounds, which can be applied as plastic additives. From the mechanisms about migration reviewed in the first chapter, it could be concluded that a good characterisation of a migrant is of prime importance. Therefore an analytical methodology to characterise this complex group of compounds was elaborated (Chapter 2). The method was based on an extensive liquid and gas chromatographic separation of the main constituents of these esters. It essentially consisted of two steps: the characterisation of the polyglycerol moiety after saponification of the esters and the analysis of the esters themselves. Using capillary gas chromatography, the trimethyl silyl ethers of the polyglycerols could be determined quantitatively up to the tetruglycerols. Since standards with an acceptable purity of tri- and tetruglycerol were not available however, these were obtained via a column chromatographic fractionation. A further elaboration of the gas chromatographic analysis by using cold-on column injection combined with the use of a short capillary column, enabled the qualitative analysis up to
heptaglycerol.
From an in depth study of the profile of the gas chromatogram from fractionated di-and triglycerols, a possible identification of the non-cyclic and cyclic diglycerol and non-cyclic triglycerols was presented. Consequently a qualitative idea about the presence of these side products in a polyglycerol sample could be obtained.

From the gas chromatographic analysis of the polyglycerol fatty acid esters using a cold-on column injection and a short capillary column, it was revealed that some of the esters present in the sample co-eluted. Therefore, for the analysis of the esters, a combined liquid and gas chromatographic analysis was necessary.

Using again a column chromatographic fractionation technique, standards of sufficient purity of the following esters could be obtained: mono-and di-esters of di- and triglycerol. Using a slightly modified column chromatographic fractionation, three fractions containing respectively the di-esters of diglycerol, the di-and mono-esters of respectively tri-and diglycerol and finally the mono-esters of tri-and tetruglycerol were obtained. These fractions could be analysed by capillary gas chromatography without any risk of co-eluting peaks. Since for all these esters, except for the mono-esters of tetruglycerol, pure standards were available, qualitative analysis of a polyglycerol esters would be possible.

Apart from the quantitative data, also qualitative data about the following components became available using the methodology presented: mono-esters of tetruglycerol and the various isomers of the mono-esters of diglycerol.

In the second part of this study, the applicability of immunological techniques to analyse bisphenol A in food matrices was evaluated. Bisphenol A is a monomer used in the production of the high quality plastic, polycarbonate and in the production of epoxy coatings. It is a compound of particular interest because of its xeno-estrogenic character and its suspected carcinogenicity. From the literature review, it was revealed that essentially classical instrumental methods are used for bisphenol A analysis, often requiring extensive sample clean-up.

In order to raise bisphenol A specific antibodies, a suitable bisphenol A hapten was prepared (Chapter 3). The hapten consisted of a bisphenol A molecule to which a five carbon membered spacer arm, containing an end-standing carboxylic acid group, was attached at one of the phenolic hydroxyl groups. Thus coupling to a protein could be achieved using the carboxylic acid moiety of the hapten and one of the phenolic hydroxyl groups was still available for immunological interactions.
The hapten was coupled to bovine serum albumin to obtain an immunizing antigen which was injected into chicken hens. The antibodies could conveniently be isolated from the egg yolk. Since eggs are produced daily and antibody concentration is relatively high, an almost infinite source of antibodies was obtained by using the presented immunization methodology.

Reactivity towards bisphenol A was evaluated in an indirect enzyme-linked immunosorbent assay using a bisphenol A-ovalbumin coating antigen. Because the isolated antibodies showed indeed reactivity towards bisphenol A, they were applied in a similar competitive indirect enzyme-linked immunosorbent assay (Chapter 4). The assay proved to be quite sensitive towards possible matrix effects such as the ionic strength and the presence of surface active components like proteins. It was more over revealed that the sensitivity of the assay was lower compared to the classical instrumental techniques or of the recently developed immunoassays for bisphenol A using mono-or polyclonal mammalian antibodies. Variation of several assay parameters resulted in an optimised \( I_{50} \) level of about 2.5 \( \mu \text{M} \) in aqueous solutions.

As a further elaboration of the indirect competitive assay, cross reactivity towards several structural analogues or other xeno-estrogenic compounds which could also migrate from food contact materials, was investigated. Essentially the assay could be considered as very specific. Only for analogues which showed strong molecular similarities significant cross reactivity was observed (maximal 43 \%).

In order to evaluate the applicability of the assay for the analysis of real food samples, milk and oil were considered. By an adjustment of the ionic strength of the buffers used throughout the assay, the loss in sensitivity could be minimized during the direct analysis of milk (\( I_{50} \) level of about 25 \( \mu \text{M} \)). For the analysis of oil, extraction of bisphenol A by an aqueous methanol solution was necessary. Methanol however affected the assay characteristics. A concentration of about 20 \% of methanol resulted in similar sensitivities as observed for the assay in milk and was therefore considered to be acceptable.

In the last part of this study (Chapter 5) the chemical interactions between active and intelligent packaging systems and food were investigated. Active food packaging can be defined as a material which changes the condition of the packed food in order to extend its shelf-life and/or improve its safety and its sensory properties. Intelligent packaging materials monitor the condition of the packed food to give information about its quality during its distribution. They can be considered as a major recent development in the packaging technology of foods. In a EU FAIR R&D research project, called ‘ACTIPAK’ (CT 98-4170), several active and intelligent packaging materials were
collected in order to evaluate their composition and their overall migration behaviour. From their compositional analysis, it seemed that most of these materials were not composed solely out of plastics. Despite this fact, EU plastic packaging legislation was considered for the classification of these materials. It could be concluded that several of the active components present in these kind of packaging materials were not on the positive lists specified in the EU legislation. From the overall migration studies in the official EU food simulants, it could be concluded that several active food packaging materials exceeded the limits specified. It was revealed however that the applied migration methodology did not correspond with the actual use of some of the active packaging materials. Therefore, the use of a moisture-rich solid food simulant was introduced. Using agar gels, more realistic overall and specific migration levels were obtained which were moreover comparable to the levels observed in real food matrices. This alternative aqueous food simulant was especially useful to quantify the specific migration from the selected active packaging systems. Despite the introduction of a more realistic migration simulation methodology, only 20 % of the investigated active and intelligent packaging systems were in agreement with current food contact material legislation taking into account the overall migration data and their composition. Since in addition for a lot of the tested systems however, the plastic directives did not apply, it is clear that an urgent need for a dedicated legislation applicable in the whole EU exists. If however the additional legislative problems, for example with the food additive legislation, are considered which can be expected if such packaging materials are introduced on the European market, it seems that a case by case evaluation seems appropriate when application of these new technologies on the EU-level should be realised within the near future without endangering food safety and quality.
Samenvatting

Uitgaande van het uitgebreide literatuuroverzicht (hoofdstuk 1) kon besloten worden dat de veiligheid en kwaliteit van het voedsel in een belangrijke mate kunnen beïnvloed worden door de chemische interacties tussen verpakkingsmaterialen en het levensmiddel. In het bijzonder werd voor plastic de materiaaloverdracht, beter gekend als migratie, vanuit het contact materiaal naar het levensmiddel intensief bestudeerd. Op basis van de fundamentele aspecten, werden belangrijke parameters welke het migratie fenomeen beheersen, geïdentificeerd. Daarenboven werden twee wettelijke benaderingen, welke de volksgezondheid moeten garanderen door de blootstelling aan migratieresiduen tot aanvaardbare niveaus te beperken, in detail besproken. Tot slot werden de courante analytische methoden om globale en specifieke migratie te bepalen besproken.

In het voorgestelde onderzoek wordt een bijkomende bijdrage aan dit specifieke domein van de levensmiddelenwetenschappen gepresenteerd. Het onderzoek spitste zich in essentie toe op de volgende drie onderwerpen:

- chemische karakterisering van polyglycerol vetzuur esters (hoofdstuk 2)
- de ontwikkeling van een immunochemische techniek voor het bepalen van bisfenol A in levensmiddelen (hoofdstuk 3 en 4)
- evaluatie van de chemische interacties tussen actieve en intelligent verpakkingsmaterialen en levensmiddelen (hoofdstuk 5)

In elk van deze delen is getracht een innovatieve analytische techniek in het domein van de chemische interacties tussen levensmiddelen en verpakkingsmaterialen te introduceren.

Polyglycerol vetzuur esters zijn een complexe groep van verbindingen, welke onder andere hun toepassing vinden in de plastiek industrie als additief. Vanuit de fundamentele kennis over het migratieverschijnsel kon besloten worden dat een goede karakterisering van de migranten van primordiaal belang is. Daarom werd een analytische methode ontwikkeld die moet toelaten de samenstelling van deze verbindingen te ontrafelen. De methode is gebaseerd op een doorgedreven vloeistof- en gaschromatografische scheiding van de belangrijkste componenten die aanwezig zijn in deze polyglycerol esters. De methode bestond in essentie uit twee delen: de karakterisering van het polyglycerol gedeelte na verzeping van de esters en de analyse van de esters zelf (hoofdstuk 2). Aan de hand van capillaire gaschromatografie, werden de trimethyl silyl ethers van polyglycerolen kwantitatief bepaald tot en met de tetraglycerolen. Aangezien zuiver tri-en tetraglycerol niet ter
beschikking bleken, werden aan de hand van een kolom chromatografische fractionatie voldoende zuivere standaarden van deze componenten bekomen. Door een verdere uitbreiding van de gaschromatografische analyse van de polyglycerolen, door gebruik te maken van de on-column injectie techniek in combinatie met het gebruik van een korte capillaire kolom, werd bovendien de kwalitatieve analyse tot en met heptaglycerol mogelijk gemaakt.

Door een diepgaande studie van het gaschromatografisch profiel dat bekomen werd na de analyse van gefractioneerde di- en triglycerol stalen werd een mogelijke identificatie naar voren geschoven betreffende respectievelijk de niet cyclische en cyclische diglycerol isomeren enerzijds en de niet cyclische triglycerol isomeren anderzijds. Bijgevolg kon een kwalitatief idee verkregen worden omtrent de aanwezigheid van deze producten in een polyglycerol ester.

Uit de gaschromatografische analyse, aan de hand van een on-column injectie techniek en een korte capillaire kolom, van polyglycerol esters, bleek dat verschillende componenten co-elueerden. Daarom werd voor de analyse van de esters een gekoppelde vloeistof- en gaschromatografische scheiding geïntroduceerd.


Naast de kwantitatieve data, was het ook mogelijk om kwalitatieve informatie te bekomen over de volgende componenten : mono-esters van tetraglycerol en de verschillende isomeren van de mono-esters van diglycerol.

In het tweede deel van dit werk, werden de mogelijkheden onderzocht om immunochemische technieken toe te passen om bisfenol A te analyseren in levensmiddelen. Bisfenol A is een belangrijk monomeer dat gebruik wordt om polycarbonaat en epoxy harsen te produceren. Het is een verbinding waaraan de laatste jaren bijzondere aandacht werd besteed gezien zijn xeno-estrogeen karakter en zijn mogelijke carcinoogene werking. Uitgaande van het literatuur overzicht kon
besloten worden dat de huidige analyse methodieken instrumentaal zijn die bovendien dienen vooraf te gaan door een uitgebreide staalvoorbereiding.

Om de productie van bisfenol A specifieke antilichamen te bewerkstelligen, diende een geschikt bisfenol A hapteen gesynthetiseerd te worden (Hoofdstuk 3). Het hapteen bestond uit een bisfenol A molecule waarvan aan één van de fenolische hydroxylgroepen een koolstof arm, bestaande uit vijf koolstof atomen en een eindstandige carbonzure groep, werd gekoppeld. Op deze manier kon de covalente koppeling van het hapteen aan verschillende eiwitten gerealiseerd worden. Bovendien was de tweede vrije fenolische hydroxylgroep nog steeds ter beschikking om immunochemische reacties te induceren.

Het hapteen werd gekoppeld aan serum albumine van runderen om een immuniserend antigeen te bekomen. Dit antigeen werd geïnjecteerd bij leghennen. De antilichamen werden op een elegante manier geïsoleerd uit het eigeel. Aangezien dagelijks een ei per kip werd bekomen en aangezien de concentratie van immunoglobulinen in het eigeel vrij hoog is, werd een nagenoeg onuitputtelijk bron van antilichamen aangeboord aan de hand van de gebruikte immunisatie strategie.

De reactiviteit van bisfenol A ten opzichte van de geïsoleerde immunoglobulinen werd onderzocht aan de hand van een indirecte enzym-gelieerde immunosorbent assay (ELISA). Hiertoe werd een coating anitigeen gesynthetiseerd door het hapteen te koppelen aan ovalbumine.

Aangezien de geïsoleerde antilichamen behoorlijke reactiviteit vertoonden ten opzichte van bisfenol A, werden ze toegepast in een analoge indirecte competitive ELISA (Hoofdstuk 4). De assay bleek vrij gevoelig te zijn aan mogelijke matrix effecten, zoals de aanwezigheid van zouten of oppervlakte actieve stoffen, zoals bijvoorbeeld eiwitten. Bovendien werd duidelijk dat de assay minder goed presteerde op het vlak van gevoeligheid in vergelijking met de huidig beschikbare instrumentele technieken en de recent ontwikkelde ELISA’s welke gebruik maken van mono-en polyclonale antilichamen, geïsoleerd uit zoogdieren. Door een afstellen van verschillende assay parameters, kon een I₅₀ waarde van ongeveer 2.5 µM bereikt worden in waterige oplossingen.

Bij een verdere evaluatie van de indirecte competitieve ELISA, werd de kruisreactiviteit van verschillende structurele aanverwante verbindingen, alsook andere xeno-estrogene verbindingen welke kunnen migreren vanuit verpakkingsmaterialen, onderzocht. De antilichamen konden als bijzonder specifiek omschreven worden. Enkel voor zeer sterk gelijkende molecules werd een belangrijke kruisreactiviteit vastgesteld (maximaal 43 %).

Om de toepasbaarheid van de assay voor de analyse van echte levensmiddelen na te gaan, werden melk en olie beschouwd. Door een aanpassing van de ionensterkte van de buffers welke gebruikt worden gedurende de assay, werd het verlies in gevoeligheid dat waargenomen werd in melk
beperkt (I_{so} van ongeveer 25 \mu M). Voor de analyse van olie was een extractie van bisfenol A met een waterige methanol oplossing noodzakelijk. Methanol evenwel had een nefaste invloed op de werking van de assay. Toch werd met oplossingen die tot 20 % methanol bevatten gelijkaardige resultaten bekomen inzake gevoeligheid als bij de analyse van melk.

In het laatste deel van dit werk (Hoofdstuk 5), werden de chemische interacties tussen actieve en intelligente verpakkingsmaterialen enerzijds en levensmiddelen anderzijds onderzocht. Actieve verpakking kan gedefinieerd worden als een materiaal dat het verpakte levensmiddel op een dusdanige manier gaat veranderen dat zijn houdbaarheid en/of veiligheid en zijn sensoriële eigenschappen verbetert. Intelligente verpakkingsmaterialen geven informatie betreffende de kwaliteit van het verpakte levensmiddel. Deze verpakkingstechnieken kunnen beschouwd worden als één van de belangrijkste recente ontwikkeling op het vlak van verpakken van levensmiddelen. In een EU FAIR R&D project (ACTIPAK, CT 98-4170), werden verschillende actieve en intelligente verpakkingsmaterialen verzameld om hun samenstelling en hun globaal migratie gedrag te evalueren.

Uitgaande van de samenstelling van deze materialen, bleek dat het merendeel uit meer dan alleen maar plastic bleek te bestaan. Desondanks werden de gecollecteerde materialen geklasseerd op basis van de EU directieven welke toepasbaar zijn op plastic contact materialen. Er kon besloten worden dat verschillende verbindingen die noodzakelijk zijn voor het werkingsmechanisme van de actieve of intelligente verpakking niet opgenomen zijn in de positieve lijsten welke vervat zijn in de EU wetgeving.

Op basis van de studies omtrent hun migratiedrag in de officiële levensmiddelen simulanten, bleek terug dat verschillende materialen niet bleken te voldoen aan de wettelijk voorziene eisen. Het bleek evenwel dat de toegepaste methodiek om de globale migratie te bepalen niet direct overeenstemmende met de te verwachten gebruiksomstandigheden van sommige systemen. Daarom werd het gebruik een vochtrijke maar vaste simulant ingevoerd om het migratie fenomeen te onderzoeken. Het gebruik van agar gels bleek inderdaad aanleiding te geven tot meer realistische globale en specifieke migratie waarden, die zelfs goed overeenstemden met de migratiewaarden waargenomen in echte levensmiddelen.

Ondanks de invoering van de meer realistische simulant om migratiestudies uit te voeren, bleek slechts 20 % van de geteste materialen te voldoen aan enerzijds de beperkingen inzake samenstelling en anderzijds de globale migratie limiet, welke van toepassing zijn voor plastic verpakkingsmaterialen. Omdat bovendien een deel van deze systemen bleek samengesteld te zijn
uit verschillende materialen, is het duidelijk dat er een dringende nood is aan een wetgevend kader omtrent de toepasbaarheid van deze nieuwe verpakkingstechnologieën binnen de Europese Unie. Indien evenwel wordt rekening gehouden met bijkomende wettelijke restricties die van toepassing kunnen zijn, zoals de wetgeving op additieven bijvoorbeeld, lijkt het dat een evaluatie van elk individueel systeem voorlopig de snelste manier is om de toepassing van deze materialen mogelijk te maken binnen de Europese markt zonder dat er een gevaar wordt gecreëerd inzake de voedseelveiligheid of voedselkwaliteit.
1. Introduction: migration from food contact materials

1.1. Food contact and packaging materials

1.1.1. Classes of food contact materials

During the handling of agricultural raw materials, during their processing and transformation into foods and during the transport of these products from the producers to the consumers, contacts with other materials frequently occur. The most common example for the end-user of the food is probably the packaging material. However, apart from a variety of packaging materials, a lot of other contact materials should be considered as well, e.g. stainless steel processing, transport or storage equipment, tubing for food transport, sealing materials in piping equipment, protection foils or lacquers used in storage facilities, etc.

Instead of classifying these materials according to their function or use, a classification based on their chemical characteristics is more convenient and appropriate. The European food legislation differentiates various classes of food contact materials as indicated in Table 1.

Table 1. Groups of contact materials requiring legislation within EU¹ (EEC, 1989)

| 1. plastics including varnish and coatings |
| 2. regenerated cellulose |
| 3. elastomers and rubber |
| 4. paper and board |
| 5. ceramics |
| 6. glass |
| 7. metals and alloys |
| 8. wood, including cork |
| 9. textile products |
| 10. paraffin and micro-crystalline waxes |

It should be stressed that this list is not involving only packaging materials, but all kinds of materials which can be in contact with an agricultural raw material or a foodstuff (Rossi, 2000).

¹ All abbreviations are summarized in Annex 1
1.1.2. Packaging materials

Packaging materials represent an important group within the food contact materials listed in Table 1. The use of these materials is very important for the food industry because packaging fulfils four essential functions (Robertson, 1993). Food packaging materials contain the food. Due to this containment, the food can be protected against a broad spectrum of deteriorating processes (e.g. light, oxygen, micro-organisms, etc.). The packaging allows moreover to create a communication with the consumer who can be informed about content of the package (e.g. brand, price, quantity, ingredients, producer, etc.). Finally it offers the possibility to increase the convenience of the packed food product (e.g. ready to eat meals, individual portions, etc.). Thus the same food can be packed in different packages in order to meet the different requirements of the various consumers (e.g. individual size, family size, etc.).

Various food contact materials are being used to pack food. In Table 2 an overview of the use of various packaging materials within the USA is represented (FDA, 1995a). It is striking that polymeric materials are predominantly used as such. This can be explained by the broad range of commercial plastics available and their diversity in functionality and applicability. In addition, polymeric materials seem to be frequently combined with other materials such as metal or paper. If these additional data are considered, it can be concluded that about 80 % of the packed food in the USA is contacted with polymeric materials. EU data with regard to the use of various packaging materials are unfortunately not available (Castle, 2000). Belgian data of Fechiplast (Fechiplast, 2000) indicate that the general plastic and elastomer production increased with 30 % from 1995 to 2000, illustrating the ever growing importance of the plastic industry in general and possibly of the plastic packaging industry in particular.

Table 2. Relative use of different food packaging materials within USA (FDA, 1995a)

<table>
<thead>
<tr>
<th>Packaging material</th>
<th>Relative use (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>10</td>
</tr>
<tr>
<td>Metal - polymer coated</td>
<td>17</td>
</tr>
<tr>
<td>Metal – not coated</td>
<td>3</td>
</tr>
<tr>
<td>Paper – polymer coated</td>
<td>20</td>
</tr>
<tr>
<td>Paper – not coated</td>
<td>10</td>
</tr>
<tr>
<td>Polymers</td>
<td>40</td>
</tr>
</tbody>
</table>
1.1.3. Plastic food packaging materials

Plastics can be regarded as macromolecular organic compounds which can be produced synthetically or by modification of naturally occurring products, like for example regenerated cellulose (Figge, 1996). The European legislator however considers plastics and regenerated cellulose as a different kind of contact material (Table 1). This is because of the special characteristics of regenerated cellulose compared to the other plastics (Rossi, 2000). Elastomers and rubbers on the other hand, are clearly different from plastics although they are also macromolecular organic compounds. In contrast to plastics however, elastomers exhibit, as the name suggests, an enormous elasticity due to a cross linked structure created by chemical vulcanisation (Sidwell, 1996). Elastomers can also be natural (e.g. rubber) or synthetic (e.g. fluorocarbon rubber). Figure 1 cites the main synthetic plastics used.

Figure 1. Overview of the main synthetic plastic materials used (Figge, 1996)

Basically, thermosets and thermoplasts can be differentiated depending on their thermal properties (Brandsch and Piringer, 2000). At sufficiently high temperatures, a thermoplastic will become liquid, as indicated in Figure 2. If this polymer is cooled down, crystallisation proceeds generally so slow that the polymer becomes super cooled or rubbery. Further lowering the temperature will finally result in a material that becomes glassy and relatively brittle, having physical properties similar to a crystalline solid, but due to the high molecular disorder is still a liquid. The temperature at which
this occurs, is called the glass transition temperature ($T_g$) (Robertson, 1993). Typical glass temperatures for some important polymers are the following: low density PE (237 K), PP (270 K); PS (373 K); PET (342 K) and PC (418 K) (Wunderlich et al., 1989; Xanthos and Todd, 1996). Thermosets on the other hand, will not become liquid upon heating due to their high cross linked structure (e.g. epoxy resins).

![Elastic modulus of a thermoplastic as a function of temperature](image)

**Figure 2.** Elastic modulus of a thermoplastic as a function of temperature (Schouten and van der Vegt, 1987)

With regard to their chemical synthesis, addition and condensation polymerisation can be distinguished. Addition polymerisation proceeds via a chain reaction between unsaturated molecules, initiated by the use of for example radical or ion formation. Condensation polymerisation however involves the reaction between two functional groups in organic molecules. Three possible reactions or groups are described: polycondensation (e.g. polyesters, like PET), polyaddition (not be confused with addition polymerisation, e.g. polyurethane) and ring opening reactions (e.g. epoxy coatings) (Figge, 1996; Brandsch and Piringer, 2000).

Apart from the fundamental component –the polymer– plastics contain other chemical components as well. Of course, the polymer may contain residual monomers and oligomers (e.g. Jickells et al., 1993; Kontominas, et al., 1985; Lickly et al., 1993). In addition to those however, also other low molecular weight substances may be present as well. Additives can be added to the polymer in

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2 All symbols, together with their units are summarized in Annex 2
order to alter the properties of the polymer in a desired way (Figge, 1996; Pospíšil and Nešpůrek, 2000). Table 3 shows the main additives used in plastic manufacture together with their function. Generally these additives are applied in relatively small concentrations, although for example fillers (e.g. silica) and plasticizers (e.g. phthalates) are used at high concentrations as well. Apart from these, also additives to the polymerisation medium should be considered, such as emulsifiers, solvents, thickening agents etc. (Pospíšil and Nešpůrek, 2000; EC, 2002). A third class of substances are the so-called aids to polymerisation, which directly influence the formation of polymers. Typical examples include catalysts, cross-linking agents, initiators, etc. (Pospíšil and Nešpůrek, 2000; EC, 2002). Finally also impurities, degradation or reaction products of plastic ingredients were found to be present in polymeric contact materials as well (Lichtenhaler and Ranfelt, 1978; Grob et al, 1999). Because most of these low molecular weight compounds are not covalently bound to the polymer chain, they are able to diffuse throughout the polymer matrix. As discussed in the following, this diffusion is one of the basic processes of the migration from plastic food contact materials.

Table 3. Main additives for plastics and their function (Pospíšil and Nešpůrek, 2000)

<table>
<thead>
<tr>
<th>Additive</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. nucleating agents</td>
<td>induce regular crystallisation</td>
</tr>
<tr>
<td>2. lubricants</td>
<td>improve processing above $T_g$, alters rheology</td>
</tr>
<tr>
<td>3. antistatic agents</td>
<td>reduce the chargeability of the plastic</td>
</tr>
<tr>
<td>4. blowing agents</td>
<td>generate inert gases to produce expanded plastics</td>
</tr>
<tr>
<td>5. plasticizers</td>
<td>gel the polymer, improve flexibility and processibility</td>
</tr>
<tr>
<td>6. antifogging agents</td>
<td>avoid water droplets on films used to pack moisture rich foods</td>
</tr>
<tr>
<td>7. dyes and pigments</td>
<td>impart colour of the plastic</td>
</tr>
<tr>
<td>8. fillers and reinforcing agents</td>
<td>increase bulk and improve physical properties</td>
</tr>
<tr>
<td>9. stabilizers</td>
<td></td>
</tr>
<tr>
<td>9.1. antioxidants</td>
<td>avoid polymer oxidation by trapping free radicals or by inducing decomposition of peroxides</td>
</tr>
<tr>
<td>9.2. UV absorbers</td>
<td>reduce harmful effect of UV radiation</td>
</tr>
<tr>
<td>9.3. heat stabilizers</td>
<td>prevent dehydrochlorination during processing of PVC</td>
</tr>
<tr>
<td>9.4. anti-acids</td>
<td>neutralise acids arising from catalysts or PVC thermo degradation</td>
</tr>
</tbody>
</table>
1.2. **Migration from plastics**

1.2.1. **Interactions in a packaging system**

In a food packaging system, three phases should be considered: the food, the package, and the environment (Figure 3). In between these phases interactions may occur, resulting in an energy or a mass transfer (Hernandez and Gavara, 1999). The mass transfer can be macroscopic as in the chipping of a glass container or microscopic as in the contamination of food by micro-organisms. The sub microscopic mass transfer however involves the diffusion of individual molecules in one phase and their sorption by the other. If mass transfer is restricted only to the food and the packaging material, the phenomenon is also known as migration (Katan, 1996a). It can be regarded as a chemical interaction between the food and its contact materials since it results in a transport of chemical substances from one phase to the other.

Figure 3. The three phases of a packaging system

Migration can take place from the contact material to the food and vice versa. The latter case is also known as negative migration, while the former is simply identified as migration. A typical example of negative migration is the flavour scalping in fruit juices due to the partial absorption of flavour compounds by the plastic contact material (e.g. Imai et al., 1990; Baner et al. 1991; Charara et al., 1992; Konczal et al., 1992). Due to this phenomenon the fruit juice aroma might be affected. Another example resulting however in an improvement of food quality, is the use of oxygen scavenging materials in the packaging of foodstuffs sensitive to oxidation (Klein and Knor, 1990).
The mass transfer from the packaging material to the food can have both deteriorating and improving consequences for the food. Migration of toxic packaging compounds to the food is a serious risk to food safety (Katan, 1996b). Similarly, migration of particular substances could induce sensorial deterioration of the food (Linssen et al., 1991; Piringer and Rütter, 2000). On the other hand, migration of particular food additives such as antioxidants (e.g. Wessling et al., 1999) and anti-microbial agents (e.g. Weng et al., 1999), could improve the shelf life of the product and at the same time minimize the direct use of these additives in the food manufacture.

The mass transfer can also involve the three phases of the packaging system. In this particular case, volatiles are transported from the environment via the contact material to the food or vice versa. This phenomenon is known as permeation (Hernandez and Gavara, 1999; Piringer, 2000a). In contrast to migration, no net uptake or removal of chemical substances from the food contact material takes place. The permeation process may significantly affect the quality of the food (Hernandez and Gavara, 1999). Mild preservation techniques such as modified atmosphere packaging, which are used successfully to prolong the shelf life of minimally processed foods (Devlieghere et al., 1999), are based on the selective permeation of particular gasses through the packaging material (Potter and Hotchkiss, 1995). Chemical contamination due to permeation of organic volatiles (e.g. solvents) through the packaging material has been reported as well (Marsili, 1997).

In the present discussion, especially the first type of sub microscopic mass transfer described – migration - is considered in more detail.

**1.2.2. Basic aspects of migration from plastic food contact materials**

**1.2.2.1. General principle**

Basically, the migration from plastics can take place in three ways as schematically illustrated in Figure 4 (Katan, 1996b). In the first case, the food is contacted single sided with the contact material on the one hand and with the environment on the other. The conveyor belt can serve as a typical example. In the second case, the food is only contacted with one or various contact materials. Typically most packed liquid foods can be seen as an example. For the final type, no direct contact between the food and the contact material exists. Direct contact however is not necessary to induce migration since a mass transfer via the headspace in the package is possible as well. A typical example could be the scavenging of secondary oxidation products by an aldehyde absorbing polymer incorporated in the contact material (Rooney, 1995).
Three important stages can be distinguished controlling the sub microscopic mass transfer in general and consequently the migration in particular: diffusion within the polymer, solvation at the polymer-food interface and dispersion into the bulk food (Lau and Wong, 2000; Hernandez and Gavara, 1999). First a low molecular weight compound will diffuse in the polymer in the direction of the food due to the presence of a concentration gradient (diffusion process). Subsequently, reaching the food-plastic interface, the migrant will be desorbed by the polymer and absorbed by the food (solvation process). Finally the migrant, currently dissolved in the food, will diffuse into the total food matrix, again due to the presence of a concentration gradient. Alternatively, the latter mass transfer can be accelerated due to a convection process inside the food matrix as will be discussed in more detail later.

The sorption and diffusion process can be described quantitatively by using the partition coefficient $K_{P/F}$ and the diffusion coefficients $D_P$ and $D_F$, where the indexes P and F refer to the polymer or plastic and the food respectively.

Figure 4. Schematic representation of migration from food contact materials. Symbols: E: environment; C/M: contact material; F: food; HS: headspace (explanation: see text)
1.2.2.2. Partition coefficient and the sorption process

The partition coefficient of a migrating compound between a polymer and a food can be defined as follows

\[ K_{P/F} = \frac{C_{P,\infty}}{C_{F,\infty}} \]  

where \( C_{P,\infty} \) and \( C_{F,\infty} \) are respectively the equilibrium concentration of the component in the polymer and the food (Franz, 2000). Basically this definition is derived from the assumption that in equilibrium conditions (time \( t=\infty \)) the chemical potential of the migrating substance in the polymer, \( \mu_P \), is equal to the chemical potential of the same compound in the food, \( \mu_F \) as described by Baner (2000).

Mainly the partition coefficient depends on the polarity of the substance and of the polarity of the two phases involved. The following simple example illustrates the importance of the partition coefficient.

At equilibrium conditions, the amount of migrated substance into the food, \( m_{F,\infty} \), can be calculated as follows. Supposing that initially no migrating substance was present in the food (\( m_{F,0}=0 \)) and that \( m_{P,0} \) represents the initial amount of migrant present in the polymer, than it can be concluded from the mass balance that

\[ m_{P,0} = m_{F,\infty} + m_{P,\infty} \]  

From equation [1] however

\[ m_{P,\infty} = \frac{m_{F,\infty} \times K_{P/F} \times V_P}{V_F} \]  

where \( V_P \) and \( V_F \) are respectively the volume of the polymer and the food.

Consequently,

\[ m_{F,\infty} = \frac{m_{P,0}}{1 + K_{P/F} \times \frac{V_P}{V_F}} \]  

Generally is can be assumed that \( V_P/V_F << 1 \). From equation [4] it can be concluded that in the case \( K_{P/F} << 1 \), the amount of migrated substance in the food in equilibrium conditions (\( m_{F,\infty} \)) equals the initial amount of substance present in the polymer (\( m_{P,0} \)). This implies that total migration of the substance out of the polymer occurred. If on the contrary, an apolar substance is applied in an apolar polymer contacted with water, it is clear that \( K_{P/F} >> 1 \) and from equation [4] it can be concluded that
This indicates that migration remains restricted. As most polymers used are (fairly) apolar (Figure 1) and because most of the low molecular weight compounds present in a plastic are apolar as well, it follows from the above example that migration will especially be important in apolar food matrices, like fatty foods. Of course, polar migrants, such as the antistatic polyethylene glycol, will preferentially migrate to more polar, so-called aqueous foods.

Partition coefficients can be determined experimentally, but this approach often is very tedious and prone to experimental errors. Therefore empirical methods were developed to estimate the partition coefficients for given polymer-migrant-food systems (Baner, 2000). Detailed discussion of these methods fall out of the scope of this work.

1.2.2.3. Diffusion coefficient and the diffusion process

The diffusion coefficient $D$ of a migrating compound in a particular matrix follows from Fick’s first law, stating that the mass flux of the compound in the direction $x$, $J_x$, during a time ‘$t$’ through an unit area is proportional to the gradient of the concentration of the compound, $C$, considered in the $x$ direction. Mathematically this gives the following

$$J_x = -D \frac{\partial C}{\partial x}$$

Of course, fluxes in the other directions can be defined similarly

$$J_y = -D \frac{\partial C}{\partial y}$$

$$J_z = -D \frac{\partial C}{\partial z}$$

Due to this flux however, the concentration in a unit cell of the matrix in which the diffusion is taking place, with dimensions $\Delta x\Delta y\Delta z$, will vary accordingly as a function of the time (Figure 5).

The net change of the concentration of the compound in this unit cell as a function of time can be found from

$$\frac{-\partial C}{\partial t} = \frac{J_x(x+\Delta x) - J_x(x)}{\Delta x\Delta y\Delta z} + \frac{J_y(y+\Delta y) - J_y(y)}{\Delta x\Delta y\Delta z} + \frac{J_z(z+\Delta z) - J_z(z)}{\Delta x\Delta y\Delta z}$$

$$\frac{\partial C}{\partial t} = \frac{\partial J_x}{\partial x} + \frac{\partial J_y}{\partial y} + \frac{\partial J_z}{\partial z}$$
If the unit cell becomes infinitely small, then equation [7] becomes

\[
\frac{\partial C}{\partial t} = \frac{\partial J_x}{\partial x} + \frac{\partial J_y}{\partial y} + \frac{\partial J_z}{\partial z}
\]  

[8a]

or if the diffusion coefficient is constant

\[
\frac{\partial C}{\partial t} = D \left( \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2} \right)
\]  

[8b]

Equation [8a] is known as the general Fick’s second law of diffusion.

Without going into detail about the practical applicability of these diffusion laws and their consequences for the migration from plastic food contact materials to food, it is clear that the amount of substance migrating to the food during a specified period of time will partially depend upon the diffusion coefficient of the migrant in the polymer (and in the food).

Because of the importance of the diffusion coefficient of the migrant in the polymer, models which are able to predict this parameter for a given thermoplastic polymer-substance system have been developed throughout the years (Mercea, 2000). It is not the intention to review those models in detail in this work, because it would fall outside its scope. Only the mechanistic principle of the most important models will be discussed to better understand the fundamental mechanisms of diffusion of low molecular weight compounds in polymers.

Basically, two kinds of models are being developed. The first group is considered as the microscopic
models. The second group are merely ‘atomistic’ models that try to estimate the diffusion coefficient by making use of computer simulated diffusional processes (Merca, 2000). For both types however, it should be emphasised that the diffusion mechanisms in thermoplasts below and above the glass transition temperature of the polymer, are totally different. Basically three different cases can be considered depending on the diffusion rate of the migrant and the relaxation rate of the polymer. Case I diffusion or Fickian diffusion occurs when the diffusion rate is much less than the relaxation rate of the polymer. Case II diffusion is characterized by a rapid diffusion in comparison with the relaxation of the polymer. The relaxation rate of the polymer is related to the time the polymer needs to adjust itself to a new equilibrium. Finally case III or anomalous diffusion occurs when both the diffusion and relaxation rates are comparable (Schlotter and Furlan, 1992). Rubbery polymers correspond quickly to changes in their physical condition so consequently the diffusion of low molecular weight compounds is considered to be Fickian. Due to the limited mobility of the polymer below its glass transition temperature, diffusion obeys mostly to case II or III in these circumstances.

**Microscopic diffusion models in rubbery polymers**

For the first approach, better known as the classical approach, two types of models are described: the molecular and the free volume models. In the molecular model for rubbery polymers (T>Tg), it is assumed that the polymer matrix consists of a random distribution of identical segments. These are cylindrical cells composed of a number of parallel polymer chains. Because of thermal vibrations, the unit cell expands and contracts. Similarly, the migrant will exhibit a molecular mobility. Of course the oscillatory movement of the polymer segments is several orders of magnitude slower than those of the migrant. If the motion of the polymer chains in a unit cell, containing a migrant, is coordinated in such a way that a void is created large enough to accommodate the molecule, a longitudinal movement along the polymer chain is created as illustrated in Figure 6a (DiBenedetto, 1963 a-b).

This movement however was estimated to be several orders of magnitude faster than the macroscopically determined diffusion rates. Hence it can be concluded that the migrating molecules merely move forward and backward along the polymer chains, without inducing effective diffusion. Apart from the longitudinal movement however, a perpendicular movement to the latter, allowing the migrant to transfer into another adjacent unit cell, can occur as well (Figure 6b). This transverse movement is much slower compared to the longitudinal one and is therefore the rate determining step in the diffusion process. In order to allow a symmetrical separation of two
polymer chains present in two adjacent unit cells, permitting the passage of the migrant, an activation energy $E_d$ is needed. This activation energy is related to the diffusion coefficient of the migrant in the polymer. This parameter can be estimated from several polymer and migrant specific parameters, which sometimes are however difficult to determine (Pace and Datyner, 1979 a-c). In addition, for the relationship between $E_d$ and the diffusion coefficient, only solutions can be found in special cases of which the practical relevance is restricted (Kloczkowski and Mark, 1989).

The free volume models for rubbery polymers, assume that the mobility of the migrant in the polymer-migrant system is primarily determined by the available free volume in the system. The free volume in a polymer is regarded as an ‘empty’ volume between the chains of the polymer. Similarly the free volume of the migrant can be regarded as the volume not occupied between these molecules. If both the polymer and migrants are regarded as hard spheres, void spaces in the liquid of spheres will originate from local fluctuations in density of these spheres. If these voids are large enough to contain a migrant and if the migrant jumps into it before the original sphere returns to its original position, diffusion occurs. Consequently redistribution of the free volume within the liquid of hard spheres provides diffusion (Cohen and Turnbull, 1959; Turnbull and Cohen, 1961). If the model allows to calculate the amount of free volume, to determine the free volume distribution and
to estimate the energy required to redistribute the free volume, realistic estimates of the diffusion coefficient can be obtained from a limited amount of experimental data, using a quite complex procedure (Vrentas and Vrentas, 1994a).

Microscopic or molecular diffusion models in glassy polymers

In glassy polymers, diffusion of migrants is much more complex. Time to reach equilibrium after a diffusional jump of a migrant in a glassy polymer is generally longer than the characteristic time involved in the diffusion of the migrant itself (Crank and Park, 1968, Stannett et al., 1979). This is because the free volume of the polymer chains is restricted. It is assumed that the holes throughout the polymer matrix are ‘frozen’ into the polymer as it is quenched from the rubbery state (Mercea, 2000).

It is speculated that below $T_g$, the fixed holes present in the polymer structure can be filled with migrating molecules, since they act as Langmuir troughs. Therefore the models describing the diffusion in glassy polymers are known as the dual sorption theories (Fredrickson and Helfand, 1985). Although it was assumed initially that these captured migrants were not participating in the diffusion process (Meares, 1957 a-b), it became clear that the normally dissolved molecules in the rubbery part of the polymer are in equilibrium with the captures ones and hence that both kind of molecules should be kept into account to estimate the diffusion coefficient (Paul, 1969; Petropoulos, 1970). Consequently, the kinetics of the immobilisation process should be kept into account and models become extremely complex. As a further result exact analytical results were only found in a limited amount of cases (Fredrickson and Helfand, 1985).

The free volume models were adjusted as well to incorporate local density fluctuations in glassy polymers. Due to the restricted polymer mobility, the redistribution of the free volume in the polymer is hindered and consequently diffusion proceeds more difficult. Complex models have been introduced assuming that the diffusion becomes dependent upon the solvent concentration (Vrentas and Vrentas, 1994b).

As indicated in Figure 7, reasonably good agreement is obtained between the theoretical and experimental values by for example the free volume models for rubbery polymers. Practical applicability in the prediction of diffusion coefficients for the estimation of migration from plastics remains however problematic for all the microscopic models discussed. The main reason for this lies in the fact that almost all models included parameters which can be determined only by fitting the experimental data to the theoretical curves obtained by the model. In addition they include a lot of simplifying assumptions, without physical relevance. Therefore these microscopic models are
considered especially useful because they offer an insight on the mechanism of diffusion, although they do not truly allow prediction of the diffusion coefficients (Gusev et al., 1994).

Figure 7. Temperature (A, at three different mass fractions of the solvent, \(\omega_1 : \Delta = 0.189 \); \(\Omega = 0.160\); \(\Box = 0.136\)) and composition dependence (B, at two different temperatures \(\Delta = 30^\circ C\); \(\Omega = 100^\circ C\)) of the diffusion coefficient of respectively toluene and ethylbenzene in polystyrene. Lines are theoretical predictions and points are experimental data (from Vrentas and Vrentas, 1994b)

Atomistic diffusion models
The second type of models, the computational ones, envisages developing an atomistic model on the basis of data about the atoms and molecules involved. From these data, simulations can predict the properties and behaviour of the polymer molecule. A molecular structure is created by a sufficient number of simulations. Once this structure is created, migrating molecules are ‘inserted’ into the model, followed again by a number of simulations. From these calculations, the actual diffusion coefficients can be estimated. In order to make such computations possible, powerful methods for the simulation of polymeric microstructure and dynamics are necessary together with a large computation capacity. Basically two methods for modelling the diffusion in amorphous
polymers are available: molecular dynamics and the transition-state approach. Although these computation methods do not assume a microscopic diffusion mechanism as those discussed above, they confirm the mechanisms laid down in these phenomenological models. Therefore they are worthwhile to mention them here and explain them briefly.

In the molecular dynamics approach a theoretical polymer structure based, on detailed mechanical equilibriums, is generated. Based on this theoretical structure an average free volume can be calculated. Subsequently, migrating molecules are inserted into the free volumes of the theoretical molecular structure, again taking into account several parameters, such as the energy state of the new structure. Finally, interaction is simulated by again creating theoretical structures by computation (Gusev et al., 1994). From these, molecular movements can be visualised as illustrated in Figure 8. As can be seen from this picture, the mechanism of the simulated diffusion behaviour is in correspondence with those proposed in the phenomenological models. These movements can be monitored as a function of time and consequently a diffusion coefficient can be estimated with a reasonable to excellent agreement to the experimentally determined values (Hofmann et al., 1997, Fritz and Hofmann, 1997). The disadvantage of this approach is situated in the applicable range of diffusion coefficients to the order of magnitude of $10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$. For diffusion coefficients ranging from $10^{-9}$ to $10^{-12} \text{ cm}^2 \cdot \text{s}^{-1}$, which are frequently encountered for plastic additives, computation infrastructure is currently not available (Mercea, 2000).

![Figure 8. A typical trace of a water molecule in a polymeric matrix (Hofmann et al., 1997)](image)

From the results obtained by the molecular dynamics, which are in agreement with the assumptions made in the previously discussed classic approaches, the jumps of migrating substances can be regarded as elementary processes thus justifying the transition-state approach to quantitatively deal with the problem (Gusev et al., 1994; Mercea, 2000). Using the molecular dynamics approach, an
upper bond for times at which the system fluctuates around its equilibrium condition can be calculated. Within these time frames, the polymer is performing so-called elastic movements and for the migrants, especially vibrational motions predominate. At longer time intervals the system will perform structural relaxations enabling migrants to diffuse in the polymer matrix (Gusev et al., 1994). Using stochastic methods and other advanced mathematical techniques in combination with a number of simplifications and suppositions, the jumps of the migrant in the polymer can be simulated without keeping into account the dynamics of the migrant during the elastic behaviour of the polymer. Thus much longer simulation intervals can be used, reducing the time needed to perform a simulation of the dynamics of the polymer-migrant system. The latter simulations enable again, as in the molecular dynamics method, to estimate the diffusion coefficient of the migrant. The transition state approach could be very useful for systems in which the interactions between the migrant and the polymer can be neglected. For systems in which the migrant causes swelling of the polymer however, molecular dynamic simulations remain the method of choice. Therefore it is considered important to use both techniques in conjunction (Gusev et al., 1994, Mercea, 2000).

Considering the diffusion in the polymer below T_g, again more complications occur. It has been reported that the molecular dynamic approach is not yet able to generate realistic estimates of the diffusion coefficient because of the large variability in the size of the crystal cells (Mercea, 2000).

As a conclusion it can be stated that none of the above mentioned diffusion models are currently able to estimate diffusion coefficients for every given polymer-migrant system.

1.2.3. Mathematical approach to estimate migration from plastics

1.2.3.1. General transport equation

The goal of the mathematical models describing migration from plastic food contact materials is to predict the concentration of the migrant in the food after contact with the plastic. In such a manner, lengthy and costly migration experiments, which are legally requested, can be avoided. In order to obtain a reliable model, all mass transfer phenomena and other processes affecting the concentration of the migrant in the food, should be considered. Basically the following processes are taken into account:

- diffusion of the migrant
- convection of the medium in which the migrant is dissolved
- chemical reactions in which the migrant is involved

It is important to realise that from a theoretical point of view all these processes can take place in
both the food and the polymer. Practically however mainly the following processes control the migration behaviour:

- diffusion of the migrant in both the polymer and the food
- chemical reaction in both the polymer and the food

Convection of the polymer is very much restricted in normal conditions of use, so will be of no influence with regard to the migration. In liquid foods, convection will cause a quick distribution of the migrant in the food favouring a uniform concentration of the migrant. In solid foods or highly viscous foods, diffusion of the migrant will be of higher importance compared to convection fluxes of the food itself.

Migrants can be subjected to chemicals reactions in the polymer itself (e.g. partial degradation of antioxidant during plastic extrusion) or in the food. The hydrolysis of bisphenol A diglycidyl ether (BADGE), a cross linking agent used in epoxy coatings for food cans, is a typical example in this respect (Tice and Mc. Guiness, 1987, Tice, 1988, Paseiro Losada et al., 1997).

Mathematically the predominant processes affecting the concentration C of the migrant at a particular place with coordinates (x,y,z) in the food-polymer system can be written as follows:

For diffusion, as introduced before as the second diffusion law of Fick

\[
- \frac{\partial C}{\partial t} = \frac{\partial J_x}{\partial x} + \frac{\partial J_y}{\partial y} + \frac{\partial J_z}{\partial z}
\]

[8a]

For the chemical reaction

\[
\frac{\partial C}{\partial t} = -k \times C^m
\]

[9]

In, equation [9], m represents the order of the chemical reaction and k is the reaction rate constant. Summation of the two equations, gives the general transport equation in which the convection in both the polymer and the food are supposed to have minor influence

\[
\frac{\partial C}{\partial t} = \left( \frac{\partial^2 DC}{\partial x^2} + \frac{\partial^2 DC}{\partial y^2} + \frac{\partial^2 DC}{\partial z^2} \right) - k \times C^n
\]

[10]

Of course the main problem in solving this equation is situated in the second order partial differential equation. Such an equation has only an analytical solution in some special cases. In addition, the diffusion coefficient should be constant. In all other cases, numerical methods should be used to solve the equation. Once this equation is solved however, the problem of a reliable estimate of the diffusion coefficient of the migrant remains. As discussed previously (paragraph 1.2.2.3), mechanistic and atomistic diffusion models are currently unable to solve this problem.

The second part in this equation is rather specific for particular migrants and will not be discussed.
here in more detail. Therefore only solutions to the second order differential equation will be presented.

As indicated before, a number of assumptions should be made to analytically solve the second order partial differential equation given in equation [10]. Primarily, the diffusion coefficient is supposed to be constant in both the food and the polymer. In addition, it is assumed that diffusion takes place in only one direction, perpendicular to the surface of the polymer. Consequently, the partial differential equation becomes

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \tag{11}
\]

Solutions to this equation have been described for finite and infinite polymers.

1.2.3.2. Diffusion from finite polymer

Further basis assumptions in addition to those mentioned above include the following:

- there is one single migrant, which is uniformly distributed in the polymer at \( t=0 \) at a concentration \( C_{F,0} \)
- the concentration of the migrant in the food at a particular time, \( C_{F,t} \), is everywhere the same, implying that the food is ideally mixed
- a constant distribution of the migrant between the polymer and the food takes place according to
- the contact material is a flat sheet
- the mass transfer is mainly controlled by diffusion taking place in the polymer

Crank (1975) developed the following solution to equation [11] (Piringer, 2000b; Hamdani et al., 1997) for a polymer in contact with a finite food

\[
\frac{m_{F,t}}{m_{F,\infty}} = 1 - \sum_{n=0}^{\infty} \frac{2 \alpha (1 + \alpha)}{1 + \alpha + \alpha^2 q_n^2} \times e^{-\left(\frac{D_p q_n^2 t}{L_p^2}\right)} \tag{13}
\]

in which \( m_{F,t} \) is the amount of migrant in the food at a particular time \( t \), \( q_n \) is the positive root of the trigonometric identity \( \tan(q_n) = -\alpha q_n \), \( L_p \) is the thickness of the polymer, \( D_p \) the diffusion coefficient of the migrant in the polymer and \( \alpha \) is given by the following formula
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in which \( V_F \) and \( V_P \) represent the volume of respectively the food and the polymer and \( m_{P,\infty} \) is the mass of the migrant present in the polymer at equilibrium conditions (\( t=\infty \)).

This rather complex equation [13] can be simplified by assuming the finite polymer is contacted with an infinite food. This implies that the concentration of the migrant in the food equals zero, since mathematically spoken, \( V_F \to \infty \). Consequently, from equation [14] it follows that \( \alpha >> 1 \).

According to Piringer (2000b) and Hamdani et al. (1997) equation [13] can then be simplified into:

\[
\frac{m_{F,t}}{m_{F,\infty}} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} e^{-\frac{(2n+1)^2 \pi^2 D_P t}{4L_P^2}}
\]

Equation [15] is reported to give the same results as equation [13] if the volume of the food (\( V_F \)) exceeds 20 times the volume of the polymer (\( V_P \)), which in practice is usually achieved, also in migration tests (Hamdani et al., 1997).

Equation [15] can further be simplified for the following two cases (Hamdani et al., 1997):

- long contact time (\( m_{F,t}/m_{F,\infty} > 0.6 \))

\[
\frac{m_{F,t}}{m_{F,\infty}} = 1 - \frac{8}{\pi^2} e^{-\frac{\pi^2 D_P t}{L_P^2}}
\]

- short contact time (\( m_{F,t}/m_{F,\infty} < 0.6 \))

\[
\frac{m_{F,t}}{m_{F,\infty}} = \frac{2}{L_P} \sqrt{\frac{D_P t}{\pi}}
\]

For all these models, it was assumed that diffusion in the polymer is the main factor controlling the migration phenomenon. If other processes, such as the dissolution and the diffusion of the migrant in the food are also important factors to consider, analytical solutions of the diffusion equation [11] are not available. Numerical methods for some cases have been described (Laoubi and Vergnaud, 1996).

A further simplification of the problem, assuming the polymer is infinite, allows in some cases to take into account the dissolution and the diffusion of the migrant in the food as explained in the following.
**1.2.3.3. Diffusion from infinite polymer**

The assumption of infinite polymer implies that the concentration of the migrant in the polymer is constant as a function of time (C_{P,0} = C_{P,t}). Of course, this does not correspond to reality since it is known that the concentration of the migrant in the polymer is affected by migration (Hamdani et al., 1997). Again several solutions of equation [11] have been proposed for a number of cases taking into account the following supplementary boundary conditions:

- there is one single migrant, which is uniformly distributed in the polymer at t=0 at a concentration C_{P,0}
- a constant distribution between the polymer and the food takes place according to

\[ K_{P/F} = \frac{C_{P,t}}{C_{F,t}} = \frac{C_{P,\infty}}{C_{F,\infty}} \]  

- the contact material is a flat sheet

Two major cases can be distinguished depending on the concentration gradient of the migrant in the food.

**No concentration gradient of the migrant in the food**

If no concentration gradient in the food is present, this implies that the food is well mixed or that the diffusion of the migrant in the food proceeds much faster compared to the diffusion in the polymer. The general solution of equation [11] is given by (Limm and Hollifield, 1995; Piringer, 2000b, Lickly et al., 1997; Hamdani et al., 1997):

\[ m_{F,t} = \frac{C_{P,0} \times A}{K_{P/F}} \left( 1 - e^{-2z^2} \operatorname{erfc}(z) \right) \]  

in which A is the contact surface between the polymer and the food and z is given by

\[ z = \frac{K_{P/F} \times \sqrt{D_{P,t}}}{A} \]  

According to Hamdani et al. (1997) this equation is valid for infinite polymers contacted with finite foods, indicating that the migrant slowly dissolves in the food as confirmed by Limm and Hollifield (1995). Consequently, diffusion is mainly governed by solvatation.

If the migrant is very well soluble in the food however (K_{P/F} << 1), equation [18] can be simplified into (Piringer, 1994; Lickly et al., 1997)

\[ \frac{m_{F,t}}{A} = 2C_{P,0} \sqrt{\frac{D_{P,t}t}{\pi}} \]  

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According to Hamdani et al. (1997) equation [20] represents the migration from an infinite polymer in contact with an infinite food (mathematically: \(C_I=0\)). In this case, diffusion of the migrant in the polymer will dominate the migration process.

**Concentration gradient of the migrant in the food**

If a concentration gradient of the migrant is present in the food, the following equation has been proposed as a solution to equation [11] (Piringer, 2000b)

\[
m_{F,t} = 2C_{P,0} \sqrt{\frac{D_P t}{\pi}} \left( \frac{\beta}{\beta + 1} \right)
\]

where

\[
\beta = \frac{1}{K_{P/F}} \sqrt{\frac{D_F}{D_P}}
\]

As can be noticed, diffusion of the migrant in both the food and the polymer are taken into account. If in this case, diffusion in the food is fast (\(\beta \gg 1\)), equation [21] is turned into equation [20], indicating that due to the high diffusion in the food, the concentration gradient of the migrant in the food is negligible.

If on the other hand, \(\beta \ll 1\), because of the poor solubility of the migrant in the food, migration will be especially dominated by the migration in the food as indicated in the following equation derived from equation [20] (Piringer, 2000b)

\[
m_{F,t} = \frac{2C_{P,0}}{K_{P/F}} \sqrt{\frac{D_F t}{\pi}}
\]

### 1.2.3.4. Estimation of material constants

As can be concluded from all the analytical solutions to the general diffusion equation [11], diffusion coefficients and the partition coefficient of the migrant should be known to practically apply these equations. As will be explained later, from a regulatory point of view, the ‘worst-case’ scenario for the prediction of the migration is of primary interest. Therefore, it is most frequently assumed that the solubility of the migrant in the polymer is very high, which implies that \(K_{P/F}=1\), thus avoiding difficulties for the estimation of the partition coefficient for a given migrant-polymer-food system. Consequently, the problem of a realistic estimate of the diffusion coefficient remains. Diffusion coefficients of migrants range from about \(10^{-9}\) cm².s⁻¹ down to about \(10^{-18}\) cm².s⁻¹. From the above equations it can be concluded that this large difference in magnitude will play a major role in the final migration result for most cases. Realistic estimates are therefore considered indispensable.
because underestimated diffusion coefficients will underestimate migration and overestimates will make the practical use of these migration models impossible (Brandsch et al. 2000).

The mechanistic models on diffusion currently available however can not be applied for the estimation of diffusion coefficients of migrants in polymers. Alternatively, empirical formulas such as equation [24] and [25] can be used.

\[
D_P = D_0 e^{\left(\frac{\xi}{M_r - \psi M_r^{3/2}}\right)}
\]  

[24]

in which \(D_0\) can be considered as the diffusion coefficient for a migrant at \(T=\infty\) and \(M_r=0\), \(\xi\) is a constant related to the dependence of the diffusion coefficient upon \(M_r\), \(\psi\) is a constant related to the activation energy of diffusion, \(T\) is the absolute temperature and \(M_r\) is the molecular weight of the migrant considered (Limm and Hollified, 1996).

\[
D_P = 10^4 e^{\left(\frac{A_P - b M_r - c}{T}\right)}
\]  

[25]

in which \(A_P\) is related to the effect of the polymer on the diffusion, \(b\) and \(c\) are constants related to the effect of respectively the migrants molecular weight and the temperature on the diffusion (Brandsch et al., 2000).

According to Brandsch et al. (2000) reliable diffusion coefficients for migrants having a molecular weight up to 4000 could be calculated in such a way for selected polyolefins between the melting and glass transition temperature of the polymer.

For non polyolefins however, which are characterized by a higher \(T_g\) (frequently between 323-373 K), such models are not available due to a lack in experimental data. Therefore no useful diffusion coefficient estimates are available up till now for these polymers.

**1.2.3.5. Practical use of mathematical models**

There is a general consensus about the usefulness of mathematical modelling of migration to limit laboratory tests which are tedious and costly. This is reflected by the possibility to use mathematical modelling to prove compliance with legislation as recently accepted within the EU (EC, 2001a) and as been accepted before in the USA (FDA, 1995a). Moreover, in the recently updated Practical Guide for users of the European Directives with regard to food contact materials (EC, 2002) reference is made to a specially tailored and user-friendly computer program which is available on the internet (http://www.inra.fr/Internet/Produits/securite-emballage/Fichiers/inramig.exe).

Mathematical models however are prone to a number of limitations which are important to consider. As indicated before, an important aspect in the evaluation of the models is the
correspondence between the calculated and experimental data. Because of the necessity of a reliable estimate of the diffusion coefficient of the migrant, it can be concluded from the above discussions that currently only models applicable to polyolefins are available. It should be noted on the other hand that polyolefins are currently the most frequently applied polymers for food contact. Mathematical modelling could therefore be helpful already in a large number of applications.

The models described are only able to predict the migration of known and well characterised migrants. Consequently the models will not be able to predict the total amount of substance migrated from a contact material, since the contact material may contain apart from the additives also a number of other compounds of which the identity is not completely known (e.g. ethylene oligomers or their breakdown products present in PE).

Care should be taken in using too simplified migration models. Hamdani et al. (1997) for example illustrated that the use of equation [20] should be considered with a lot of care. Although the model assumes that the polymer is infinite and therefore could suggest a ‘worst-case’ scenario, they recommended the use of equation [17] instead because of the risk of underestimating the migration levels. In addition it should be noted that the models predict the migration from an idealized geometric object (plane). Previously Chatwin (1996) indicated that the actual geometry of the polymeric object should be considered as well if mathematical models are used. Another important aspect is the possible absorption of food components by the polymer, altering the diffusion coefficients in the polymer. Consequently, a supplementary variable is introduced, making the presented models in most cases too simplistic.

Therefore, the use of a more general equation such as equation [13] is considered to be better. O’Brien et al. (1999) and O’Brien and Cooper (2001) compared specific migration of various additives from polyolefins to olive oil at various time temperature conditions with the predicted values of this migration model (Figure 9). For polypropylene, almost all the estimated values were higher then those experimentally observed. From a safety point of view this is interesting. Results for polyethylene were a bit less promising. Despite this observation, Brandsch et al. (2000) considered the overestimation of the predicted migration levels too high! In order to obtain a more accurate estimation, it was believed that the use of a more realistic \( K_{R/F} \), especially at lower temperatures, would be appropriate.

For the sake of completeness it should be mentioned that mathematical modelling is also applied with regard to so-called functional barrier concept. Although this concept is of particular importance with regard to the migration from recycled and re-used polymers, it is applicable to any
multilayer structure. It should be stressed that a functional barrier is not an absolute physical barrier preventing migration as such. It is a migration barrier to a so-called functional quantity of material present in the polymer (Franz et al., 1993). A lot of models are published tackling the functional barrier concept, which cannot be discussed here in more detail (Laoubi and Vergnaud, 1995-7; Laoubi et al., 1995; Franz et al., 1997). Again the usefulness of mathematical modelling within this respect should be noted.

![Comparison between predicted and experimental migration levels in olive oil for several additives out of high density PE and PP.](image)

**Figure 9.** Comparison between the predicted and experimental migration levels in olive oil for several additives out of high density PE (■) and PP (○) (Based on the experimental data reported in O’Brien et al., 1999; O’Brien and Cooper, 2001)

As a conclusion, most of the migration models described, are only applicable in particular polymer-migrant-food systems because of the basic assumptions always made. As indicated before, more complex situations exist in reality (e.g. no constant diffusion coefficient, crystallinity, laminated structures etc.). In addition, from the fundamentals about diffusion, it could be concluded that the exact estimation of the diffusion coefficient of a particular migrant in a specified polymer is also an impossible task at the moment. Since the current migration models generally overestimate migration, an upper bond approach for the estimation of the diffusion coefficient would also be more practical and more economical. Specialised software, combining both aspects (diffusion coefficient and migration prediction) is reported to be available (Brandsch et al., 2000).
1.2.4. Parameters of importance for the migration from plastics

From the above discussed mathematical models describing migration and diffusion, important parameters affecting migration can be derived. In the following, experimental confirmation of the influence of these parameters will be presented. This confirmation is of importance with regard to the validity of the models discussed. In addition however, identification of such factors are a key issue as well if regulatory aspects are considered. By making use of the fundamental knowledge on migration, relevant test methods can be developed and legally imposed to simulate, in the laboratory, migration to food in a practical and economic way.

1.2.4.1. Time

Because of transport equation [10], migration should be regarded as a dynamic process. Therefore time is of major importance determining the amount of substance migrating from the polymeric contact material to the food. From the simplified models (equations [17], [20], [21] and [22]) it can be concluded that, especially at the beginning of the migration process, the migrated amount is proportional to $t^{1/2}$. This is confirmed by numerous experiments, especially involving migration from polyolefins as reviewed by Figge (1996). Of course at longer time intervals migration will flatten off as illustrated in Figure 10. These curves are in correspondence with the general exponential trend reflected in the more general equations describing migration mentioned above (paragraph 1.2.3). The plateau is reached in equilibrium conditions and depends on the partition coefficient $K_{P/F}$.

Since the migration models described previously are based on a number of simplifications, it is not surprising however that other relationships with regard to time could be observed as well. This could be due to for example a non Fickian diffusion mechanism, like a class II diffusion which proceeds linear as a function of time as indicated previously by Schlotter and Furlan (1992). Evaluation of the migration dynamics could therefore reveal the nature of the diffusion mechanisms involved.

Irrespective of the true nature of the controlling mechanisms of the migration process, it should be clear that migration from a contact material to a food will increase as a function of time as long as no equilibrium between these two materials is reached.
1.2.4.2. Factors affecting the diffusion and partition coefficient

The migration process is highly dominated by the diffusion coefficients of the migrant and its partition coefficient in a particular food-polymer system. Consequently, parameters exerting an effect on these main factors are of prime importance as well.

Temperature will highly influence the migration process as illustrated in Figure 10. Because in this particular example, the initial migration rate is dependent upon \( D_P \) (equation [17]), it is clearly demonstrated that by increasing the temperature an increase of \( D_P \) is observed resulting in a higher initial migration speed. It is also obvious that at equilibrium conditions, higher migration levels are observed at higher temperatures, confirming the expected change of the partition coefficient \( K_{P/F} \).

Generally an Arhenius type of relationship is observed between migration and temperature (Figge, 1996). However, if one of the components involved, undergoes a change in its physical state due to a change in temperature, deviations from this relationship can be expected. Consequently migration and its dynamics in polymers below and above glass transition temperatures will be highly different. Similarly, migration will be influenced by the crystallinity of the food (Figge, 1996).

In addition to this extrinsic factor, a number of intrinsic factors of the polymer-food-migrant systems affect the partition and diffusion coefficients as well.
For the partition coefficient especially the interactions between the three phases should be considered. Generally migration of an apolar migrant to an apolar food from an apolar polymer or vice versa, can be considered as a worst-case. (Franz, 2000). So the polarity of each phase present in the system is of extreme importance. This was illustrated with numerous experimental data (e.g. Figge, 1996; Scott, 1988). Since most of the polymers used are apolar (Figure 1) it follows that especially fatty foods are susceptible for migration. In this regard it should be stressed however that the fat content of a food is considered of less importance compared to the so-called fat-releasing properties, which for example are enhanced if fat is present on the foods surface (Figge, 1996; Castle et al., 1994).

For the diffusion coefficients the migrants molecular weight is of prime importance as could be concluded from the empirical equations [24] and [25]. In addition however, also steric effects should be considered. As can be expected from the fundamentals of diffusion in polymers, especially polymer crystallinity and intrinsic factors affecting it (e.g. orientation, side chains, polarity, presence of plasticizers, etc.) should be considered with regard to the polymer. Similarly, crystallinity of the food can be of importance as well (Figge, 1996), as already stressed before.

Interactions between the polymer, the migrants and the food are of importance as well (Franz, 2000). If due to a significant interaction between the polymer and the food (e.g. oil in contact with polyolefins) polymeric absorption of food components occurs, two extreme cases can be distinguished, depending on the diffusion coefficient of the migrant ($D_{P,m}$) and of the absorbed food component ($D_{P,f}$) in the polymer:

- $D_{P,m} >> D_{P,f}$, which implies that the migration from the packaging is not affected by the uptake of the food because of the high mobility of the migrant. For oil this is the case for migrants having a molecular weight up to 600-1000.
- $D_{P,m} << D_{P,f}$, which means that the migrant will be ‘overrun’ by the food penetrating the polymer. This is for example the case if a polyolefin is contacted with a solvent, serving as a food simulant. Again two cases can be distinguished, depending on the interaction between the solvent and the polymer:
  - If the simulant is readily absorbed by the polymer, causing severe swelling, total extraction of the migrant is possible if the partition coefficient of the migrant for the particular polymer-solvent system allows it. This is for example the case for iso-octane in contact with polyolefin containing some apolar additives. In order to obtain comparable migration levels to those in real foods (e.g. oil), the contact time between the solvent and the polymer should be restricted.
If the simulant is not very well absorbed by the polymer, migration is not significantly affected. This is for example the case for a polyolefin contacted with the polar ethanol at moderate temperatures. Consequently if migration tests with such volatile simulants is used, contact time should not be adjusted compared to a test carried out in oil.

These considerations are the basis for the use of alternative volatile food simulants to check migration in oil as described more in detail elsewhere (Freytag et al., 1984; De Kruijf and Rijk, 1988; Lickly et al., 1990; Piringer, 1990; Figge and Hilpert, 1991; Baner et al., 1992; Indiramma et al., 1992; Van Battum, 1996).

### 1.2.4.3. Other factors

In addition to the factors mentioned above, the following parameters affect the migration from plastic food contact materials as well.

From the models related to infinite polymers (paragraph 1.2.3.3) it is clear that the initial concentration of the migrant in the polymer influences the migrated amount to the food. This relationship has been supported by several experimental studies (e.g. Figge and Hilpert, 1990; O’Brien et al., 1997).

For the polymer, thickness of the tested material is of importance as well. For polyolefins a linear relationship between the wall thickness and the migration has been reported. From a particular limiting thickness however, migration remains constant (Figge et al., 1988a-c, Figge et al., 1989). For other polymers however, more complex relationships were reported (Figge, 1988).

The volume of the contacted food may influence the migration as well, since the volume can affect the migrants concentration in the food. As reported by Murthy et al. (1990), too low volumes underestimate migration. A minimal amount of 50 mL of solvent per square decimetre of contact material is proposed to be used. The difference in single sided or double sided contact between the food simulant and the polymer was investigated by the same group. Per unit of contact surface, single sided contact resulted in higher migration levels, especially if the food simulant caused swelling of the polymer (Vijayalakshmi et al., 1992).

Finally it should be mentioned that the shape of the test object is reported to be of importance as well. Figge (1988) observed higher antioxidants migration from high impact polystyrene into a fat if the cut edges were exposed to the fat as well.
1.2.5. Legislative aspects about migration from plastics

1.2.5.1. Introduction

From the principles of migration from plastics to foods and as already indicated before, it is obvious that this phenomenon can be a food safety issue. Plastics may contain compounds which should be considered as carcinogens (e.g. vinylchloride, acrylonitrile) or which exhibit another type of toxicity. In order to ensure consumer protection, legislation has been developed through the years in various countries with regard to food contact materials in general and plastic food contact materials in particular.

Within the European Union, a process of harmonisation, started in 1972, tries to bring all existing legislation in the various member states in correspondence or implies new directives (Rossi, 2000). Especially with regard to the plastic food contact materials most initiatives were elaborated. This resulted in a fairly detailed legislation as will be discussed below. In addition to the European legislation however, also the United States’ legislation will be discussed to illustrate different legislative approaches to ensure consumer protection in this particular field.

1.2.5.2. General aspects of European food contact material legislation

As already indicated before, the European legislator differentiates several types of contact materials (Table 1). Two kinds of directives can be distinguished: directives applicable to all materials considered on the one hand and those applicable to individual substances and materials on the other hand.

Two general directives apply to all food contact materials. The framework directive (EEC, 1989) specified a list of food contact materials to which the directive applies (Table 1). In addition two general principles are established:

(1) The principle of inertness, specifying that a material to come into contact with food shall not “endanger human health and bring about an unacceptable change in the composition of the foodstuff or a deterioration of the organoleptic characteristics thereof”.

(2) The principle of ‘positive labelling’, specifying that contact materials should be accompanied by the words “for food” or an appropriate symbol, unless it is obvious that materials are clearly intended to come into contact with food. Eventually restrictions in use should be specified as well.

In addition to these basic principles, criteria and procedures to be followed in drafting specific directives have been pointed out.
The symbol which can be used to indicate that a material is intended for food contact is specified in the second general directive (EEC, 1980) (Figure 11).

![Figure 11. Symbol identifying materials intended to come into contact with food (EEC, 1980)](image)

As already stressed before, not only packaging materials are considered in these general directives, but all materials and articles intended to come into contact with food except public water installations.

The other directives on food contact materials relate to individual substances and materials. As can be observed from Table 4 most of the currently issued directives relate to plastics.

In the following, specific EU directives with regard to plastic food contact materials will be discussed in more detail.

1.2.5.3. **European directives on plastic food contact materials**

As indicated before, plastic food contact materials represent a very important group among the different food contact materials used. Moreover it is a very complex group because several kinds of polymers are used in addition to an enormous diversity of low molecular weight compounds added to the polymer (Table 3). The directives discussed below only relate to materials solely composed of plastic. Consequently, coated materials such as plastic coated board or polymer coated metals fall out of their scope. In addition, Directive 2001/62/EC, changing the former 90/128/EEC for plastic materials intended to come into contact with foods, specify that the following substances are not considered as plastics:

- regenerated cellulose materials
- elastomers or synthetic rubbers
- paper and board
- coatings from paraffin’s or mixtures of paraffin’s with plastics
- ion exchangers
- silicones
On the other hand, materials composed out of two or more layers of materials, each consisting exclusively out of plastics, fall into the scope of the mentioned legislation.

Table 4. Main directives adopted on materials intended to come into contact with food applicable to individual materials and substances

<table>
<thead>
<tr>
<th>Subject</th>
<th>Directive number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st amendment</td>
<td>92/39/EEC</td>
<td>EEC, 1992</td>
</tr>
<tr>
<td>2nd amendment</td>
<td>93/9/EEC</td>
<td>EEC, 1993a</td>
</tr>
<tr>
<td>3rd amendment</td>
<td>95/3/EEC</td>
<td>EC, 1995</td>
</tr>
<tr>
<td>4th amendment</td>
<td>96/11/EEC</td>
<td>EC, 1996</td>
</tr>
<tr>
<td>5th amendment</td>
<td>99/91/EC</td>
<td>EC, 1999</td>
</tr>
<tr>
<td>Directives on basic rules for migration tests</td>
<td>82/711/EEC</td>
<td>EEC, 1982a</td>
</tr>
<tr>
<td>1st amendment</td>
<td>93/8/EEC</td>
<td>EEC, 1993b</td>
</tr>
<tr>
<td>2nd amendment</td>
<td>97/48/EC</td>
<td>EC, 1997</td>
</tr>
<tr>
<td>Directives on list of simulants</td>
<td>85/572/EEC</td>
<td>EEC, 1985</td>
</tr>
</tbody>
</table>

**Regenerated cellulose film**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Directive number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base directive</td>
<td>93/10/EEC</td>
<td>EEC, 1993c</td>
</tr>
</tbody>
</table>

**Ceramics**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Directive number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base directive</td>
<td>84/500/EEC</td>
<td>EEC, 1984</td>
</tr>
</tbody>
</table>

**Elastomers**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Directive number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosoamines in teats and soothers</td>
<td>93/11/EEC</td>
<td>EEC, 1993d</td>
</tr>
</tbody>
</table>
The legislation established, deals with:

1. a list of authorized substances
2. a restricted amount of migration
3. a system of checking migration

### EU list of authorized substances

Throughout the years various lists concerning monomers, other starting substances and most types of additives (except colorants and catalysts) have been approved and published (see Directives in Table 4). These lists rule out the use of unlisted materials, so they should be considered as a so-called positive lists. The lists specified do not include monomers or other starting substances, which are only used for the production of

- surface coatings obtained from resinous or polymerised products in liquid, powder or dispersions form, such as varnishes, lacquers, paints, etc.
- epoxy resins
- adhesives
- printing inks

New substances can be added to the lists on request. The Scientific Committee for Food (SCF) of the European Union advises the European Commission on the safety-in-use of these substances. Therefore, a petition for enlarging the positive lists with a particular substance should contain a technical file enabling the SCF to evaluate the risk associated with the use of the substance (Table 5).

<table>
<thead>
<tr>
<th>Table 5. Data necessary for the evaluation of a new substance by the SCF (Barlow, 1994)</th>
</tr>
</thead>
<tbody>
<tr>
<td>identity</td>
</tr>
<tr>
<td>physical, chemical and other properties</td>
</tr>
<tr>
<td>use</td>
</tr>
<tr>
<td>migration data</td>
</tr>
<tr>
<td>toxicological data</td>
</tr>
</tbody>
</table>

The full set of toxicity data required are summarized in Table 6. As indicated in Table 7, the toxicity data needed, will depend upon the expected migration level.

---

*Chemical interactions between packaging materials and foodstuffs*
Table 6. Full set of toxicological test needed for the authorization of a new substance (Barlow, 1994)

- 90 day study of oral administration
- three mutagen studies
  - a mutagenicity test on bacteria (Ames test)
  - a mutagenicity test on mammalian cell culture
  - a test to detect chromosomal aberrations in mammalian cell culture in vitro
- long-term toxicity and/or carcinogenicity studies
- reproduction studies
- teratogenicity studies
- studies of absorption, distribution, metabolism and excretion

If a new substance is found to be genotoxic or is a genotoxic carcinogen, it is very unlikely that their use will be accepted (Barlow, 1994). The use of some genotoxic or carcinogenic compounds such as acrylonitrile and vinylchloride is nevertheless tolerated.

Table 7. Reduced set of toxicological tests needed for the authorization of new substances (Barlow, 1994; Rossi, 2000)

<table>
<thead>
<tr>
<th>Migration data (ppm)</th>
<th>Toxicological tests required by the SCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-0.05</td>
<td>3 mutagenesis tests</td>
</tr>
<tr>
<td>0.05-5</td>
<td>3 mutagenesis tests</td>
</tr>
<tr>
<td>- no bioaccumulation</td>
<td>90 day oral administration test</td>
</tr>
<tr>
<td>- no toxic effect presumed</td>
<td>bioaccumulation test</td>
</tr>
<tr>
<td>5-60</td>
<td>Full set of essential toxicological tests as indicated in Table 6, unless there are good reasons for dispensing with them</td>
</tr>
</tbody>
</table>

On the basis of the toxicological evaluation, the SCF classifies the substances in 10 lists as indicated in Table 8.

All substances listed in lists 0-4 are authorized by the European Commission for use, provided they comply with specified restrictions. Compounds listed in list 5 are prohibited for use. The use of substances listed in lists 6-9 is temporarily tolerated by the Commission until the Scientific
Committee has been able to evaluate them properly.

1.2.5.3.2. **Migration restrictions**

Apart from the fact that compositional restrictions are imposed on plastic materials, also the migrated amount of substances to the food should be limited. Two general restrictions are applied:

- an overall migration limit
- a specific migration limit

The overall migration limit, set at 60 mg.kg⁻¹ of food or 10 mg.dm⁻² contact material is the total amount of substances which can migrate out of a plastic material to the food. This limit is set to ensure the inert character of the packaging material as foreseen in the framework directive. In addition it avoids that for every listed compound a specific migration limit should be specified.

The specific migration refers to the restricted migration of particular substances of toxicological relevance. The specific migration limit (SML, [mg.kg⁻¹]) is calculated on the basis of the acceptable daily intake (ADI) or tolerable daily intake (TDI) laid down by the SCF for the particular substance. Supposing that 1 kg of the food, containing the migrating substance, is consumed daily by a 60 kg adult, it is obvious that the specific migration limit is given by:

\[
\text{SML} = 60 \times \text{ADI}
\]

or

\[
\text{SML} = 60 \times \text{TDI}
\]

The specific migration limit for a particular substance is specified in the positive lists issued by the European legislator.
Table 8. SCF classification scheme for substances used in plastic food packaging (Barlow, 1994)

<table>
<thead>
<tr>
<th>List</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>list 0</td>
<td>food ingredients or normal metabolites for which no need for acceptable daily intake (ADI) is established</td>
</tr>
<tr>
<td>list 1</td>
<td>substances for which an ADI, a t-ADI (temporary ADI), maximum tolerable daily intake (MTDI), a provisional maximum tolerable daily intake (PMTDI), a provisional tolerable weekly intake (PTWI) has been established by the SCF or the Joint Expert Committee on Food Additives (JECFA)</td>
</tr>
<tr>
<td>list 2</td>
<td>substances for which a TDI or t-TDI has been established by the SCF</td>
</tr>
<tr>
<td>list 3</td>
<td>substances for which no ADI or TDI could be established, but of which their use is self-limiting, their migration is very low or which are inert</td>
</tr>
<tr>
<td>list 4</td>
<td>substances for which no ADI or TDI could be established, but of which the migration is not detectable</td>
</tr>
<tr>
<td>list 5</td>
<td>substances which bioaccumulate or are too toxic for use</td>
</tr>
<tr>
<td>list 6A</td>
<td>substances suspected to have carcinogenic properties, but of which data are lacking or insufficient</td>
</tr>
<tr>
<td>list 6B</td>
<td>substances suspected to have toxic properties (other than carcinogenic), but of which data are lacking or insufficient</td>
</tr>
<tr>
<td>list 7</td>
<td>substances for which toxicological data exist, but for which an ADI or TDI could not be established</td>
</tr>
<tr>
<td>list 8</td>
<td>substances for which no adequate data are available</td>
</tr>
<tr>
<td>list 9</td>
<td>substances which could not be evaluated because of a lack of specifications or inadequate description</td>
</tr>
<tr>
<td>list w</td>
<td>waiting list, substances not yet included in the other lists</td>
</tr>
</tbody>
</table>

1.2.5.3.3. Migration testing

Based on the theoretical and experimental considerations discussed before (paragraphs 1.2.2-1.2.4), the European legislator has implemented a number of rules concerning migration testing. Because of the diversity in foods contacted with plastics, migration testing should be simplified to minimize the number of tests to a practical and economic level. Therefore, simulants are proposed to be use instead of real foods. In addition, the migration tests become analytically better feasible. The simulants are based on four food types (Table 9). Dry foods do not require migration testing, although migration to dry foods has been reported (Schwope and Reid, 1988; Boccacci Mariani et
Chemical interactions between packaging materials and foodstuffs

al., 1999).

Table 9. Food types and simulants used in the EU (EEC, 1982a, EC, 1997)

<table>
<thead>
<tr>
<th>Food type</th>
<th>Food simulant</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>aqueous foods (pH &gt;4.5)</td>
<td>simulant A</td>
<td>water</td>
</tr>
<tr>
<td>acidic foods (pH &lt;4.5)</td>
<td>simulant B</td>
<td>3% acetic acid (w:v)</td>
</tr>
<tr>
<td>alcoholic foods</td>
<td>simulant C</td>
<td>10 % ethanol (v:v)</td>
</tr>
<tr>
<td>fatty foods</td>
<td>simulant D</td>
<td>olive oil or alternatives:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- sunflower or corn oil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- synthetic oils such as HB307 or Miglyol 812™</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- iso-octane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 95 % ethanol (v:v)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- modified polyphenoloxide (MPPO, Tenax®)</td>
</tr>
<tr>
<td>dry foods</td>
<td>none</td>
<td></td>
</tr>
</tbody>
</table>

Especially the fatty food simulant and their alternatives require some additional comments. Because of analytical restrictions, specific migration testing in olive oil is sometimes impossible. Therefore, alternative simulants are being proposed (iso-octane and ethanol) which in contrast to oil are volatile and therefore are easy to be separated from the analyte after the migration test is performed. These volatile simulants are also of practical importance in routine overall migration testing, because the analytical input is much more restricted compared to an overall migration test in oil rendering the tests more economical. Basically however for legislative purposes, the volatile fat simulants should be used if indeed technical reasons require their application or if the values obtained in the alternative fatty food simulants are higher or equal then those obtained in one of the food oils.

The alternative oils are especially foreseen to enable overall migration measurements if analytical interferences occur between the test specimen and the olive, sunflower or corn oil. HB307 is a synthetic mixture tri-acylglycerols (main fatty acids: capric, lauric and myristic acid). Miglyol 812™ is a fractionated cocos fat (main fatty acids are caprylic and capric acid) (Figge et al., 1972; FDA, 1995b).

If the contact material is intended to come into contact with all kinds of foodstuff, migration tests in simulants B, C and D have to be accomplished. If the material is intended to come in contact with specific foodstuffs, a restricted amount of tests can be performed as indicated in Table 10.
Table 10. Food simulants to be selected for testing in special cases (EC, 1997)

<table>
<thead>
<tr>
<th>Contact foods</th>
<th>Simulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>only aqueous foods</td>
<td>A</td>
</tr>
<tr>
<td>only acidic foods</td>
<td>B</td>
</tr>
<tr>
<td>only alcoholic foods</td>
<td>C</td>
</tr>
<tr>
<td>only fatty foods</td>
<td>D</td>
</tr>
<tr>
<td>all aqueous and acidic foods</td>
<td>B</td>
</tr>
<tr>
<td>all aqueous and alcoholic foods</td>
<td>C</td>
</tr>
<tr>
<td>all acidic and alcoholic foods</td>
<td>C and B</td>
</tr>
<tr>
<td>all fatty and aqueous foods</td>
<td>D and A</td>
</tr>
<tr>
<td>all fatty and acidic foods</td>
<td>D and B</td>
</tr>
<tr>
<td>all fatty and alcoholic and aqueous foods</td>
<td>D and C</td>
</tr>
<tr>
<td>all fatty food and alcoholic and acid foods</td>
<td>D, C and B</td>
</tr>
</tbody>
</table>

It should be noted as well that if the packaging material is to be contacted with alcoholic foods with a higher ethanol content of 10 % (v:v), the ethanol concentration of simulant C has to be adjusted accordingly.

Alternatively groups of food products can be specified which are intended to come into contact with the material. In Directive 85/575/EEC (EEC, 1985), lists are specified for particular food products laying down the lists of simulants to be used in the migration tests. This is illustrated in Table 11.

As can be observed, for some foodstuffs a so-called reduction factor can be applied for the migration tests in simulant D. This is because migration in these particular foods is supposed to be lower then the migration in the fatty food simulants. Therefore, the obtained migration levels in simulant D may be divided by the reduction factor to evaluate compliance with the legislation.

Apart from the simulants to be used, the legislator specifies as well the time and temperature conditions at which the migration test should be performed as indicated in Table 12.

It is possible that the food packaging material undergoes different temperatures regimes during its use. The principle is always that the experimental conditions should simulate a worst-case scenario. In addition to this table, Directive 97/48/EC specifies the time temperatures conditions to be applied with the alternative fat simulants (Table 13).
Table 11. Some examples taken from Directive 85/575/EEC laying down the simulants to be used if the contact material is contacted with specific food groups (X=migration test in specified simulant necessary ; - = migration test in specified simulant not necessary)

<table>
<thead>
<tr>
<th>Foodstuffs</th>
<th>Simulants to be used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>non alcoholic beverages</td>
<td>X</td>
</tr>
<tr>
<td>chocolate, chocolate-coated products, etc.</td>
<td>-</td>
</tr>
<tr>
<td>meat of zoological species (chilled, fresh, salted, smoked)</td>
<td>X</td>
</tr>
<tr>
<td>egg yolk, liquid</td>
<td>X</td>
</tr>
<tr>
<td>egg yolk, powdered or frozen</td>
<td>-</td>
</tr>
<tr>
<td>animal fats and vegetable fats and oils</td>
<td>-</td>
</tr>
<tr>
<td>cheese, non processed, no rind</td>
<td>X</td>
</tr>
<tr>
<td>cheese, whole with rind</td>
<td>X</td>
</tr>
</tbody>
</table>

* as explained in the text

The recent directive 2001/62/EC (EC, 2001a) indicates that apart from conducting migration experiments, specific migration can also be estimated by using general accepted migration models based on the concentration of the migrant in the polymer. These models should of course be based on scientific data, as those introduced before. Due to this adjustment, time consuming and expensive migration tests can be avoided if indeed appropriate models are present for the selected polymer-migrant-food system.
Table 12. Time-temperature conditions for migration tests (EC, 1997)

<table>
<thead>
<tr>
<th>Conditions of actual use</th>
<th>Test conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contact time</strong></td>
<td></td>
</tr>
<tr>
<td>$t \leq 0.5$ hour</td>
<td>0.5 hour</td>
</tr>
<tr>
<td>$0.5 &lt; t \leq 1$ hour</td>
<td>1 hour</td>
</tr>
<tr>
<td>$1 &lt; t \leq 2$ hours</td>
<td>2 hours</td>
</tr>
<tr>
<td>$2 &lt; t \leq 24$ hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>$t &gt; 24$ hours</td>
<td>10 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Contact temperature</strong></th>
<th><strong>Test temperature</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>$T \leq 5^\circ C$</td>
<td>5$^\circ C$</td>
</tr>
<tr>
<td>$5^\circ C &lt; T \leq 20^\circ C$</td>
<td>20$^\circ C$</td>
</tr>
<tr>
<td>$20^\circ C &lt; T \leq 40^\circ C$</td>
<td>40$^\circ C$</td>
</tr>
<tr>
<td>$40^\circ C &lt; T \leq 70^\circ C$</td>
<td>70$^\circ C$</td>
</tr>
<tr>
<td>$70^\circ C &lt; T \leq 100^\circ C$</td>
<td>100$^\circ C$ or reflux temperature</td>
</tr>
<tr>
<td>$100^\circ C &lt; T \leq 121^\circ C$</td>
<td>121$^\circ C$*</td>
</tr>
<tr>
<td>$121^\circ C &lt; T \leq 130^\circ C$</td>
<td>130$^\circ C$*</td>
</tr>
<tr>
<td>$130^\circ C &lt; T \leq 150^\circ C$</td>
<td>150$^\circ C$**</td>
</tr>
<tr>
<td>$T &gt; 150^\circ C$</td>
<td>175$^\circ C$**</td>
</tr>
</tbody>
</table>

* use simulant C at reflux temperature
** use simulant D at 150$^\circ C$ or 175$^\circ C$ in addition to simulants A, B and C as appropriate at 100$^\circ C$ or at reflux temperature
Table 13. Time-temperature conditions for migration tests using alternative fatty food simulants (EC, 1997)

<table>
<thead>
<tr>
<th>Test conditions with</th>
<th>Test conditions with isooctane</th>
<th>Test conditions with 95% ethanol</th>
<th>Test conditions with MPPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>10d – 5°C</td>
<td>0.5 d – 5°C</td>
<td>10d – 5°C</td>
<td>-</td>
</tr>
<tr>
<td>10 d – 20°C</td>
<td>1d – 20°C</td>
<td>10d – 20°C</td>
<td>-</td>
</tr>
<tr>
<td>10 d – 40°C</td>
<td>2d – 20°C</td>
<td>10 d- 40°C</td>
<td>-</td>
</tr>
<tr>
<td>2 h – 70°C</td>
<td>0.5 h – 40°C</td>
<td>2 h – 60°C</td>
<td>-</td>
</tr>
<tr>
<td>0.5 h – 100°C</td>
<td>0.5 h – 60°C*</td>
<td>2.5 h – 60 °C*</td>
<td>0.5 h – 100°C</td>
</tr>
<tr>
<td>1h – 100°C</td>
<td>1 h – 60°C*</td>
<td>3 h – 60°C*</td>
<td>1h –100°C</td>
</tr>
<tr>
<td>2h – 100°C</td>
<td>1.5 h – 60°C*</td>
<td>3.5 h – 60°C*</td>
<td>2h –100°C</td>
</tr>
<tr>
<td>0.5 h – 121°C</td>
<td>1.5 h- 60°C*</td>
<td>3.5 h – 60°C*</td>
<td>0.5h – 121°C</td>
</tr>
<tr>
<td>1 h – 121°C</td>
<td>2 h- 60°C*</td>
<td>4 h – 60°C*</td>
<td>1h – 121°C</td>
</tr>
<tr>
<td>2 h – 121°C</td>
<td>2.5 h- 60°C*</td>
<td>4.5 h – 60°C*</td>
<td>2h – 121°C</td>
</tr>
<tr>
<td>0.5 h – 130°C</td>
<td>2.0 h – 60°C*</td>
<td>4.0 h – 60°C*</td>
<td>0.5 h – 130°C</td>
</tr>
<tr>
<td>1 h – 130°C</td>
<td>2.5 h – 60°C*</td>
<td>4.5 h – 60°C*</td>
<td>1 h – 130°C</td>
</tr>
<tr>
<td>2 h – 150°C</td>
<td>3.0 h – 60°C*</td>
<td>5.0 h – 60°C*</td>
<td>2 h – 150°C</td>
</tr>
</tbody>
</table>

* Volatile test media are used up to a maximum temperature of 60°C. Materials should withstand mechanically test conditions applied in simulant D

1.2.5.4. Aspects of US food contact material legislation

In contrast to the European legislation, in which migrants from plastic food contact materials are considered as contaminants, the US legislation considers migrants as indirect food additives. Indirect food additives are compounds “used in the processing, packaging, holding and transporting of foods that have no functional effect in the food but which may reasonably be expected to become components of the food” (FDA, 1995b). So in contrast to direct food additives they do not exhibit a functional effect in the food.

In order to use an indirect additive that does not confirm to an existing regulation, a petition proposing the issuance of a new regulation should be filed. In this petition the following aspects should be documented:

- identification of the indirect food additive
- proposed conditions of use of the indirect food additive
- intended technical effect of the indirect food additive
- analytical methods for the indirect food additive
- migration data with regard to the indirect food additive
- exposure assessment of the indirect food additive

This file is submitted to the US Food and Drug Administration (FDA) for approval.

Especially the two latter aspects merit some more explanation to allow a proper comparison with the legislative approach within the EU. If migration data indicate however that the indirect food additive concentration is less or equal to 0.5 ppb in the daily diet or the daily intake is less or equal to 1.5 µg/person, no issue of regulation is required (Begley, 1997; Begley, 2000). The substance should not be a carcinogen or should not contain carcinogenic impurities (Code of Federal Regulations, 1999). This policy is better known as the threshold of regulation. Because of its importance, it will be discussed in the first place.

1.2.5.4.1. **Threshold of regulation and threshold of toxicological concern**

The threshold of regulation is based on the so-called threshold of toxicological concern for chemicals present in the diet. This threshold can be defined as a level of exposure to chemicals below which no significant risk is expected to exist (Kroes et al., 2000). The concept goes further than the practice of setting acceptable daily intake because it proposes a threshold of exposure for any chemical, including those of unknown toxicity, below which no significant risk to the human health is observed.

The threshold of regulation is based on a database of Gold et al. (1984) containing potent carcinogenic compounds. By making use of the probabilistic distribution of carcinogenic potencies, the dietary concentration of most carcinogens which would give rise to a one in a million upper bound life-time risk of cancer was estimated to be 0.5 ppb, from which a human exposure of 1.5 µg/person/day was derived. Enlarging the database did not alter the original carcinogenic potency distribution significantly (Gold et al., 1995).

Apart from the carcinogenic endpoint of toxicity, the applicability of the threshold of toxicological concern was also evaluated for other kinds of toxicity. Therefore a more extensive database (Munro et al., 1996) based on the chemical structure of various chemicals was used in a study concerning neuro-, immuno-, developmental- and endocrine toxicity together with allergenicity (Kroes et al., 2000). From this study it could be concluded that generally the threshold set based on carcinogenicity data is considered conservative enough to ensure safety with regard to the other types of toxicity mentioned. In such a way a supplementary safety factor could be attributed to the threshold of regulation. The threshold of toxicological concern could moreover be used in other contexts as well.
applications as well (Kroes et al., 2000).

Barlow et al. (2001) however, considered additional data necessary to come to a final conclusion for the endocrine disrupting chemicals, the immunotoxic compounds and the allergens. The concept can furthermore be questioned because of variations in human sensitivity or because it is inappropriate to deal with bioaccumulating compounds. It should be noted as well that outliers to the set threshold of 1.5 µg/person/day were already found as well. If the concept is to be applied for other kinds of chemical exposure, supplementary toxicological data should be considered as well.

Despite of these points of criticism, the threshold of toxicological concern is generally considered as a useful instrument to evaluate the relevance of toxicity of chemical substance. In such a way limited resources can be concentrated to those chemicals of concern. More especially, it has been applied successfully in the US in the presented threshold of regulation (Barlow et al., 2001).

1.2.5.4.2. Migration testing

In contrast to the European legislation, FDA differentiates five food types and four kinds of food simulants as indicated in Table 14.

<table>
<thead>
<tr>
<th>Food type</th>
<th>Recommended food simulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>aqueous foods</td>
<td>10 % ethanol (v:v)</td>
</tr>
<tr>
<td>acidic foods</td>
<td>10 % ethanol (v:v)</td>
</tr>
<tr>
<td>low alcoholic foods</td>
<td>10 % ethanol (v:v)</td>
</tr>
<tr>
<td>high alcoholic foods</td>
<td>50 % ethanol (v:v)</td>
</tr>
<tr>
<td>fatty foods</td>
<td>food oil (e.g. corn oil), HB 307 or Miglyol 812™</td>
</tr>
</tbody>
</table>

Water and 3 % acetic acid are not recommended to be used because these simulants seem to underestimate migration into aqueous foods. If on the other hand it is expected that migration levels in 3% acetic are higher compared to those in 10 % ethanol, like in the case of acid sensitive indirect food additives, tests in the former simulant should be performed (FDA, 1995b). Simulants should be selected to simulate a worst-case scenario.

As in the EU, alternative fatty food simulants are proposed to deal with analytical problems. According to the FDA, not one solvent can effectively simulate food oil for all polymers. The use of alternative fatty food simulants is specified and restricted to some polymers as indicated in Table
15. As can be seen, only ethanolic solutions are used. Previously also heptane was used, but this simulant was thought to be too aggressive and therefore its use is not recommended anymore.

Table 15. Alternative fatty food simulants for specified polymeric materials (FDA, 1995b)

<table>
<thead>
<tr>
<th>Polymeric material</th>
<th>Alternative fatty food simulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>polyolefins and ethylene-vinylacetate copolymers</td>
<td>95% ethanol (v:v)</td>
</tr>
<tr>
<td>rigid polyvinyl chloride</td>
<td>50% ethanol (v:v)</td>
</tr>
<tr>
<td>polystyrene and rubber modified polystyrene</td>
<td>50% ethanol (v:v)</td>
</tr>
</tbody>
</table>

Also with regard to the selected time temperature conditions a different approach as the one in the EU is used. Generally migration tests are also conducted for 10 days at 40°C if the packaging material is used at room temperatures. For polymers used below their glass transition temperature, migration data obtained over 10 days should be extrapolated to 30 days to better approximate migration levels expected after extended time periods at 20°C. For frozen or refrigerated food applications, a test temperature of 20°C during 10 days is used. Other time temperature combinations are proposed if the time-temperature regime does not correspond to the ones indicated above (e.g. hot fill applications, …). The rather rational approach of the EU legislation is not followed, resulting in number of selected migration testing protocols, which will not be discussed in detail here (FDA, 1995b).

A very important difference with regard to the EU legislation concerns the fact that only the specific migration of the indirect food additive is to be monitored. No overall migration needs to be determined and no limit in this regard is specified.

Similar to EU legislation, reliable migration models may be used to replace or extrapolate migration experiments (Begley, 2000). In addition to these models, the FDA compiled a migration database from various sources that can be used to minimize the migration experiments to be performed. If migration experiments are to be executed, additional data with regard to the migration dynamics are requested to extend the FDA database. (e.g. for a ten day test, migration data at 2, 24, 96 and 240 hours are requested).

1.2.5.4.3. **Exposure assessment**

From the migration data, obtained by experiment or simulation, the exposure to the indirect food additive needs to be calculated by the petitioner. As indicated above, if migration data reveal that
the daily dietary intake is lower then the threshold value of 1.5 µg/person, the substance is of no legal concern.

If migration results in higher concentrations then the threshold, exposure assessment is necessary. These calculations are based on the migration experiments conducted, the consumption factors of food packaging materials and the so-called food type distribution factors.

The consumption factors (CF) of food packaging materials have been introduced before (Table 2). These factors represent the weight percentage of the daily diet contacted with a specific kind of a food contact material and are based on market surveys. In addition to this, a more detailed table is available for polymeric food contact materials as indicated in Table 16. The minimal consumption factor used is 0.05.

Table 16. Consumption factors (CF) for polymeric food contact materials (FDA, 1995b; Begley, 2000)

<table>
<thead>
<tr>
<th>Polymeric contact material</th>
<th>CF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>polyolefins</td>
<td>33</td>
</tr>
<tr>
<td>low density polyethylene</td>
<td>18</td>
</tr>
<tr>
<td>high density polyethylene</td>
<td>13</td>
</tr>
<tr>
<td>polypropylene</td>
<td>2</td>
</tr>
<tr>
<td>acrylics, phenolics, etc.</td>
<td>15</td>
</tr>
<tr>
<td>polystyrene</td>
<td>10</td>
</tr>
<tr>
<td>PVC</td>
<td>10</td>
</tr>
<tr>
<td>all others</td>
<td>5</td>
</tr>
</tbody>
</table>

The food-type distribution factor (fT) reflects the fraction of all food contacting each material that is aqueous, acidic, alcoholic and fatty. Again these data are derived from market surveys and tabulated data are available (Table 17).

On the basis of the two factors introduced above and by the data obtained from the migration experiments, the concentration of the indirect food additive in the daily diet, CF, daily, can be calculated as follows.
Table 17. Food type distribution factors (in percent) (FDA, 1995b; Begley, 2000)

<table>
<thead>
<tr>
<th>Package category</th>
<th>Aqueous(^a)</th>
<th>Acidic(^a)</th>
<th>Alcoholic</th>
<th>Fatty</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. General</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>8</td>
<td>36</td>
<td>47</td>
<td>9</td>
</tr>
<tr>
<td>metal-polymer coated</td>
<td>16</td>
<td>35</td>
<td>40</td>
<td>9</td>
</tr>
<tr>
<td>metal- uncoated</td>
<td>54</td>
<td>25</td>
<td>1(^b)</td>
<td>20</td>
</tr>
<tr>
<td>paper-polymer coated</td>
<td>55</td>
<td>4</td>
<td>1(^b)</td>
<td>40</td>
</tr>
<tr>
<td>paper-uncoated</td>
<td>57</td>
<td>1(^b)</td>
<td>1(^b)</td>
<td>41</td>
</tr>
<tr>
<td>polymer</td>
<td>49</td>
<td>16</td>
<td>1(^b)</td>
<td>34</td>
</tr>
<tr>
<td><strong>B. Polymer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>polyolefins, polystyrene</td>
<td>67</td>
<td>1(^b)</td>
<td>1(^b)</td>
<td>31</td>
</tr>
<tr>
<td>PVC</td>
<td>17</td>
<td>40</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>acrylonitrile, ionomers, PVDC</td>
<td>1(^b)</td>
<td>1(^b)</td>
<td>1(^b)</td>
<td>97</td>
</tr>
<tr>
<td>polycarbonates</td>
<td>97</td>
<td>1(^b)</td>
<td>1(^b)</td>
<td>1(^b)</td>
</tr>
<tr>
<td>polysters</td>
<td>1(^b)</td>
<td>97</td>
<td>1(^b)</td>
<td>1(^b)</td>
</tr>
<tr>
<td>ethylenevinlyl alcohol</td>
<td>30</td>
<td>28</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>wax</td>
<td>47</td>
<td>1(^b)</td>
<td>1(^b)</td>
<td>51</td>
</tr>
<tr>
<td>cellophane</td>
<td>5</td>
<td>1(^b)</td>
<td>1(^b)</td>
<td>93</td>
</tr>
</tbody>
</table>

\(^a\) for aqueous, acidic foods, generally 10 % ethanol is used as a simulant, therefore the food type distribution factors should be summed

\(^b\) 1 % or less

The concentration of the migrant in a food contacted with a material \(j\), \(C_{F,j}\) is equal to

\[
C_{F,j} = \sum_{i=1}^{4} f_i \times M_i \tag{27a}
\]

where \(M_i\) represents the concentration of the indirect food additive in the food simulant used in the migration experiment and \(f_i\) is the food type distribution factor of material \(j\). Since the food simulant for aqueous and acidic foods is generally the same equation [27a] can be rewritten as follows

\[
C_{F,j} = (f_{\text{aqueous}} + f_{\text{acidic}}) \times M_{\text{10\% ethanol}} + f_{\text{alcoholic}} \times M_{\text{50\% ethanol}} + f_{\text{fatty}} \times M_{\text{fatty}} \tag{27b}
\]

The average concentration of the migrant in the daily diet, \(C_{F, \text{daily, } j}\) due to migration of a single contact material \(j\), can be calculated by combining \(C_{F,j}\) and the percentage of the daily diet in contact with the contact material ‘\(j\’\) (CF)
Because the migrant can be present in several contact materials, the total concentration in the daily diet should be calculated by taking into account the migration from all contact materials \( j \) involved. Therefore, \( C_{F, \text{daily}} \) becomes

\[
C_{F, \text{daily}} = \sum_j C_{F,j} \times CF_j
\]

The estimated probable daily intake of the indirect food additive (EDI) is then determined by multiplying the dietary concentration by the total weight consumed by an individual per day, which is supposed to be 3000 g:

\[
\text{EDI} = 3000 \frac{g \text{ food}}{\text{person.day}} \times C_{F, \text{daily}}
\]

This EDI represents the cumulative exposure to the indirect food additive present in all contact materials and is used for risk evaluation.

### 1.2.6. Analytical approach to study migration

#### 1.2.6.1. Introduction

As a final part of this review on migration from plastic food contact materials, analytical methodologies to study this phenomenon will be discussed. Two approaches should be distinguished:

- the approach of an enforcement laboratory, who’s tasks consists to check whether a contact material used on the market complies with legislation
- the approach of other analytical laboratories which are requested by a producer of contact materials to prove compliance with legislation.

The second approach is far more easier because the laboratory generally can use the confidential compositional information of the material to decide which tests are necessary (overall migration, specific migration for selected compounds). In contrast, the enforcement laboratory has no information at all about the kind and composition of the contact material, making the analytical task far more complex.

Once the material and composition have been revealed, the enforcement laboratory can also evaluate the migration behaviour. Therefore tests in simulants can be used or alternatively, concentration of the migrants in the food can be assessed. This latter approach again is much more difficult because of the additional matrix affects. On the other hand it is indispensable if exposure studies are to be performed. Such exposure studies are an essential tool in the analysis of risks.
arising from the exposure of migrants to the consumer. As indicated in a recent review however, such exposure studies and risk evaluations related to migration from food contact materials are still scarce (De Meulenaer and Huyghebaert, 2001a).

The following general approach can be presented for a migration study:

- analysis of the material
  - identification of the material
  - compositional analysis of the material
- selection of the appropriate contact conditions
- migration testing
  - overall migration
  - specific migration

1.2.6.2. Analysis of the material

The first task is to identify the polymeric object. An experienced person may often identify the polymer by appearance or by using simple tests such as a burning experiment. For a more detailed analysis, the method of choice is infra red analysis, using a Fourier transform infrared spectrometer (FTIR). Because of the complexity of the spectra obtained, only a limited part of the spectrum is used to assign specific structural units of the polymer. In addition, spectral libraries for comparison and confirmation are indispensable. These are commercially available but supplementation with own spectra gathered from the analysis of known samples is recommended. In addition to FTIR analysis, also the use of other advanced analytical methods such as pyrolisis-GC or GC-MS, $^1$H or $^{13}$C NMR and Raman spectroscopy is reported. Although some of them are very powerful, their use is still restricted (Castle, 1996).

The second step consists of the compositional analysis. In principle it is not only required to test for the hundreds of authorised substances but also for unapproved ingredients and contaminants. This enormous task is clearly too resource intensive for enforcement. Pragmatic approaches are considered to be more useful than a detailed analytical analysis of every possible compound. Preliminary functional group identification of mixtures of additives using $^1$H NMR has been suggested by Feigenbaum et al. (1994) as a useful technique.

The Netherlands Food Inspection Service extracts the material with diethyl ether and analyses the extract subsequently with IR and GC-MS (Van Battum and Van Lierop, 1988; Van Lierop, 1994). An additional analysis using LC-MS could be useful as well to include less volatile ingredients (Castle, 1996). A library of spectral data (MS, IR, NMR) has been published (Bush et al., 1993) and an even
more extensive collection of spectra is available on the internet ([http://cpf.jrc.it/smt/home.htm](http://cpf.jrc.it/smt/home.htm)). The German approach consists of a solvent extraction and a fractionation technique followed by a chromatographic analysis using thin layer, gas, liquid or size exclusion chromatography (Mücke, 1988). This approach is considered especially useful if an idea about the expected compounds is available (Castle, 1996).

### 1.2.6.3. Contacting food simulant and the test specimen

A polymer can be contacted with a food simulant to
- achieve a total extraction of the polymer
- achieve a simulation of a food contact at a particular time-temperature combination

Experimental conditions have been normalized for a rapid extraction test as an alternative for overall migration assessment in fatty food simulants (Table 18)

<table>
<thead>
<tr>
<th>Polymer type</th>
<th>Solvent</th>
<th>Extraction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>polyolefins</td>
<td>iso-octane</td>
<td>24h at 40°C</td>
</tr>
<tr>
<td>polyamides</td>
<td>95% ethanol (v:v)</td>
<td>24h at 40°C</td>
</tr>
<tr>
<td>polystyrene</td>
<td>iso-octane and 95% ethanol (v:v)</td>
<td>24h at 40°C</td>
</tr>
<tr>
<td>polyethylene terephthalate</td>
<td>95% ethanol (v:v)</td>
<td>24h at 50°C</td>
</tr>
<tr>
<td>polyvinyl chloride (plasticised)</td>
<td>iso-octane and 95% ethanol (v:v)</td>
<td>24h at 40°C</td>
</tr>
<tr>
<td>polyvinyl chloride (rigid)</td>
<td>95% ethanol (v:v)</td>
<td>24h at 50°C</td>
</tr>
<tr>
<td>others</td>
<td>iso-octane and 95% ethanol (v:v)</td>
<td>24h at 50°C</td>
</tr>
</tbody>
</table>

It should be noted that these rapid extraction tests have not been focused on specific migration experiments. Especially if specific migrants are labile, care should be taken to use these tests for specific migration measurements (Franz, 2000).

General contact conditions to carry out overall (CEN, 1994a) and specific (CEN, 1999a) migration tests using the simulants and conditions as foreseen by legislation have been normalised. Contact between the plastic and the food can be simulated by total immersion, article filling or by using a migration cell. Such a migration cell is a stainless steel cell, consisting of two plates and provided in between with a spacer, in which the material (e.g. plastic film) is attached. The void space, due to the presence of the spacer, is filled with the appropriate simulant (Figure 12.)
Overall migration testing

In addition to the general contact conditions for overall migration testing, test methods for overall migration assessment from plastics have been normalised as well (CEN, 1994b-j; CEN, 1995a-b, CEN, 1999b-c) (Table 19). These methods are available from CEN or some of them can be consulted on the internet (http://cpf.jrc.it/webpack/analytic.htm). Only some general aspects will be discussed.

Overall migration testing in volatile food simulants involves the evaporation of the simulant to dryness. The residue contains all non volatile migrants. Loss of volatiles can be evaluated by incubating the test specimen in analogue conditions without contacting it with the food simulant and measuring the weight loss after contact.

Overall migration testing in non volatile fatty food simulants is a much more complicated procedure since the simulant cannot be removed after contact. Therefore mass changes in the test specimen are monitored (Rossi, 1981). Two important drawbacks should be taken into account using this methodology:

- absorption of oil by the test specimen
- moisture loss/uptake by the test specimen in the case of polar materials such as polyamide, coated paper etc.

The first drawback can be solved by a total extraction of the polymer after contact and subsequent gas chromatographic quantification of the absorbed amount of fat. Again problems may occur due to the presence of interfering compounds in the polymer which are co-extracted with the oil (Ledegen and Vergallen, 1995; Ledegen and Vergallen, 1996). The use of other food oils than olive oil could provide a solution.
The second drawback can be solved by ensuring a constant moisture content of the test specimen by equilibration in a moisture controlled atmosphere or by measuring the moisture content of the material using the Karl-Fisher method (Castle et al., 1992). Because of the more complex analytical methodology compared to overall migration assessment in volatile simulants, the analytical tolerance is 3 mg.dm$^{-2}$ instead of 1 mg.dm$^{-2}$.

Table 19. Normalised overall migration test methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>overall migration in olive oil by total immersion</td>
<td>CEN, 1994b</td>
</tr>
<tr>
<td>overall migration into aqueous simulants by total immersion</td>
<td>CEN, 1994c</td>
</tr>
<tr>
<td>overall migration olive oil by cell</td>
<td>CEN, 1994d</td>
</tr>
<tr>
<td>overall migration into aqueous simulants by cell</td>
<td>CEN, 1994e</td>
</tr>
<tr>
<td>overall migration in olive oil using a pouch</td>
<td>CEN, 1994f</td>
</tr>
<tr>
<td>overall migration into aqueous simulants using a pouch</td>
<td>CEN, 1994g</td>
</tr>
<tr>
<td>overall migration into olive oil by article filling</td>
<td>CEN, 1994h</td>
</tr>
<tr>
<td>overall migration into aqueous simulants by article filling</td>
<td>CEN, 1994i</td>
</tr>
<tr>
<td>overall migration into olive oil (modified method for use in case where incomplete extraction of olive oil occurs)</td>
<td>CEN, 1994j</td>
</tr>
<tr>
<td>test methods for overall migration into mixtures of $^{14}$C-labelled synthetic tri-acyl glycerols</td>
<td>CEN, 1995a</td>
</tr>
<tr>
<td>test methods for overall migration at low temperatures</td>
<td>CEN, 1995b</td>
</tr>
<tr>
<td>test methods for overall migration at high temperatures</td>
<td>CEN, 1999b</td>
</tr>
<tr>
<td>test methods for ‘substitute tests’ for overall migration using iso-octane and 95% ethanol</td>
<td>CEN, 1999c</td>
</tr>
</tbody>
</table>

For the sake of completeness it should be mentioned that BCR/IRRM reference materials and ring tests (e.g. FAPAS programme of the Central Science Laboratory, UK, http://ptg.csl.gov.uk/current.cfm) are available to allow analytical laboratories to check the validity of their analytical methods in overall migration assessment.

1.2.6.5. Specific migration testing

1.2.6.5.1. General remarks

Specific migration testing is not always a necessity, even if a specific migration limit for a particular
migrant is specified. By assuming total migration of the migrant from the polymer to the food, agreement with the specific migration limit can be checked if some product specifications are known (e.g. migrant concentration in the polymer, thickness of the polymer). Provided that the partition coefficient is known, a similar calculation using the concentration of the migrating substance in the polymer in equilibrium conditions can be another method not requiring advanced analytical methods. Alternatively, mathematical modelling as indicated before can be a valuable option for specific migration testing in particular circumstances.

Most frequently however chemical analysis is necessary to demonstrate compliance of a polymer-migrant system. Most laboratories apply their own methods to tackle the analysis of specific migrants in foods or food simulants. As a consequence, results obtained in different laboratories for the same sample may vary in such a way that there is a need for uniform, standardized and validated analytical methods (Franz, 2000). Such a standardization and validation program has been undertaken already in a Standards Measuring and Testing Program funded by the EU for 36 monomers. For 7 substances, normalized methods for their analysis in foods or in plastics are already available (CEN, 1999d-j). In addition, other methods are available on the internet (http://cpf.jrc.it/smt/home.htm). In addition, reference materials and ring tests (e.g. FAPAS programme) are available to allow analytical laboratories to check the validity of some of their analytical methods to study specific migration.

It is impossible to discuss every possible analytical method in detail. In order to illustrate some of the major techniques used in specific migration analysis, a review is presented with regard to the analytical methodologies related to xeno-estrogenic migrants from food contact materials.

1.2.6.5.2. Analytical methodologies to study migration of xeno-estrogens from food contact materials (adopted from De Meulenaer and Huyghebaert, 2001b)

Xeno estrogens from food contact materials

Although various xeno-estrogenic compounds are described, with regard to food contact materials, especially the compounds shown in Figure 13 are of interest: bisphenol A 1, di-n-butyl phthalate 2, butyl benzyl phthalate 3 and bisphenol A diglycidyl ether 4 (BADGE). In this review, only attention towards the migration from these compounds is considered, although other estrogenic compounds from contact materials (e.g. dioxins in paper), could enter into the food chain (Lafleur et al., 1990; Lafleur et al., 1991; Kardinaal et al., 1997).
Figure 13. Xeno estrogenic compounds from food contact materials

Phthalic acid esters
For the phthalic acid esters, especially di-n-butyl and butyl benzyl phthalate have been reported to be estrogenic.

Phthalate esters in general are extensively used in polyvinyl chloride (PVC) for food packaging and other applications. Within this respect it should be emphasised that PVC is a very brittle material in its pure state and requires the incorporation of various additives such as plasticizers to give its required properties. These additives, which are not part of the polymer matrix, are added in concentrations that may exceed 50% of the total weight of the material. The most frequently applied phthalate esters is di-(2-ethyl-hexyl) phthalate (DEHP) and di-n-butyl phthalate (Lhuguenot, 1997). Besides PVC, the phthalates are reported to plasticise polyvinyl acetate and polyvinylidene chloride, which are both important barrier polymers used in multiple layered films. Furthermore their use in polystyrene, acrylic type resins, epoxy and phenolic alkyls etc. is reported (Fishbein and Albro, 1972). Apart from their use as a plasticiser in various polymers, phthalate esters are used in printing inks used in food packaging applications in which they contribute to the adhesion of the ink, imparting improved flexibility and wrinkle resistance (Nerin et al., 1993). Due to their extensive use, not only in the food packaging industry as already stressed, phthalates have been found to contaminate animal tissues, human blood, sediments etc. (Fishbein and Albro, 1972). Because of the reported toxicity of especially DEHP, the use of PVC in food packaging applications has been reduced extensively the last decade and other plasticizers have been introduced as well.
Bisphenol A
For food applications, bisphenol A is used for the production of polycarbonate. This amorphous thermoplastic possesses a balance of functional properties such as toughness, optical clarity and fairly high temperature resistance. Polycarbonate is especially used for the packaging of dairy products in refillable bottles and for the manufacture of tableware such as baby bottles (Howe and Borodinsky, 1998). Apart from their use in polycarbonate materials, bisphenol A is used for the polymeric coating of food cans. Such a coating is applied to prevent the can from corroding. These polymeric coatings are usually highly cross-linked thermoset resins. The most widely used types of internal coating, if heat resistance is required, are epoxyphenolic resins, PVC organosols and cross-linked polyester resins. The epoxyphenolic resins are the most important within this respect because bisphenol A is used to make most types of epoxy resins. These resins are cross-linked before being applied to the can interior to decrease possible migration (MAFF, 2001).

BADGE
The bisphenol A epoxy derivative BADGE is used as well for the manufacture of epoxy, acrylic, polyesters and organosol coatings both as a stabilizer (e.g. PVC organosols to scavenge hydrogen chloride) and cross linking agent (Sharman et al., 1995, Grob et al., 1999). These coatings are applied at the interior part of cans, water storage installations, pipes etc. Its use as an adhesive component is also reported (Sharman et al., 1995).

Analytical methodologies for phthalates

Analytical techniques
For the analysis of estrogenic phthalates, methodologies described for the analysis of phthalate esters can be applied. Giam and Wong (1987) reviewed at the end of the eighties some older analytical methodologies such as packed column gas chromatographic analysis, thin layer chromatography, UV spectrometry, fluorescence measurements etc. Due to the development of high resolution gas chromatography however, capillary high resolution GLC analysis of a mixture of phthalates is the method of choice nowadays. In order to facilitate identification and to allow quantification up to the ppb level, hyphenation of the gas chromatographic equipment to a mass spectrometer allowing the detection of selected ions, is indispensable. Generally, phthalates are easily separated on apolar GLC columns such as HP-1 or HP-5, Restek XTI-5, CP-Sil 5CB or similar (Nerin et al., 1993; Castle et al., 1988, Castle et al., 1989, Castle et al., 1990, Petersen and Breindahl, 2000, Hirayama et al., 2001). For the sake of completeness it should be mentioned that some commercially important phthalate esters, such as di-iso-octyl phthalate (DIP) and others are
complex mixtures of isomers that are dispersed over a wide range of retention times in capillary GLC. Therefore, LC-MS analysis under which the isomers of these phthalates are eluted as single peaks, may facilitate the quantification of these particular mixtures (MAFF, 1996). Complete chromatographic resolution of all phthalate esters is however reported to be difficult using HPLC. Moreover, limits of quantification are higher compared to GC-MS methodologies. Therefore, the possibilities of HPLC remain limited except for the quantification of particular phthalates (Lhughuenot, 1997).

Apart from the identification and quantification of individual phthalates, determination of total phthalates is reported as well. Phthalate esters are saponified in the presence of methanolic potassium hydroxide. Subsequently, phthalic acid is esterified to dimethyl phthalate by making use of boron trifluoride as a catalyst. Subsequently, the sample can be analysed by GC-MS (MAFF, 1996).

**Extraction**

Prior to the identification and quantification of phthalate esters, they need to be extracted from their matrix. If the matrix consists of the packaging material itself, chloroform extraction is frequently reported to determine phthalates in printing inks or plasticised films (Castle et al., 1989). For the analysis of PVC materials, dissolution of the polymer in tetrahydrofurane and subsequent precipitation of the polymer in methanol is reported (Castle et al., 1990). If food is to be analysed, various extraction methods are reported in the review of Giam and Wong (1987). Apart from those, the use of acetone-hexane (Castle, et al., 1988), pentane (Petersen and Breindahl, 2000), dichloromethane-acetonitrile (MAFF, 1995), dichloromethane-cyclohexane (MAFF, 1995) have been reported. For milk samples, prior to the extraction with hexane, the addition of potassium hydroxide and methanol for destabilisation of the emulsion is reported (Castle, 1990).

**Clean-up methods**

For all the extraction methods applied on foods cited above, part of the fat is co-extracted together with the phthalate esters. In order to reach low detection levels, these interfering tri-acyl-glycerols need to be removed from the extract. Although some other techniques have been used (Burns et al., 1981; Giust et al., 1990, Page and Lacroix, 1992), the method of choice is gel permeation chromatography using for example a porous styrene-divinylbenzene copolymer such as Bio-Beads S-X3 enabling the separation of for example 63 mg of fat in a single chromatographic run on a 40 cm x 1.5 cm column (Castle et al., 1990). Recently, Tsumura et al. (2001) presented a clean-up procedure involving the combined use of Florisil and Bondesil PSA columns.
Special precautions in phthalate analysis

Because phthalates are ubiquitous environmental contaminants and in particular can be a problem in the laboratory due to the contamination of chemicals and reagents, it is always necessary to take extensive precautions for cleaning of glassware, checking for possible sources of contamination and running frequent control blank samples. All glassware used, should be washed prior to use with solvents. All solvents used should be checked for phthalate contamination by evaporation of for example 100 mL of solvent to near dryness and subsequent GC analysis (Castle et al., 1990). Impure solvents should be rejected because of the multiple concentration steps throughout the analytical procedure, which transform the traces of phthalates present into significant concentrations distorting the final result (Lhuguenot, 1997). Therefore contact with other materials than glass, stainless steel or PTFE should be avoided as much as possible throughout the total analytical procedure (Castle et al., 1988).

Analytical methodologies for bisphenol A

Analytical techniques

In contrast to the phthaltic esters, bisphenol A is generally chromatographed in the liquid phase. The use of C18 (Howe and Borodinsky, 1998; Takino et al., 1999; Yoshida et al., 2001) C8 and cyano (Mountfort et al., 1997) bonded phases have been reported in combination with respectively methanol-water, acetonitrile-water or hexane-isopropanol-dichloromethane as mobile phases. For detection UV (230, 280 nm) is reported (Takino et al., 1999; Franz and Rijk, 1996) although with fluorescence detection (Ex 285 or 235 nm, Em 300 or 317 nm, Howe and Borodinsky, 1998; Mountfort et al., 1997) better sensitivity can be obtained for some matrices. Fatty matrices however, such as the official EU fatty food simulant olive oil, show interference using fluorescence detection rendering the applicability of this detector restricted (Simoneau et al., 2000).

Recently, gas chromatographic methods for the analysis of bisphenol A are reported. Lee and Peart (2000) derivatised bisphenol A from sewage, sludges and waste water into its pentafluoropropionyl or its acetylated analogue. Similarly MAFF (2001) reported in a recent food survey the use of acetylation to quantify bisphenol A in different food matrices by GC-MS analysis. Alternatively, Kawamura et al. (1999) reported trimethylsilylation prior to GC-MS analysis. Similarly, Takao et al. (1999a) reported an improved peak area by on column silylation after solid phase micro extraction of the phenol out of different matrices. Biles et al. (1997) used GC-MS without derivatisation to confirm the presence of bisphenol A in infant formulae liquid concentrates. As a stationary phase for GC analysis, HP-5 (MAFF, 2001) or similar seems appropriate.

Apart from the use of classical instrumental techniques such as chromatography, bio-analytical
methods are developed for the measurement of bisphenol A. In Japan, three groups recently developed an enzyme linked immunosorbent assay (ELISA) based respectively on the immunization of rabbits (Kodaira et al., 2000; Ohkuma et al., 2002) and on the use of mice monoclonal antibodies (Nishii et al., 2000).

**Extraction and clean-up**

For the quantification of residual bisphenol A in polycarbonate, dissolution of the polymer in chloroform or dichloromethane is reported (Sugita et al., 1994). Afterwards Mountfort et al. (1997) precipitated the polymer with isopropanol and added hexane which was subsequently analysed by HPLC. This is quite surprising, since bisphenol A is not very well soluble in hexane. Sugita et al. (1994) precipitated the polymer with acetone and dissolved the clean upper layer in water-acetonitrile to precipitate remaining oligomers. Howe and Borodinsky (1998) extracted the dissolved polymer with a diluted sodium hydroxide solution, which was subsequently analysed on HPLC.

In contrast to the phthalates, a CEN method has been prepared for the quantification of bisphenol A in the official EU food simulants (Franz and Rijk, 1996). For the non fatty food simulants, direct analysis by HPLC-UV is recommended. For olive oil, extraction of the oil with an aqueous methanol solution (1:1) and subsequent HPLC-UV analysis was found to be appropriate. Within laboratory detection limits were reported to be in the range of 0.05-0.7 mg.kg⁻¹ of food simulant. As indicated above, using fluorescence detection, better detection limits can be obtained as reported by Howe and Borodinsky (1998) and Mountfort et al. (1997) : respectively 5 30 µg.kg⁻¹. For the sake of completeness it should be noted that in the former case the fatty food simulant used, Miglyol 812™, was extracted using an aqueous tetrabutylammonium hydroxide solution and that in the latter case, solid phase clean-up was used for the extraction of the analysed infant feed samples.

In a recent MAFF survey (MAFF, 2001) a general methodology to determine bisphenol A is presented. Fat containing homogenised samples were blended with heptane-acetonitrile while for non fatty matrices heptane was omitted. After drying and concentrating the acetonitrile fraction, the sample was diluted with water prior to derivatisation with acetic anhydride. The acetylated bisphenol A was extracted with heptane, which was analysed by GC-MS. Limit of detection was 2 ppb and recoveries ranged from 81-103 %.

Yoshida et al. (2001) extracted vegetables and fruits with acetonitrile, which was afterwards applied on a Florisil cartridge for further purification prior to HPLC-UV analysis. The aqueous fraction was directly applied on an Oasis HLB extraction cartridge followed by a cleaning on a Florisil column.
For canned fish, Takino et al. (1999) extracted the sample with ethyl acetate followed by defatting with hexane-acetonitrile partitioning. Subsequently further sample purification was accomplished by applying the sample on a silicagel column prior to HPLC-UV analysis. Kawamura et al. (1999) used polystyrene solid phase cartridges to extract bisphenol A from coffee and thee drinks. Similarly Biles et al. (1997) reported the use of styrene-divinyl benzene solid phase extraction cartridges for direct application of diluted infant food formulae. After removal of the apolar interference with hexane, bisphenol A was eluted with chloroform prior to HPLC fluorescence detection.

Analytical methodologies for BADGE

Analytical techniques

Similar to bisphenol A, BADGE can be analysed both by gas and liquid chromatographic techniques. Sharman et al. (1995) for example confirmed the presence of BADGE using a CPSil 5CB column in conjunction to a mass spectrometer. Similarly Simoneau et al. (1999) used GC-MS for confirmation purposes. Most reported analytical methods however, use reversed phase HPLC using C18 columns. Earlier methods (Crathorne et al., 1986) apply UV detection (275 nm), but better performances are obtained using fluorescence detection. Previously, 275 nm was employed for excitation and 300 nm for emission ( Paseiro Losada et al., 1991a, b), but better sensitivity was later obtained at an excitation and emission wavelength of respectively 225 nm and 305 nm (Paseiro Losada et al., 1997). Currently, the latter conditions are mostly used. The same authors reported the use of both gradient and isocratic elution designed for different sets of analytical priorities (Paseiro Losada et al., 1997). Simial Gandara et al. (1993) reported the use of LC-MS as well. The proposed CEN method however describes the use of reversed phase HPLC with fluorescence detection as indicated above (Franz and Rijk, 1997). For the sake of completeness it should be mentioned that Biederman and Grob (1998) presented a gradient normal phase HPLC method coupled with fluorescence detection to enable detection of other contaminants which may originate from epoxy coatings apart from BADGE and to avoid prior clean-up steps for fatty samples.

Extraction and clean-up

For the analysis of BADGE in the package, Paseiro Losada et al. (1991b) described a fairly drastic reflux method (10h, chloroform-methanol 25:75) for epoxy amine formulations, while Sharman et al. (1995) extracted susceptor board containing a BADGE adhesive, with chloroform at room temperature. Biedermann and Grob (1998) on the other hand used acetonitrile at room temperature to extract BADGE from can coatings.
For the analysis of BADGE in foods and food simulants, it should be kept into account that BADGE undergoes hydrolysis with a half life of less than 2 days (Tice and Mc. Guiness, 1987, Tice, 1988, Paseiro Losada et al., 1992). Apart from these hydrolysis products however, the presence of chlorohydroxy derivatives as well as oligomers of BADGE have been reported to be present in canned foods due to migration from the can coating (Biedermann et al., 1997, Grob et al., 1999). Because hydrolysis products can be produced out of migrating BADGE during migration tests in aqueous simulants, their presence in the simulant should be kept into account to calculate the total specific BADGE migration from the contact material (Franz and Rijk, 1997). The advanced HPLC fluorescence method cited above (Paseiro Losada et al., 1997) is able to quantify BADGE and its hydrolysis products in water without a need of sample purification. In fatty food simulants such as olive oil, BADGE hydrolysis is not a problem. A CEN method is available (Franz and Rijk, 1997) for the quantification of BADGE in oil. The sample is dissolved in a dichloromethane-heptane mixture before it is applied on a Florisil cartridge. BADGE is eluted from the column using tetrahydrofurane. Limit of detection have been reported to be in the range of 1 to 10 ppb, depending on the food simulant used.

For oil or other fatty foods such as canned fish, a direct normal phase HPLC method is described (Biederman and Grob, 1998). Alternatively, reversed phase HPLC can be used as well. In their first survey MAFF (1997) developed a method to determine BADGE in foods. After homogenisation, the sample was extracted using dichloromethane. The extract was dried and dissolved in hexane. Finally BADGE was extracted with acetonitrile and further cleaned up. In their modified procedure recently published (MAFF, 2000), extraction of the sample with heptane-acetonitrile was followed by HPLC-fluorescence analysis. Oil of canned fish was extracted with acetonitrile and filtered over a C18 cartridge. Alternatively, florisil clean-up (Franz and Rijk, 1997), methanol (Rauter et al., 1999; Philo et al., 1997) or acetonitrile (Simoneau et al., 1999) extraction are reported to remove possible interferences from the sample extract.

1.3. Migration from other food contact materials

As indicated in the beginning of this review, apart from plastics also other contact materials are being used in the food industry (Table 1). Plastics however are clearly the most dominant materials used and in addition, migration from plastics is generally more important as well considering the total exposure to the consumer. This however does not implicate that other food contact materials are inert nor that migration from these materials is considered to be of no importance. A complete overview of the migration mechanisms however from these materials fall out of the scope of this
work. A summarizing table indicating major migrating compounds, relevant legislation and reference to more detailed literature is presented here for the sake of completeness (Table 20).

1.4. Relevance of the presented research

Three main parts can be distinguished in the experimental work of this doctoral thesis:

- a study on the chemical characterization of a complex group of plastic additives
- the development of an immunochemical method for bisphenol A analysis in foodstuffs
- migration and legislative aspects of active and intelligent packaging materials

In these three major parts, basically three innovative analytical approaches in the particular field of chemical interactions between packaging materials and foodstuffs are introduced.

1.4.1. Development of an analytical protocol for the characterisation of polyglycerol fatty acid esters

As could be concluded from the extensive literature review on migration from plastic food contact materials, a full identification of plastic additives is of prime importance. Polyglycerol fatty acid esters are a complex group of compounds for which the analytical methodology is currently not fully developed. Since these compounds can be used as additives in plastic food contact materials, their chemical characterisation is of interest. Therefore a hyphenated analytical approach will be presented in chapter 2 enabling the characterization of polyglycerols and polyglycerol fatty acid esters.

1.4.2. New developments in specific migration assessment

Two new approaches for the assessment of specific migration are presented. In the first approach, the development of an enzyme linked immunosorbent assay for the analysis of bisphenol A is discussed. This immunochemical approach is rather unusual in this particular field of food contaminant analysis as could be concluded from the literature review (paragraph 1.2.6.5.2). In chapter 3, the production of bisphenol A specific antibodies in an animal host is discussed. In the next chapter, studies on the usefulness of the isolated antibodies are presented (chapter 4).

Immunochemical methods could have some distinct advantages compared to the classical instrumental methods used to study migration. No expensive chromatographic or mass spectrometric equipment is necessary. Sample clean-up can be avoided or restricted to an important extend, because of the specificity of the antibodies used. Therefore, use of chemicals and typical devices such as solid phase extraction columns can be reduced. Immunochemical methods are reported to be quite sensitive as well. Finally, the number of samples which can be analysed in one
analytical run can be much higher.

In chapter 5, dealing with two new kinds of packaging technologies (active and intelligent packaging materials), the use of alternative aqueous food simulants is discussed to study the specific migration from selected active packaging materials.

1.4.3. **New development in overall migration assessment**

Similarly as for the specific migration assessment, the usefulness of alternative aqueous food simulants is discussed for overall migration measurement from selected active packaging materials (Chapter 5). The need for such alternative simulants is derived from overall migration studies of several active and intelligent packaging materials in the framework of a European FAIR project (ACTIPAK, CT98-4170). Apart from those findings, some legal drawbacks with regard to food packaging, food additive and other relevant legislation are discussed as well (Chapter 5).
### Table 20. Migration from other food contact materials than plastics

<table>
<thead>
<tr>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>regenerated cellulose</td>
</tr>
<tr>
<td>elastomers and rubber</td>
</tr>
<tr>
<td>paper and board</td>
</tr>
<tr>
<td>ceramics</td>
</tr>
<tr>
<td>glass</td>
</tr>
<tr>
<td>tin coated steel plate</td>
</tr>
<tr>
<td>aluminium</td>
</tr>
<tr>
<td>wood and cork</td>
</tr>
<tr>
<td>textile products</td>
</tr>
<tr>
<td>paraffins and micro-crystalline waxes</td>
</tr>
</tbody>
</table>
2. Chemical characterisation of complex plastic additives: polyglycerol esters

2.1. Introduction

2.1.1. Polyglycerol esters

Polyglycerol esters are non-ionic surfactants with an inherent complexity. They can be considered as esters from polyglycerol and fatty acids. Polyglycerol originates from an alkaline catalysed random polymerisation of glycerol via the epoxy intermediate 6 (Dolhaine et al., 1984) which is produced via a so-called neighbouring group reaction mechanism (March, 1992). Subsequently, the reactive intermediate 6 reacts with a glycerol molecule. Because of its asymmetric structure, epoxide 6 will preferentially be attacked at the less highly substituted carbon atom (March, 1992). Since additionally, the primary hydroxy group of glycerol 5 is most likely to attack the epoxide 6, it can be concluded that the non branched diglycerol isomer 7 will be preferentially produced. Similarly, it can be supposed as well that the branched isomer 8 is obtained in preference compared to isomer 9. Thus, three different non-cyclic diglycerol isomers can be produced in different quantities as indicated in Figure 14.

Figure 14. Polymerisation of glycerol to various diglycerol isomers (isomers are produced in different quantities, see text, De Meulenaer et al., 2000a)
If the polymerisation proceeds to tri-, tetra- or higher glycerols, the number of linear and branched isomers increases exponentially. Moreover, once a dimer is formed, cyclic products can result from intra-molecular ring closure reactions (Figure 14) via a similar epoxide reaction mechanism as explained above. Thus out of the non-branched isomer 7 cyclic diglycerols 10, 12, and 13 can be produced. Similarly, out of isomer 8, diglycerol 12 can be obtained as well, together with isomer 11. Since out of isomer 9 no epoxide derivative can be produced, it cannot give rise to supplementary cyclic diglycerol isomers. Predicting which cyclic isomer is preferentially produced becomes very difficult. Apart from the previous considerations with respect to the production of linear diglycerol isomers, it should be kept into account that 6 membered rings are produced preferentially compared to 7 or 8 membered rings. From the above cited principles on epoxide ring opening reactions (nucleophilic attack of the primary hydroxyl group on the less highly substituted carbon) it can be concluded that the cyclic isomer 13 would be preferentially produced. This however would be very unlikely because of the low probability 8 membered cyclic molecules are produced. Nevertheless it can be concluded that the composition of a polyglycerol mixture is extremely complex, even if a low degree of polymerisation is reached (Dolhaine et al., 1984).

Esterification is achieved using isolated fatty acids or tri-acyl glycerols in an ad random process, which may be catalysed by alkalics. This results in a very complex mixture of esters that vary in polymerisation degree, polyglycerol isomers, degree of esterification, kind of esterified fatty acids and position of esterification.

For the analysis of polyglycerols, several chromatographic techniques have been used: thin layer chromatography (Seher, 1964; Sahasrabudhe, 1967; Dallas and Stewart, 1967), gas chromatography (Sahasrabudhe, 1967), high performance liquid chromatography (Aitzemüller et al., 1979; Chaimbault et al., 1999) and super critical chromatography (Macha et al., 1994). Although some of these analyses were able to produce quantitative data, full resolution of the isomers of the lower polyglycerols was not achieved. Interestingly, pure standards of polyglycerols could be obtained using fractionated distillation (Sahasrabudhe, 1967; Schütze, 1977) or by selective chemical synthesis (Aitzemüller et al., 1979; Neissner, 1980). Pure standards of polyglycerols are necessary for quantification purposes. Many researchers have tried to analyse polyglycerols fatty acid esters using several chromatographic techniques as well, including paper (Behrens and Mieth, 1984), thin layer (Neissner, 1980), column (Schütze, 1977), gas (Sahasrabudhe, 1967; Schütze, 1977), high
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performance liquid (Garti and Aserin, 1981a; Garti and Aserin, 1982; Cassel et al., 2001) and super critical fluid chromatography (Chester and Innis, 1986). All these techniques were qualitative and most of them did not succeed to resolve the different esters present. Isolation of pure compounds was not reported as well.

Polyglycerol esters can be used as plastic additives. Their use as for example an antifogging or an antistatic agent in different kinds of films has been reported. It is evident that the composition of the final mixture will be of prime importance with regard to its functionality. In addition, the presence of side product, such as the cyclic isomers, could be of toxicological concern as well. Therefore, appropriate analytical methods to characterise these complex mixtures should be available. For the sake of completeness, it should be noted that apart from their use in the plastic industry, polyglycerol esters have an important potential for use as additives in the food (Hemker, 1981; Babayan and McIntyre, 1971; Garti and Aserin, 1981b; Dobson et al., 1993; Krog, 1990), cosmetic and pharmaceutical industry (Behrens and Mieth, 1984; Saad, 1975) as well. Especially the use of di-, tri- and tetrarglycerol mono- and di-esters is relevant (Krog, 1990).

From the previous chapter, it can be concluded that a thorough chemical identification of plastic additives is an important issue. Apart from the legal and safety aspects concerned however, it should be noted that the functionality of the polyglycerol fatty acid esters is largely dependent upon their composition (Cassel et al., 2001). So also from a functional point of view, the elucidation of the chemical composition of polyglycerol fatty acid esters is an important issue.

### 2.1.2. Research strategy

Because of the complex nature of the compounds studied, a systematic approach is proposed to tackle their chemical identification. As a first step, the chemical analysis of the polyglycerol backbone was studied using gas chromatographic techniques (De Meulenaer et al., 2000a). For the characterisation of the esters themselves, a hyphenated chromatographic separation including both liquid and gas chromatographic analysis was evaluated (De Meulenaer et al., 2000b). In combination with qualitative chromatographic analysis, the possibilities of a quantitative approach were elaborated as well. Therefore, it was necessary to obtain standards with an acceptable purity which could be used for calibration purposes.
2.2. Materials and methods

2.2.1. Reagents
Silica gel plates (60, F254, 5x10cm, film thickness 0.25 mm), silica gel 60, dry pyridine (max 0.01 % water), ethyl acetate and 2,7 dichlorofluoresceine were obtained from Merck, Germany. Hexane, chloroform, isopropanol, methanol, ethanol and potassium hydroxide were from Chemlab, Belgium. Sodium periodate, benzidine, sorbitol, benzene, methanol, ethanol, hexane, potassium hydroxide, butanol and glycerol were from Acros Organics (Belgium). Acetone (pesticide analysis grade), petroleum ether and hydrochloric acid (25%) were purchased from Vel, Belgium. Hexamethyldisilazane (HMDS) and trifluoracetic acid (TFA) were from Sigma, Belgium. Polyglycerol fatty acid esters, diglycerol and polyglycerols were provided by Beldem, Belgium. Diglycerol and glycerol were pure enough to be used without any further purification (checked by gas chromatography). Two polyglycerol samples were produced under various experimental conditions to obtain different polymerisation degrees. The two samples are further identified respectively as the triglycerol and polyglycerol sample. All reagents were of analytical grade or better unless otherwise mentioned.

2.2.2. Liquid chromatography
For the thin layer chromatographic (TLC) experiments on polyglycerols, the mobile phase consisted of ethyl acetate-isopropanol-water (5:2:1) (v:v), unless otherwise stated. The sample was dissolved in the mobile phase (typical concentrations 1 mg.mL⁻¹) and 10 µL of the solution is brought on the plate using a syringe. After development, the plates were dried by exposure to air and sprayed with a 0.1 % (w:v) aqueous sodium periodate solution. After 5 minutes, the plates were sprayed with another spray reagent, which was prepared as follows. Benzidine (2.8 g) was dissolved in 50 mL methanol, after which the solution was diluted with 50 mL water, 20 mL acetone and 10 mL 0.2 N hydrochloric acid. This spray reagent should be kept in the refrigerator. The polyglycerols are visible as white spots on a blue background (Stahl, 1967).

For open column chromatography of polyglycerols, 100 g silica gel inactivated with 5% (w:w) water packed in glass columns (internal diameter 3.2 cm) was used together with ethyl acetate-isopropanol-water (5:2:1) (v:v) as mobile phase. An appropriate amount of sample (approximately 0.5 g), dissolved in a few mL of methanol, was used for each experiment and samples of about 15 mL were collected and checked for their composition and purity using TLC. Samples containing
pure polyglycerol species were combined, dried under a gentle stream of nitrogen during one night at about 50°C to remove all traces of water. Triglycerol and tetragnlycerol could be collected from the following fraction respectively: 800-1400 mL and 1600-2400 mL.

A pure glycerol and diglycerol standard was obtained from glycerol and diglycerol samples that were pure enough to be used without any further purification (checked by gas chromatography). For the preparation of the pure triglycerol and tetragnlycerol standard, the triglycerol and the polyglycerol sample, as introduced previously (paragraph 2.2.1), were used respectively.

For TLC separation of polyglycerol esters, a mobile phase of chloroform-acetone 94:4 (v:v) was used unless otherwise mentioned. Detection was achieved by spraying the developed plates with a 0.125 % (w:v) 2,7-dichlorofluoresceine solution in ethanol.

For open column chromatographic experiments on polyglycerol esters, 50 g silica gel was inactivated with 5% (w:w) water and introduced as a slurry with the mobile phase (chloroform-acetone 94:4 (v:v)) in a glass column (2.1 cm ID, 40 cm height). Samples, immobilised on silica gel (2 g, 10 % (w:w) sample) were introduced in the column before starting the separation. Elution was first performed with chloroform-acetone 94:4 (v:v) and subsequently with acetone and methanol.

LC-MS experiments on polyglycerols and polyglycerol esters were performed on a quadrupole HP 1100 Series LC-MSD set-up (Hewlett Packard, USA). Samples were dissolved in methanol, 5 µL samples were injected and a flow rate of 1 mL MeOH.min⁻¹ was used. Electro spray-MS parameters were as follows: positive polarity, gas temperature (N₂) 340°C, nebulizing gas pressure: 50 psi, drying gas flow rate 12 L.min⁻¹, voltage at capillary: 4000 V, Quadrupole temperature: 100°C, scan range: 150-1000 amu, fragmentor (CID) 100 V. Molecular ions with a m.e⁻¹ of M+23 (due to the addition of sodium ions) are obtained. These experimental conditions were used, unless otherwise stated.

### 2.2.3. Gas chromatography

Gas chromatographic analysis of polyglycerols was performed on a Carlo Erba GC 8000 (Interscience, Belgium), provided with a FID detector. Chromatographic parameters were: stationary phase: CP Sil 5 CB WCOT, film thickness 0.25 µm, internal diameter 0.25 mm, external diameter 0.39 mm, length 25 m (Chrompack, The Netherlands); mobile phase: He 0.6 mL.min⁻¹ (controlled with a DPFC module (Carlo Erba, Interscience, Belgium); split 1/100; injector temperature: 250 °C; detector temperature: 340 °C; injection volume: 1-2 µL; temperature
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Program: 100°C for 1 min — ramp 10°C.min⁻¹ — 290°C for 10 min. The FID detector was operated with hydrogen and air at 30 mL.min⁻¹ and 300 mL.min⁻¹ respectively.

Alternatively, a Perkin Elmer GC 8700 (Perkin Elmer, USA), provided with a FID detector was used as well for cold on-column injection experiments. Chromatographic parameters were: stationary phase: CP Sil 5 CB WCOT, film thickness 0.25 µm, internal diameter 0.25 mm, external diameter 0.39 mm, length 5 m (Chrompack, The Netherlands); mobile phase: He 1 mL.min⁻¹; cold on-column injection (1-2 µL), detector temperature: 350°C, temperature program: 100°C for 1 min — ramp 10°C.min⁻¹ — 350°C for 30 min. The FID detector was operated with hydrogen and air at 30 mL.min⁻¹ and 300 mL.min⁻¹ respectively.

A dried polyglycerol sample (0.1-10 mg) was dissolved in 1 mL dry pyridine containing sorbitol as internal standard (concentration ± 1 mg.mL⁻¹). To this solution, 1 mL HMDS and 0.1 mL TFA were added to derivatise the polyglycerols. The solution was shaken vigorously and subsequently kept for 15 min at room temperature. From the upper solvent layer 1-2 µL was injected.

Preliminary gas chromatographic analysis of polyglycerol fatty acid esters was performed on a Carlo Erba GC 8000 (Interscience, Belgium), provided with a FID detector. Chromatographic parameters were: stationary phase: CP Sil 5 CB WCOT, film thickness 0.25 µm, internal diameter 0.25 mm, external diameter 0.39 mm, length 25 m (Chrompack, The Netherlands); mobile phase: He 0.6 mL.min⁻¹ (controlled with a DPFC module (Carlo Erba, Interscience, Belgium); split 1/100; injector temperature: 340°C; detector temperature: 350°C; injection volume: 1-2 µL; temperature program: 100°C for 1 min — ramp 10°C.min⁻¹ — 325°C for 66 min. The FID detector was operated with hydrogen and air at 30 mL.min⁻¹ and 300 mL.min⁻¹ respectively. Further gas chromatographic experiments were performed on a Perkin Elmer GC 8700 (Perkin Elmer, USA), provided with a FID detector. Chromatographic parameters were as described for the analysis of polyglycerols on the same instrument. Sample preparation was similar as for the polyglycerols except that no internal standard was added.

Chromatographic parameters for the fatty acid analysis were as follows: Carlo Erba GC 5160 (Interscience, Belgium), stationary phase: CP Sil 88 CB WCOT, film thickness 0.20 µm, internal diameter 0.25 mm, external diameter 0.39 mm, length 30 m (Chrompack, The Netherlands); mobile phase: He 1 mL.min⁻¹; split-splitless injector, split ratio 1/100 (1-2 µL), injector temperature 200°C, detector temperature: 250°C, temperature program: 120°C for 1 min — ramp 5°C.min⁻¹ — 200°C for 20 min. The FID detector was operated with hydrogen and air at 30 mL.min⁻¹ and 300 mL.min⁻¹.
respectively. Correction factors were derived from the injection of a reference oil (CRM 162, IRRM, Belgium).

Fatty acid analysis was performed by transesterification of the esters in a methanolic KOH solution (2N) and subsequent extraction of the fatty acid methyl esters in hexane.

Data collection of all gas chromatographic analyses and subsequent data processing were performed using the Gilson Unipoint software (Middleton, USA). These experimental conditions were followed unless otherwise stated.

2.2.4. Other methods

Continuous vacuum distillation experiments on polyglycerols, were performed using the KDL 1 equipment of Leybold-Heraeus provided with a vacuum pump, which allowed to control the pressure in the system. Typically distillation experiments were conducted between 170-240°C and at pressures of 101-266 Pa.

1H-NMR experiments on polyglycerols were performed on a Jeol PMX 270Si (270 MHz) NMR instrument. Samples were dissolved in deuterated water, using acetonitrile as reference (CH₃CN, δ=2.00).

Saponification experiments on 1 to 2 g of polyglycerol fatty acid esters were performed by refluxing the sample for at least 30 min in 40 mL of water in the presence of 8 mL 50 % aqueous (w:v) KOH. After the reaction was completed, the reaction mixture was quickly cooled and the precipitated potassium salts of the fatty acids were removed by filtration on a paper filter. The filtrate was incubated in a refrigerator (T between 0-6 °C) for one night and filtered again. One mL of a 1/25 (v:v) methanolic dilution of the filtrate was dried under nitrogen, derivatised and analysed gas chromatographically according to the method described previously (paragraph 2.2.3). For the polyglycerols the same protocol was used.

The degree of esterification of the polyglycerol esters was determined based on the determination of the saponification (Firestone, 1995) and the acetyl value (De Vleesschouwer, 1948) of the polyglycerol esters.

2.3. Results and discussion

2.3.1. Isolation of suitable polyglycerol standards

Since glycerol and diglycerol standards with an acceptable purity were available as such and
because tri- and tetraglycerol are the other important polyglycerols to consider, a column chromatographic purification for these compounds was developed. Therefore, the following solvent mixtures were evaluated in preliminary TLC experiments: ethyl acetate – isopropanol – acetone – methanol – water (50:15:15:16:4) (v:v) (Aitzemüller et al., 1979), benzene – methanol (8:3) (v:v) (Sahasrabudhe, 1967), acetone – butanol – water (5:4:1) (v:v) (Kirk and Sawyer, 1991) and ethyl acetate – isopropanol – water (7:2:1), (6:2:1), (6:3:1) and (5:2:1) (v:v) (Dallas and Stewart, 1967; Kirk and Sawyer, 1991). Only the latter solvent mixture (ethyl acetate – isopropanol – water, 5:2:1 v:v) seemed to be successful for an adequate separation of the polyglycerols present in the mixtures analysed.

The results of these preliminary experiments were used to develop and optimise a preparative column-chromatographic separation to enable the isolation of tri-and tetraglycerol standards with sufficient purity. The purity of the different isolated fractions was determined using gas chromatographic analysis. Typically, about 170 mg of triglycerol (±95 % purity) and 70 mg tetraglycerol (±90 % purity) could be obtained from 500 mg of a commercial polyglycerol sample. The impurities, identified on basis of their gas chromatographic elution behaviour, were other polyglycerols (di-tri- or tetraglycerol). Penta- and higher polyglycerols could be detected in very low amounts in the last fractions collected of such a polyglycerol sample. This indicated that probably pure standards could be obtained as well from these samples, using the proposed or a similar chromatographic method, although it would be advisable to change the solvent composition (during the experiment) to reduce solvent consumption.

Trials to isolate the different isomers of individual polyglycerols were not successful using the chromatographic set-up described above. Attempts to isolate pure standards using a continuous distillation technique failed as well. However, this latter technique was useful to obtain fractions enriched with particular isomers, as will be discussed later.

For the identification of the isolated polyglycerols the use ¹H-NMR was considered. No characteristic spectra were observed for di, tri- or tetraglycerols respectively. All spectra exhibit two multiplets at δ = 3.5 and 3.8 with a ratio of approximately 5 over 1 (data not shown). Gas chromatographic analysis of the silylated polyglycerols, coupled to mass spectrometry did not allow to confirm the identification as well, since no characteristic ions were obtained for all the samples analysed (data not shown).

Proper identification of the isolated tri- and tetraglycerols could be achieved using an LC-MS technique, obtaining mass spectra of the underivatised polyglycerols species. Both spectra are displayed in Figure 15. The molecular ion is clearly observed at a m.e⁻¹ of M+23 due to the addition
of sodium to the molecular ion. Furthermore some impurities were observed in both spectra (approximately 20%). This level of impurities was higher compared to the purity as could be calculated from the gas chromatograms. A remarkable peak at \( M+23+56 \) is however observed in both spectra as well. This compound can be attributed to the presence of acrolein that is an impurity present in polyglycerols. It seems that this aldehyde reacts with the polyols to obtain the corresponding hemi-acetal, which is detected in the mass spectra. Possibly, the corresponding acetal could be formed as well, but it is known that these type of acetals are unstable and decompose to the corresponding hemi-acetal during the mass spectral analysis (Verhé, personal communication).

\[
\begin{align*}
\text{Max: 84797} \\
\text{Max: 56540}
\end{align*}
\]

Figure 15. Mass spectra of tri- and tetraglycerol purified using column chromatography.

### 2.3.2. Gas chromatographic identification of polyglycerols

Like sugars, polyglycerols are polyalcohols. Thus it was tried to derivatise them to trimethyl silyl derivatives, using the HMDS/TFA derivatisation method. These compounds are known to be more volatile and more heat stable compared to the polyalcohols. A similar protocol was already described by Sahasrabudhe (1967) and is frequently applied for the analysis of mono- and disaccharides (Southgate, 1991). A chromatogram of a polyglycerol sample to which an internal standard was added (see paragraph 2.3.3) is shown in Figure 16.
Glycerol, which is not present in the chromatogram shown in Figure 16, eluted as one peak at about 7.3 minutes.

Since diglycerol has the possibility to form different isomers (Figure 14), also different trimethyl silyl derivatives should be observed in the chromatogram. In fact two major peaks could be distinguished at 12.9 and 13.1 min respectively. The ratio between these two peaks was constant for a certain batch of polyglycerol analysed. Based on reaction probabilities, the $\alpha,\alpha$-diglycerol 7 (Figure 14) will be more readily synthesised compared to the branched isomer $\alpha,\beta$-diglycerol 8, as discussed before (see paragraph 2.1.1). Therefore, the highest peak (13.1 min) can be attributed to the linear and the lower peak (12.9 min) to the branched isomer. Similarly the third, very small peak in front of the two major peaks (visible in the enlargement of a chromatogram from a fractionated sample which is enriched in particular isomers, Figure 17) was attributed to the branched isomer $\beta,\beta$-diglycerol 9. Again the lower concentration of this compound in the mixture was expected, as explained before (see paragraph 2.1.1). An additional confirmation of this identification, is the typical elution order of these compounds. As the $\beta,\beta$-diglycerol 9 is a more compact molecule compared to the linear isomer 7, it can be expected to be more volatile as well, because of the smaller molecular interactions possible. Because of the gas chromatographic set-up used (apolar column), the molecules are essentially separated on basis of their volatility. Consequently, the observed elution order of the non cyclic diglycerol isomers is in correspondence with their
molecular structure. So it can be concluded that all the possible non-cyclic isomers of diglycerol were identified on the chromatogram.

![Gas chromatogram](image)

**Figure 17.** Detail of a gas chromatogram of a derivatised diglycerol sample obtained by fractionated distillation (enriched in cyclic isomers) - Identification of peaks: 1. cyclic diglycerol $10$ or $12$, 2. cyclic diglycerol $11$, 3. cyclic diglycerol $10$ or $12$, 4. cyclic diglycerol $13$, 5. $\beta,\beta$-diglycerol $9$, 6. $\alpha,\beta$-diglycerol $8$, 7. $\alpha,\alpha$-diglycerol $7$

In the detailed chromatogram (Figure 17), a number of small peaks eluting before the non-cyclic isomers were observed as well (retention time around 10 min). It seemed reasonable to attribute these peaks to the cyclic diglycerol isomers. Several reasons are supporting this hypothesis. First of all, it can be expected that the retention time of the cyclic-diglycerol isomers will be lower compared to the linear isomers, since the molecular weights of the trimethyl silyl derivatives is lower. Furthermore during a fractionated distillation experiment of a polyglycerol mixture an enrichment of these cyclic compounds was obtained in the first (most volatile) fractions collected, while afterwards fractions containing higher amounts of linear diglycerol isomers were isolated (results not shown). This can be explained by the higher volatility of the cyclic isomers compared to the non-cyclic species because of the lower number of (polar) hydroxyl groups and their more compact molecular structure. Finally, Sahasrabudhe (1967) proposed a similar elution profile of the isomers of diglycerols, although in these experiments no complete resolution of the different isomers could be obtained.
A remarkable observation was made, studying in detail the chromatogram displayed in Figure 17: the chromatogram seemed to consist of four pairs of peaks, of which one pair is extremely small (pair 4). Since four cyclic isomers of diglycerol can be formed (Figure 14) and because from each compound two stereoisomers exist due to the 3-dimensional orientation of the ring substituents, eight possible compounds can be obtained. This corresponds with the number of peaks eluting before the non cyclic diglycerol isomers in the chromatogram shown in Figure 17. Since Dolhaine et. al (1984) stated that 8-12 membered cyclic polyglycerols are rarely formed, the smallest couple (peak 4, Figure 17) is most probably due to the eight membered cyclic isomer 13, which seemed to be formed in very low concentrations. The largest couple (peak 3, Figure 17) can be due to the six membered cyclic isomer 10 or the seven membered cyclic isomer 12. Normally, 6-membered ring structures are preferably produced compared to 7-membered rings. Because in addition isomer 10 is produced out of the most abundant linear isomer 7, its concentration can be expected to be relatively high as well (compared to the other cyclic isomers). One the other hand however, it should be noted that although the 7-membered isomer 12 is probably not preferentially produced, it can be obtained via two synthetic routes of which one proceeds as well via the most abundant linear diglycerol 7. Therefore it cannot be excluded that this peak (peak 3, Figure 17) can be due to isomer 12 instead. Similarly for the second most intense couple (peak 2, Figure 17), no complete certitude about its identity can be obtained (isomer 10 or 12). Since the six-membered isomer 11, is produced however out of the less abundant isomer α,β-diglycerol 8, it seems logic to assign peak 2 (Figure 17) to this particular isomer.

Confirmation of this identification using GC-MS experiments was not successful since no characteristic ions of the different isomers could be detected. Moreover, isolation of each of these isomers was not feasible. Consequently this proposal of identification could not be checked by injection of purified standards. In contrast to the non cyclic isomers, it is very difficult to predict the volatility of these cyclic products on basis of their molecular structure. So the hypothetical identification could not be evaluated in such a manner either.

For the identification of the different triglycerol isomers, an even more complex chromatogram was obtained as represented in Figure 18. Five major peaks are clearly separated from a number of smaller peaks (retention times respectively 14.5-16.0 and 16.5-17.5 min). It should be noted that this chromatogram originates from a fraction obtained by distillation. As a result, it is enriched with particular isomers.
Figure 18. Detail of a gas chromatogram of a derivatised triglycerol sample obtained by fractionated distillation (enriched in certain isomers) - Identification of peaks: 1. internal standard (sorbitol), 2. cyclic triglycerol isomers, 3; triglycerol 21, 4. triglycerol 20, 5. triglycerols 17, 18 and 19, 6. triglycerols 15 and 16, 7. α,α-α,α-triglycerol 14.

It can be supposed that the peaks eluting just after the internal standard (14.5-16.1 min, grouped as peak 2, Figure 18) represent the cyclic triglycerol isomers. This is supported by the fact that in the original polyglycerol sample, the relative intensity of these peaks compared to the later eluting group (16.5-17.5 min, Figure 18) was much smaller (Figure 16). Taking into account the earlier observations with regard to the elution behaviour of diglycerol isomers, it can be expected that the cyclic triglycerol isomers would elute also before the non-cyclic triglycerol isomers. In addition, it was observed that the cyclic triglycerol isomers were enriched in the more volatile fractions obtained via fractionated distillation. The high number of peaks is in correspondence with the very complex mixture of cyclic isomers as proposed by Dolhaine et al. (1984). Due to this complexity, an identification of peaks is impossible.

Regarding the linear isomers, five peaks can be observed clearly (Figure 18), although eight different linear isomers can arise from the synthesis of triglycerol (Figure 19). Consequently some of the isomers are co-eluting. Identification can be proposed based on the results from the diglycerol analysis. The peak with the highest retention time (17.4 min) is most probably compound 14 (peak 7, Figure 18). Considering Figure 16, originating from a common polyglycerol that was not
fractionated, it is clear that this isomer is the most abundant triglycerol isomer present. This corresponds with the highest probability to obtain the $\alpha,\alpha-\alpha,\alpha$-triglycerol 14. If the chromatogram is compared with the previous results obtained for diglycerol, it can be observed that the analogue linear $\alpha,\alpha$-diglycerol 7 eluted as the last diglycerol isomer as well.

Figure 19. Linear isomers of triglycerol

By further comparison of the chromatograms obtained for the diglycerol (Figure 17) and the triglycerol isomers (Figure 18) respectively, a possible identification of all the peaks can be proposed. Comparing the retention time between the $\alpha,\alpha$-diglycerol 7 and the $\alpha,\beta$-diglycerol 8 in Figure 17, a difference of 0.19 min is observed. Similarly the shift between the $\alpha,\alpha$-diglycerol 7 and the $\beta,\beta$-diglycerol 9 amounts 0.54 min. Comparing these characteristic shifts with those observed in the chromatogram obtained from the analysis of the triglycerols (Figure 18), some remarkable similarities can be noticed. The difference between peak 7 and 6 amounts 0.22 min, which is similar to the shift in retention time observed between the diglycerol isomers 7 and 8. It can therefore be supposed that the shift between peak 7 ($\alpha,\alpha-\alpha,\alpha$-triglycerol 14) and peak 6 is due to a change of one $\alpha,\alpha$-bound into an $\alpha,\beta$- or $\beta,\alpha$-bound. Consequently peak 6 probably corresponds to the triglycerol
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Similarly, the retention time difference between peaks 5 and 6 amounts 0.25 min, so possibly corresponding to a supplementary $\alpha,\alpha$- to $\alpha,\beta$- or $\beta,\alpha$-bound change with respect to isomers 15 and 16, giving rise to the isomers 17, 18 and 19. The rather low intensity of peak 5 compared to peaks 6 and 7 (Figure 18), can be explained by the fact that isomers 17, 18 and 19 are synthesised out of diglycerol 8. In contrast, isomer 14 (peak 7, Figure 18) is synthesised out of $\alpha,\alpha$-diglycerol 7 and isomers 15 and 16 (peak 6, Figure 18) are synthesised out of diglycerol isomers 7 and 8. The fact that peaks 5 and 6 originate most probably from several compounds, is supported by the fact that in the detailed chromatogram (Figure 18) asymmetric peaks were observed, in contrast to the other peaks, which were perfectly symmetrical.

As the difference in retention time between peak 4 and 7 ($\alpha,\alpha,\alpha,\alpha$-triglycerol 14) amounts 0.54 min, it can be supposed that this shift corresponds with an $\alpha,\alpha$-to $\beta,\beta$-bound change, as for the diglycerol isomers. Consequently, peak 4 may be attributed to $\alpha,\alpha,\beta,\beta$-triglycerol 20. Finally a similar shift (0.58 min) can be observed between peak 3 and 6 ($\alpha,\alpha,\beta,\alpha$-triglycerol 15 and $\alpha,\alpha,\alpha,\beta$-triglycerol 16), indicating that peak 3 could correspond to $\beta,\alpha,\beta,\beta$-triglycerol isomer 21. The low intensity of peak 4 is due to the low reactivity of the $\beta$-hydroxyl groups involved, while the even lower intensity of peak 3 is a combination of this lower reactivity with the lower concentration of the branched diglycerol 8.

So despite the complexity of the chromatogram obtained, a possible identification of the linear and branched triglycerol isomers can be presented based on the chromatographic data of the diglycerol isomers. Validation of these logic assumptions is not feasible because no pure standards of each isomer could be obtained.

The chromatogram of tetraglycerol became so complex that no comparison with those of di- and triglycerol was feasible (Figure 20). This is not surprising because of the very high number of linear tetraglycerol isomers possible (27) compared to the 8, also not completely separated linear triglycerol isomers. Moreover, a very high number of cyclic isomers can be present as well (Dolhaine et al., 1984). Consequently no clear identification of the different peaks could be presented. Only for the last eluting peak (20.8 min) a possible identification as the $\alpha,\alpha,\alpha,\alpha,\alpha,\alpha$-tetraglycerol is proposed. This is supported because this peak is the most dominant (Figure 16) from all the tetraglycerol peaks concerned. In addition, the analogue diglycerol and triglycerol isomers 7 and 14 behaved similarly. Furthermore, it can be expected that the non branched tetraglycerol isomer has the lowest volatility compared to the other isomers. Consequently it would be the last
eluting peak of the tetraglycerol isomers. It should be noted that the chromatogram shown in Figure 20, again originates from a sample obtained via fractionated distillation. Consequently, it can be enriched with particular isomers.

For pentaglycerol no individual peaks could be identified anymore. It should be noted however that for the pentaglycerol, poor resolution and low peak intensity was observed as well. This is probably due to the low concentration of this particular polyglycerol in the sample and its high molecular weight.

![Chromatogram of derivatised tetraglycerol obtained via fractionated distillation](image)

Figure 20. Chromatogram of derivatised tetraglycerol obtained via fractionated distillation (Peak identification : 1= tetraglycerol; 2 = pentaglycerol)

Another interesting observation with respect to the chromatogram shown in Figure 20, relates to the fact that no clear separation was observed between the cyclic and linear isomers. A first explanation is that due to the high number of cyclic isomers, their individual concentrations becomes so low that they can not be detected. This is in correspondence with the lower intensity of the cyclic isomers of di- and tri-glycerol detected. Another explanation is that the resolution power of the used column is too low to resolve the cyclic and linear isomers form each other.

As a further elaboration of the gas chromatographic identification of polyglycerols, a sample was analysed by cold column injection on a short capillary column. As can be observed from Figures 21 a-b, resolution of the isomers of the respective polyglycerols is decreased as could be expected. So
this analytical approach is less appropriate if the presence of cyclic and branched di-and triglycerol molecules should be investigated. Interestingly however and in contrast to the chromatogram shown in Figure 16, it should be noted that the higher polyglycerols, up to heptaglycerol are much better detected. This is probably due to the modified injection technique and the shorter column used, making the gas chromatographic separation of these high boiling substances possible. It should be emphasised that because of this additional chromatographic analysis, a clear qualitative idea can be obtained about the presence of these higher polyglycerols in a particular sample. Since the peak, probably corresponding to octaglycerol was not completely baseline resolved, analysis up to heptaglycerol was considered to be certainly feasible.

Figure 21a. Chromatogram of derivatised polyglycerol analysed on a short capillary column by on-column injection (Peak identification : 1= diglycerol; 2 = triglycerol; 3 = tetraglycerol; 4 = pentaglycerol; 5 = hexaglycerol; 6 = heptaglycerol; 7= octaglycerol)
2.3.3. Quantitative gas chromatographic analysis of polyglycerols

In order to allow quantitative analysis, an internal standard needed to be added to the sample. Since polyalcohols were studied, preference was given to similar compounds like 1,2-propanediol, mannitol, 1,5-pentanediol, arabitol and sorbitol. Only the latter seemed to be clearly distinguished from all the other peaks present in a chromatogram resulting from the analysis of a typical polyglycerol sample (Figure 16).

From the column chromatographic experiments, pure standards of tri- and tetrarglycerol were obtained from two polyglycerol samples. These were used together with pure standards of glycerol and diglycerol in order to determine calibration curves, allowing a quantitative analysis of a polyglycerol sample, within a concentration range of 0.1-1.5 mg.mL⁻¹. Only the most abundant \( \alpha,\alpha \)-and \( \alpha,\beta \)-diglycerol isomers 7 and \( \delta \) were taken into account for the calculation of the diglycerol calibration curve, since those were present in the standard with a calculated concentration of at least 95 percent. Similarly, for the triglycerols, the most abundant isomers 14, 15 and 16 were taken into account. As no complete identification of the tetrarglycerol isomers was achieved, all the peaks within a certain retention time interval (19-21 min) were taken into account to calculate the calibration line. Also the calibration line for each polyglycerol going through the origin is indicated (Table 21). Remarkably, the tangents of the regression lines are gradually increasing if the...
polymerisation degree increases as well. Previously Sahasrabudhe (1967) observed the same phenomenon for di- and triglycerol.

Table 21. Calibration curves (0.1-1.5 mg.mL⁻¹) for the gas chromatographic analysis of polyglycerols, using sorbitol as internal standard (X = area polyglycerol / area internal standard; Y = concentration polyglycerol / concentration internal standard)

<table>
<thead>
<tr>
<th>Type of polyglycerol</th>
<th>Calibration curve</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycerol</td>
<td>[31] Y = 1.0604X + 0.0258</td>
<td>0.9905</td>
</tr>
<tr>
<td></td>
<td>[32] Y = 1.0895X</td>
<td>0.9892</td>
</tr>
<tr>
<td>diglycerol</td>
<td>[33] Y = 1.3307X + 0.0376</td>
<td>0.9933</td>
</tr>
<tr>
<td></td>
<td>[34] Y = 1.3894X</td>
<td>0.9903</td>
</tr>
<tr>
<td>triglycerol</td>
<td>[35] Y = 2.3632X + 0.0195</td>
<td>0.9923</td>
</tr>
<tr>
<td></td>
<td>[36] Y = 2.4018X</td>
<td>0.9918</td>
</tr>
<tr>
<td>tetruglycerol</td>
<td>[37] Y = 4.9521X + 0.0065</td>
<td>0.9885</td>
</tr>
<tr>
<td></td>
<td>[38] Y = 4.9052X</td>
<td>0.9884</td>
</tr>
<tr>
<td>cyclic diglycerol</td>
<td>[39] Y = 1.2395X</td>
<td>*</td>
</tr>
<tr>
<td>cyclic triglycerol</td>
<td>[40] Y = 1.8956X</td>
<td>*</td>
</tr>
</tbody>
</table>

* no correlation coefficients could be calculated since these equations were obtained by calculating the average of equations [32] and [34] and of equations [34] and [36] respectively

From these results, the tangent of the calibration curves for cyclic di- and triglycerol isomers were estimated as well, as explained in Table 21. This procedure was used because no pure standards of the cyclic isomers were obtained. It should be realised that these values are estimates for all the isomers together. Probably, the real coefficients will be different. In addition each of the individual isomers could be characterised with a different coefficient as well.

In the following experiments the reliability of these calibration curves and the analytical method were evaluated. This was achieved by analysing a known amount of several polyglycerol samples coded as samples 001 to 004. The concentration of all the constituents was calculated based on the proposed gas chromatographic analysis and calibration lines. By comparing the total sum of these calculated concentrations with the actual amount of sample applied, an idea of the reliability of the method could be obtained. The results of these experiments are summarised in Table 22. If the ratio of the calculated total mass over the applied total mass is lower then one, then gas chromatographic
method underestimates certain compounds present in the mixture and if the ratio is higher than one, some compounds are overestimated.

Table 22. Analysis of polyglycerol samples obtained from fractionated distillation experiments (higher polyglycerol concentrations in all samples were lower than 1 area percent and were not taken into account; all data are given in weight percent and are the average of two independent measurements)

<table>
<thead>
<tr>
<th>sample</th>
<th>glycerol</th>
<th>cyclic diglycerol</th>
<th>linear diglycerol</th>
<th>cyclic triglycerol</th>
<th>linear triglycerol</th>
<th>tetraglycerol</th>
<th>ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>55.58</td>
<td>4.64</td>
<td>31.16</td>
<td>3.29</td>
<td>6.12</td>
<td>0.00</td>
<td>1.008</td>
</tr>
<tr>
<td>002</td>
<td>59.34</td>
<td>4.44</td>
<td>31.87</td>
<td>0.00</td>
<td>2.33</td>
<td>0.00</td>
<td>1.004</td>
</tr>
<tr>
<td>003</td>
<td>49.13</td>
<td>20.93</td>
<td>7.87</td>
<td>0.78</td>
<td>2.44</td>
<td>0.68</td>
<td>0.822</td>
</tr>
<tr>
<td>004</td>
<td>5.05</td>
<td>2.46</td>
<td>70.09</td>
<td>9.33</td>
<td>11.21</td>
<td>3.72</td>
<td>1.037</td>
</tr>
</tbody>
</table>

* ratio = ratio of the mass of sample present calculated from the amounts of the polyglycerols obtained from the gas chromatographic analysis over the actual amount of sample applied; maximal standard deviation on calculated polyglycerol mass amounted 0.035

Considering the samples that were obtained from a distillate of synthetic mixtures containing especially glycerol and diglycerol (samples 001 and 002), a very good agreement between the actual applied amount of sample and the calculated amount of sample was obtained. No tetra- or higher glycerols could be detected in these samples, which could be expected because of the polymerisation reaction parameters applied. The low polymerisation degree is also proven by the rather high concentrations of glycerol. Sample 003 contained appreciable amounts of cyclic diglycerol. Remarkably, a serious underestimation of the calculated amount of polyglycerols present in samples was obtained as well. This illustrates that equation [39] underestimates the cyclic diglycerol content, which taking into account that the equation was obtained via interpolation, is not surprising.

In order to check the reliability of the method for tri- and tetraglycerol, another synthesis was made using more stringent polymerisation conditions to obtain a mixture containing higher amounts of tetraglycerol. Unfortunately, the reaction mixture itself contained quite high amounts of other higher polyglycerols as well (more than 10% on area basis, results not shown). Because no calibration curves for these species were available, a fractionated distillation experiment was performed (240°C and 263 Pa, sample 004), reducing the content of higher polyglycerols up to 2%. Again, the calculated amount of sample corresponded quite well to the actual amount present.
As a conclusion, it can be stated that the proposed calibration lines and the used analytical method are reliable to quantitatively estimate the concentration of non-cyclic polyglycerols up to tetraglycerol in a sample. For the cyclic isomers, semi-quantitative data can be obtained.

### 2.3.4. Polyglycerol composition of polyglycerol fatty acid esters

In order to analyse the polyglycerol content of polyglycerol fatty acid esters, an isolation of the polyglycerol fraction was necessary. It was therefore proposed to saponify the polyglycerol esters liberating the polyglycerol moiety from them. Subsequently the polyglycerols should be isolated from the reaction mixture to prepare them for gas chromatographic analysis.

Since glycerol is polymerised using alkaline catalysis it was checked if the saponification process did not alter the polymerisation degree of a polyglycerol sample. Therefore, the saponification procedure was applied on two polyglycerol samples and the composition of the resulting reaction mixture was compared with the composition of the unsaponified samples. The results (Table 23) indicate that for both investigated samples, no differences in the composition between the saponified and unsaponified samples could be detected. It was concluded that the applied saponification procedure did not induce any polymerisation or depolymerisation of the polyglycerols. Therefore it is reasonable to conclude that by saponifying a polyglycerol fatty acid ester and subsequent analysis of the polyglycerol moieties, compositional data of the polyglycerols can be obtained which correspond with the polyglycerol content of the esters themselves.

#### Table 23. Polyglycerol content of virgin and saponified polyglycerols (given in area percent, based on duplicate saponification experiments; average ± standard deviation)

<table>
<thead>
<tr>
<th>sample</th>
<th>glycerol</th>
<th>diglycerol</th>
<th>triglycerol</th>
<th>tetraglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>diglycerol-virgin</td>
<td>0.8 ± 0.8</td>
<td>97.4 ± 0.7</td>
<td>1.8 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>diglycerol-saponified</td>
<td>1.05 ± 0.45</td>
<td>96.7 ± 0.1</td>
<td>2.25 ± 0.55</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>polyglycerol-virgin</td>
<td>0.0 ± 0.0</td>
<td>26.85 ± 0.45</td>
<td>50.0 ± 0.2</td>
<td>23.2 ± 0.7</td>
</tr>
<tr>
<td>polyglycerol-saponified</td>
<td>0.0 ± 0.0</td>
<td>26.75 ± 0.75</td>
<td>49.9 ± 0.4</td>
<td>23.35 ± 1.15</td>
</tr>
</tbody>
</table>

The following step consisted of analysing the polyglycerol content of polyglycerol fatty acid esters. After saponification, the free fatty acids needed to be removed to avoid the presence of large peaks due to palmitic and stearic trimethyl silyl derivatives in the chromatogram. Extraction with petroleum ether after acidification of the reaction mixture was not successful. Precipitation of the potassium salts of the free fatty acids by cooling the reaction mixture and subsequent filtration was
found to be effective and easier. Thus the free fatty acids were almost completely removed (residual concentration of about 1 to 2 percent palmitic and stearic acid on basis of their peak area).

Four different polyglycerol fatty acid esters were used for further analysis. The characteristics of these polyglycerol fatty acid esters were summarised in Table 24. The first types of ester (samples 134 and 145) were synthesised from the triglycerol mixture, while the other samples (138 and 147) were made from the polyglycerol mixture. The esters were also characterised by a different degree of esterification.

Table 24. Characteristics of the synthesised polyglycerol fatty acid esters

<table>
<thead>
<tr>
<th>sample</th>
<th>type polyglycerol</th>
<th>degree of esterification (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>134</td>
<td>triglycerol</td>
<td>32</td>
</tr>
<tr>
<td>145</td>
<td>triglycerol</td>
<td>48</td>
</tr>
<tr>
<td>138</td>
<td>polyglycerol</td>
<td>38</td>
</tr>
<tr>
<td>147</td>
<td>polyglycerol</td>
<td>48</td>
</tr>
</tbody>
</table>

* the degree of esterification is equal to the ratio of the esterified hydroxyl groups to the total amount of hydroxyl groups present in the polyglycerol

After the saponification, the polymerisation degree of the polyglycerols was determined and compared with the producers data of the original polyglycerols from which the esters were made. For the first two types of polyglycerol fatty acid esters, with the lowest polymerisation degree (samples 134 and 145), good agreement between the data from the analysis and from the producer was obtained for all the polyglycerol species present (Table 25).

For the other two samples (138 and 147) fairly good agreement between the di- and triglycerol data obtained from the producer and the analysis results could be observed. With regard to the tetraglycerol content however, some remarkable differences could be seen between the producers and the experimental data. Moreover, a striking difference in the experimental tetraglycerol content of sample 138 and 147 could be observed as well, although the same type of polyglycerol was used to prepare both esters.

As the polyglycerol sample from which esters 138 and 147 were synthesised, was still available for analysis, its polyglycerol composition was determined to compare it with the other available data. Better agreement between the diglycerol and triglycerol contents obtained from this analysis and those obtained for the esters was observed. This was also the case with regard to the tetraglycerol
content of sample 138. Comparing these results with the producers data, especially with regard to the tetraglycerol content, remarkable differences were still observed. Therefore these latter data were considered to be less reliable.

Table 25. Polyglycerol content of the synthesised polyglycerol fatty acid esters (in weight percentages, based on independent triplicate measurements, a 95 % confidence interval ($\alpha<0.05$) was used for the statistical evaluation)

<table>
<thead>
<tr>
<th>sample</th>
<th>glycerol</th>
<th>cyclic diglycerol</th>
<th>triglycerol</th>
<th>cyclic triglycerol</th>
<th>tetraglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>134</td>
<td>0.46 ± 0.05</td>
<td>0.85 ± 0.42</td>
<td>7.45 ± 0.30</td>
<td>1.00 ± 0.32</td>
<td>77.56 ± 2.31</td>
</tr>
<tr>
<td>145</td>
<td>0.68 ± 1.02</td>
<td>1.61 ± 1.12</td>
<td>7.78 ± 0.64</td>
<td>1.55 ± 0.39</td>
<td>76.66 ± 2.72</td>
</tr>
<tr>
<td>triglycerol*</td>
<td>-</td>
<td>10 ± 6</td>
<td>&gt; 80</td>
<td>10 ± 6</td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>0.27 ± 0.20</td>
<td>0.88 ± 0.18</td>
<td>16.77 ± 0.76</td>
<td>0.56 ± 0.47</td>
<td>47.08 ± 1.79</td>
</tr>
<tr>
<td>147</td>
<td>0.21 ± 0.26</td>
<td>2.28 ± 1.01</td>
<td>22.95 ± 0.37</td>
<td>0.25 ± 0.43</td>
<td>47.08 ± 3.07</td>
</tr>
<tr>
<td>polyglycerol*</td>
<td>-</td>
<td>15-30</td>
<td>33-55</td>
<td>10-25</td>
<td></td>
</tr>
<tr>
<td>polyglycerol§</td>
<td>0.03</td>
<td>0.18</td>
<td>14.44</td>
<td>1.41</td>
<td>44.81</td>
</tr>
</tbody>
</table>

* producers data
§ gas chromatographic data, using calibration curves of Table 23

It seems that, probably due to the more stringent esterification procedure used for the synthesis of ester 147, a depolymerisation of the polyglycerol occurred. This led to a degradation of tetraglycerol and triglycerol. Consequently, a decrease in concentration of tetraglycerol and an increase of the diglycerol concentration is expected. Indeed, although the diglycerol content of ester 138 was in close agreement with the analysis data of the polyglycerol used for the synthesis of the ester, a higher diglycerol content for the ester 147 was obtained, indicating a degradation reaction of the polyglycerols leading to a considerably higher diglycerol content, as previously reported by Charlemagne and Legoy (1995). This degradation reaction cannot be due to the previously applied saponification procedure, as was proved before.

It can be concluded that the proposed method is able to determine the polymerisation degree of polyglycerol fatty acid esters in a two-step procedure: saponification of the esters and a subsequent gas chromatographic analysis of the isolated polyglycerols. Quantitative analysis up to the tetraglycerols is possible, while qualitative data can be obtained using the modified gas
Chemical characterisation of complex plastic additives: polyglycerol esters

chromatographic analysis. It should be noted however that from a practical point of view, especially the di-tri-and tetrarglycerols are the most important polyglycerols used.

2.3.5. Gas chromatographic analysis of polyglycerol fatty acid esters

In preliminary gas chromatographic experiments, silylated esters were analysed on a 25 m apolar column, using a split-splitless injector. Results indicated that injection temperatures should be as high as 340°C to allow sufficient sample evaporation (not shown). Even at those experimental conditions, analysis was restricted up to the mono-esters of triglycerol, indicating that discriminating evaporation and possible column adsorption took place during these experiments. Therefore, cold-on column injection was combined with a separation on a short column (5m) with the same stationary phase. In addition to the samples mentioned in Table 24, two esters (encoded as 044 and 055), derived from a diglycerol were analysed as well. For sample 044, the polyglycerol composition as revealed by the presented method was as follows (all mass percentages): glycerol (30.18 %); diglycerol (38.06 %); triglycerol (23.61 %); tetrarglycerol (8.37 %). The degree of esterification was 33 %. For sample 055, which was synthesised in similar conditions, no such data were collected because of the limited sample amount available. Typical chromatograms for samples sample 044, 145 and 138 are shown in Figures 22-24.

It is obvious from these chromatograms that an increasing polymerisation degree renders the chromatograms more complex. Differences in the degree of esterification did not result in more complex chromatograms, but merely changed the relative intensity of the peaks. Therefore, only a selected amount of chromatograms is shown, including an ester derived from each polyglycerol studied.

Another interesting observation is the presence of the free polyglycerols in the chromatograms (retention times: diglycerol 5 min; triglycerol 10 min, tetrarglycerol 13 min, see also Figure 21a), which is most probably due to a partial hydrolysis of the samples upon their storage. From this observation, a first idea of the polyglycerol content of an ester studied can be obtained.

In order to identify more peaks in the chromatograms, a column chromatographic separation of these polyglycerol fatty acid esters was achieved.
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Figure 22. Gas chromatogram of derivatised polyglycerol fatty acid 044

Figure 23. Gas chromatogram of derivatised polyglycerol fatty acid ester 145
2.3.6. **Column chromatographic fractionation of polyglycerol fatty acid esters and their gas chromatographic characterisation**

2.3.6.1. **Column chromatographic fractionation**

In the preliminary experiments, esters were analysed using thin layer chromatography on silica gel. Basically two solvent mixtures were evaluated: chloroform-acetone 94:4 (v:v), and chloroform-acetone-methanol 94:4:2 (v:v:v). The first mixture allowed separation in three distinct groups of constituents according to their polarity (Rf values 0.02, 0.1-0.25 and 0.67-0.92). The second solvent mixture allowed a more detailed separation, especially for the more complex triglycerol and tetraglycerol fatty acid esters for which the spots were spread out over the total TLC plate (results not shown). As a restricted group separation was thought to be more appropriate for the goal of this research, the acetone chloroform 94:4 mixture was selected instead of the methanol containing mix. As these experiments revealed the presence of very polar compounds (Rf 0.02), elution of these compounds in the column chromatographic separation was achieved by using acetone and subsequently methanol.

The separation scheme as outlined in Figure 25 was followed throughout the analysis of polyglycerol fatty acid ester samples 044, 055, 134, 145 and 138. Thus three fractions, eluted with chloroform-acetone 94:4 (v:v) were obtained. In addition, two polar fractions were collected as well. All fractions obtained were analysed by gas chromatography. By superposition of the

![Figure 24. Gas chromatogram of derivatised polyglycerol fatty acid ester 138](image-url)
chromatograms of the different fractions and comparison with those of the original samples, it could be concluded that qualitatively, all kind of compounds eluted from the column. In the following, additional details about the gas chromatographic analysis of these fractions will be given.

**Figure 25.** Fractionation scheme followed for the isolation of chromatographically pure polyglycerol fatty acid esters

### 2.3.6.2. Gas chromatographic analysis of fractions 1-2

#### 2.3.6.2.1. Di-esters of non cyclic diglycerol

The chromatogram of fraction 1 obtained from sample 055 is given in Figure 26, indicating two distinct peak groups at respectively 14.5-16.5 and 22-27 min. For sample 044, the first eluting group in this fraction was not intense. Polyglycerol analysis revealed the presence of diglycerol as principle component together with an earlier eluting compound, which presently could not be identified. Again in sample 044, this unidentified peak was not so intense compared to sample 055 (not shown). Similar results were obtained for the esters 134 and 145 (not shown).

Because of the results of the polyglycerol analysis it was concluded that fraction 1 contained particularly esters derived from diglycerol. Considering the major peaks in this fraction (Figure 26, triplets between 22-27 min), it was concluded that these were di-esters of diglycerol. This is based on their low polarity (early elution during LC prefractonation) and their lower concentration in the original sample compared to the earlier eluting peaks (Figure 22, moderate degree of esterification). Final certitude was obtained from LC-MS experiments. In Table 26, mass spectral data of a number of fractionated samples are summarised. As can be seen, fraction 1 of sample 044 seemed to be composed essentially of diglycerol di-esters. As stearic acid is the most abundant fatty acid (60%) in
this sample, the triplet with the highest intensity, eluting at 26.5-27 min, correspond to the C18:0-C18:0 di-esters. The triplet eluting before is most probably due to the C16:0-C18:0 di-esters, while the C16:0-C16:0 di-ester triplet is only present in small quantities.

![Figure 26. Gas chromatogram of fraction 1 isolated from sample 055 after trimethyl silyl derivatisation](image)

Two possible explanations can be given why the di-esters of diglycerol elute as triplets. A first possibility is the presence of various place isomers of the ester. Out of diglycerol Z, four different di-stearic-diglycerol esters can be obtained. Preliminary gas chromatographic experiments with a mixture of palmitic and stearic di-acyl glycerol revealed that the chromatographic set-up used, was able to separate the 1,2- and 1,3-di-acyl glycerol from each other. So a similar separation of diglycerol di-esters could be possible as well. Of course, in case of a complete separation, four peaks should be distinguished, while in the experiments presented, only three different peaks could be observed. This could be explained by a co-elution of the peaks or to a too low concentration of one of the isomers. It should be noted as well that only one diglycerol isomers is considered, while in fact three non-cyclic isomers are present (Figures 14 and 17). So in fact more complex chromatograms could be expected if this hypothesis is accepted.

A second possible explanation could be the presence of di-esters of three non-cyclic diglycerol isomers. This would imply that no separation of the place isomers of the various di-esters of a particular diglycerol isomer is achieved. If the relative intensity of the peaks is considered in this
case, it would imply that the relative concentration of the \( \beta, \beta \)-diglycerol \( 9 \) is relatively high, which is not in correspondence with the results of the polyglycerol analysis.

Table 26. Mass spectral data of isolated liquid chromatographic fractions

<table>
<thead>
<tr>
<th>M+23.e-1</th>
<th>Relative intensity</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>721.5</td>
<td>100</td>
<td>C18:0 – C18:0 di-esters diglycerol</td>
</tr>
<tr>
<td>693.5</td>
<td>38</td>
<td>C16:0 – C18:0 di-esters diglycerol</td>
</tr>
<tr>
<td>681.5</td>
<td>18</td>
<td>unknown</td>
</tr>
<tr>
<td>455.30</td>
<td>12</td>
<td>C18:0 mono-esters diglycerol</td>
</tr>
<tr>
<td>777.60</td>
<td>10</td>
<td>C20:0 – C20:0 di-esters diglycerol</td>
</tr>
</tbody>
</table>

Fraction 3 – Sample 134

| 823.6    | 100                | C18:0 – C20:0 di-esters triglycerol |
| 851.6    | 87                 | C20:0 – C20:0 di-esters triglycerol |
| 795.50   | 38                 | C18:0 – C18:0 di-esters triglycerol |
| 767.50   | 7                  | C18:0 – C18:0 di-esters triglycerol |

Fraction 4 – sample 044

| 455.30   | 100                | C18:0 mono-esters diglycerol        |
| 427.20   | 14                 | C16:0 mono-esters diglycerol        |
| 795.60   | 6                  | C18:0 – C18:0 di-esters triglycerol |

Fraction isolated from chromatographic separation of fraction 5, sample 134 (10-35 mL)

| 529.30   | 100                | C18:0 mono-esters triglycerol       |
| 501.30   | 56                 | C16:0 mono-esters triglycerol       |
| 603.4    | 8                  | C18:0 mono-esters tetracylglycerol  |
| 557.30   | 7                  | C20:0 mono-esters triglycerol       |
| 473.30   | 7                  | C14:0 mono-esters triglycerol       |

In a supplementary column chromatographic separation of this fraction (2.125 g 5% inactivated silica, 1.2 cm ID, chloroform-acetone 94:4 (v:v)), it was revealed that in the more apolar fractions, the highest peak of each triplet (Figure 26) was dominantly present, while in the more polar fractions, the central peak of each triplet became dominant. Both hypotheses explained above, fit in these
observations: place isomers of di-acyl glycerol could be separated in a similar way and it can be supposed that also an analogue separation is possible for the di-esters of the various diglycerol isomers as well.

Since both hypotheses have some inconsistencies, total certitude about the complete identification on the isomer level of these di-esters of diglycerol is not possible.

2.3.6.2.2. Esters of cyclic di-glycerol

In addition to the non-cyclic diglycerol esters, esters of an unidentified alcohol seemed to be present as well (Figure 26, 14.5-16 min). Polyglycerol analysis revealed that the unidentified alcohol eluted between glycerol and the non-cyclic diglycerol isomers. Probably this unidentified peak can be attributed to cyclic diglycerol isomers, because its elution behaviour is in correspondence with the earlier reported observations of the gas chromatographic separation of diglycerol isomers (Figure 17). Because the esters were collected in the first (apolar) fraction of the column chromatographic separation of the total sample, it was supposed that those were di-esters of cyclic diglycerol.

Analysing this fraction by gas chromatography without silylation, remarkable differences of the peaks of interest (14.5-16 min) were observed compared to the chromatogram shown in Figure 26: peaks eluted a bit earlier and even more important, the number of peaks and their relative intensity changed dramatically (Figure 27). These observations indicate that the compounds considered can be silylated, implementing the presence of a free hydroxyl group. Considering the various possible cyclic diglycerol isomers (Figure 14), it is obvious that di-esters cannot contain a free hydroxyl group. Consequently, the compounds considered are mono-esters of cyclic diglycerol instead of di-esters of diglycerol.

This hypothesis is in correspondence with the results obtained in a supplementary TLC separation of this fraction (hexane-ethyl acetate 1:1 (v:v)) in two spots with a totally different polarity (Rf 0.24 and 0.62). Gas chromatographic analysis of the polar spot revealed the presence of the compounds considered in addition to the peaks attributable to the di-esters of diglycerol.

Additional information with regard to the fatty acid composition of each ester eluting in the chromatogram shown in Figure 26 can be obtained as well. Considering the differences in peak intensity of the two couples of peaks (14.5-16.5 min), the elution order and the fatty acid composition of the total sample (C18:0 : 82 %; C16:0 : 16%) it can be concluded that the most intense couple is due to the stearic acid mono-esters and the other couple to the palmitic esters. The fact that only couples are observed for each ester, despite of the high number of cyclic diglycerol isomers
possible (Figure 14 and 16), is probably due to the lower resolution power of the short column used. Therefore a more detailed identification is not possible.

![Gas chromatogram of fraction 1 isolated from sample 055 without trimethyl silyl derivatisation](image)

**Figure 27.** Gas chromatogram of fraction 1 isolated from sample 055 without trimethyl silyl derivatisation

During the gas chromatographic analysis of the apolar spot, observed in the supplementary TLC separation described above (Rf 0.62), revealed the presence of some additional non-identified peaks eluting just before the di-esters of non-cyclic diglycerol. Therefore the sample was fractionated by column chromatography (2.125 g 5% inactivated silica, 1.2 cm ID) using chloroform-acetone 94:4 (v:v) as a mobile phase.

Comparing the gas chromatogram of an apolar (Figure 28) and a more polar (Figure 29) fraction, it is obvious that the composition of the latter is comparable to that of the total fraction (Figure 26). The peaks detected in the apolar fraction however, could not be distinguished in the chromatogram of the total sample (Figure 26), probably because of their low concentration. Based on the results obtained, it could be concluded that the composition of the isolated apolar fraction corresponded with the composition of the apolar spot detected in the described TLC experiment. Because the molecular weight of these compounds is comparable to the molecular weight of di-esters of diglycerol (similar gas chromatographic behaviour) and because of its more intense apolar character, it was concluded that these compounds are the di-esters of cyclic diglycerol. Their elution pattern, which is very similar to the one observed for the mono-esters of cyclic diglycerol, confirms...
this hypothesis. Of course, in this case three doublets are observed, corresponding respectively to the C16:0-C16:0, C16:0-C18:0 and C18:0-C18:0 di-esters.

Figure 28. Gas chromatogram of a derivatised fraction isolated by column chromatographic separation of fraction 1 of sample 044 (6-8 mL) containing di-esters of cyclic diglycerol

Figure 29. Gas chromatogram of a derivatised fraction isolated by column chromatographic separation of fraction 1 of sample 044 (18-20 mL) containing di-esters of non-cyclic diglycerol isomers
It should be noted that confirmation of these assumptions with regard to the identification of esters of cyclic diglycerol by mass spectral data was impossible because of the low concentrations of these compounds.

Finally, fraction 2 revealed the presence of mono-palmitine and mono-stearine glycerol (12.5-14.5 min), as confirmed by the injection of pure standards. These mono-acyl glycerols were only present in minor quantities in samples 044 and 055. The presence of di-esters of diglycerol could be observed as well in this fraction.

2.3.6.3. **Gas chromatographic analysis of the other fractions**

2.3.6.3.1. **Di-esters of triglycerol**

The chromatogram obtained from the third fraction of sample 044 and 055 revealed the presence of mono-acyl glycerols. For the triglycerol derived ester however, only the peaks eluting between 26-34.5 min (Figure 23) were observed. As can be seen from Figure 23, groups of triplets can be differentiated. Polyglycerol analysis revealed the presence of triglycerol and its branched isomers (not shown). Because the isolated components were more polar compared to the di-esters of diglycerol, it was supposed that di-esters of triglycerol were isolated. This hypothesis was confirmed by the LC-MS data (Table 26). These indicated that especially esters of C20:0 were present, which is quite surprising since C20:0 was only present in minor quantities (2% w:w) in the original ester. A possible explanation for this observation will be presented later (paragraph 2.3.6.3.3). Despite this inconsistency, it can be supposed that the last eluting triplet is due to the di-esters of the most abundant fatty acid (C18:0). Similar as for the di-esters of diglycerol, the earlier eluting groups of peaks are the C16:0-C18:0 and the C16:0-C16:0 di-esters of triglycerol. Identification of the place isomers is even more complex compared to the di-esters of diglycerol and was therefore considered as impossible.

It can be observed from the total chromatogram of the polyglycerol ester studied (Figure 24), that the di-esters of triglycerol are the last eluting components that are clearly resolved. Peaks eluting at higher retention times could be observed, but these were broad and low in intensity. Most probably these are originating from di-esters of tetruglycerol. They are not due to for example tri-esters of diglycerol, since these should then also be present in the first fraction collected during the column chromatographic separation because of their low polarity, which was not the case. Consequently, the proposed gas chromatographic technique is restricted up to the analysis of di-esters of triglycerol. In order to enlarge the scope of the presented method, an alternative stationary phase,
which can be used at even higher temperatures as the ones used in this research, could be applied although possibly liquid chromatographic separation would probably become more appropriate.

**2.3.6.3.2. Mono-esters of non-cyclic diglycerol**

Further elution with chloroform-acetone 94:4 (v:v) was not useful, since no components could be detected in the eluate. Therefore the polarity of the mobile phase was drastically increased. By eluting with acetone, a fourth fraction was obtained. For the diglycerol fatty acid esters (samples 044-055), the only peaks observed were those eluting between 16 and 17.5 min (Figure 22) Because of the fairly high polarity of these compounds and their abundance in the original sample, it was supposed that these were the mono-esters of diglycerol. This supposition could be confirmed based on the polyglycerol analysis (not shown) and on the LC-MS data obtained for this particular fraction (Table 26). Consequently it can be concluded that from this fraction, pure mono-esters of diglycerol were isolated. The two major couple of peaks in the gas chromatogram are respectively due to the stearic ester (most intense, Figure 22, 17.5 min) and palmitic ester of diglycerol, which is in correspondence with their elution behaviour and fatty acid composition.

As can observed, the mono-esters of diglycerol did not elute as one single peak. In order to have a better idea about the identity of each peak, a supplementary separation of this fraction was achieved using a longer column (Figure 30). Five peaks could be distinguished for each ester. Three major peaks (peaks 3-5 for stearic acid ester, Figure 30) of which two form a couple with equal intensity and two minor peaks (peaks 1-2 for stearic acid ester, Figure 30). For the palmitic acid esters, the same pattern is repeated at an earlier elution time (Figure 30, 22.8 min-23.3 min). Of course, due to the lower concentration, smaller peaks become more difficult to detect.

Again the question arises whether these peaks can be identified. Therefore, an idea about the possible isomers of mono-esters of diglycerol should be obtained. Isomers differing in the place of esterification and in the type of diglycerol isomer should be distinguished, giving rise to six different esters out of one single fatty acid (Figure 31). Since the α,α-diglycerol is the most abundant and the α-hydroxyl group has the highest reactivity due to the low steric hindrance, the most abundant peak 5 (Figure 30) could be attributed to isomer 22.

From the analysis of a mono-acyl glycerol sample under the same experimental conditions, it was revealed that the 2-acyl glycerol eluted before the 1-acyl glycerol and that the difference in retention time amounted 0.27 min. This is correspondence with the difference in retention time between
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peaks 5 and 3 (0.27 min). Therefore, probably peak 3 is due to the α,α-diglycerol esterified on the β-hydroxyl group, corresponding to isomer 23.

Figure 30. Gas chromatographic analysis of the derivatised fraction 4 of sample 044 on a long capillary column (25m). Peaks represent stearic esters of diglycerol as shown in Figure 28 (1. ester 24, 2. ester 26, 3. ester 23, 4. esters 25 and 27, 5. ester 22)

Figure 31. Possible isomers of mono-esters of diglycerol

Remarkably, it can be observed that the retention time difference between peak 4 and peak 5 (0.20
min) is very similar to the retention time difference observed earlier between the $\alpha,\alpha$-diglycerol and the $\alpha,\beta$-diglycerol (Figure 17, 0.19 min) in similar experimental conditions. Therefore it seems logic to assume that peak 4 originates from the isomers 25 and 27, which would then co-elute. It is interesting to stress in this respect that peaks 3 and 4 have an almost equal intensity, although peak 3 is due an ester of the most abundant $\alpha,\alpha$-diglycerol. Since peak 4 would be due to the co-elution of two esters derived from the less abundant $\alpha,\beta$-diglycerol, its intensity is increased and seems to become comparable with the intensity of peak 3.

Similar to the shift observed between isomer 22 and isomer 23, a shift of 0.29 min is observed between peak 4 ($\alpha,\beta$-diglycerol esters 25 and 27) and peak 2. This indicates that peak 2 is probably due to the $\alpha,\beta$-diglycerol esterified on the $\beta$-hydroxyl group (isomer 26). Finally, the remaining peak 1, should be isomer 24, which is only present in very low concentration, because of the low concentration of the $\beta,\beta$-diglycerol.

So despite the complexity of the chromatogram obtained, a possible identification of the various mono-esters of diglycerol can be presented. This is based on the chromatographic behaviour of the analogue mono-acyl glycerol isomers combined with the previously reported chromatographic data on non-cyclic diglycerol isomers. Validation of these logic assumptions was not feasible because no pure standards of each isomer could be obtained.

Comparing the more detailed chromatogram of Figure 30, with the one obtained on a shorter column (Figure 22), it is obvious that in the latter case, peak resolution is not as good as in the former case. Despite this fact however, it can be concluded that also in the latter chromatogram information about the various isomers of the di-esters of non cyclic diglycerol can be obtained since probably peaks 3 and 4 (Figure 30) will co-elute as one single peak, eluting just in front of the most intense peak (Figure 22), corresponding to peak 5 in Figure 30.

2.3.6.3.3. Mono-esters of triglycerol

In the acetone fraction of the tri- and polyglycerol esters, similar peaks were observed as those present in fraction 3. Polyglycerol analysis proved that both di- and triglycerol were present in this fraction. Because of the earlier observations, it could be concluded that mono-esters of diglycerol were present together with the di-esters of triglycerol. Consequently, it should be concluded that the chloroform-acetone mixture was not able to elute the di-esters of triglycerol and the mono-esters of diglycerol completely. This indicates that this particular solvent mixture is able to fractionate for
example the di-esters of triglycerol according to their polarity. Possibly this could be the reason why in the LC-MS data obtained for the isolated di-esters of triglycerol (Table 26), the fatty acid composition is not completely in correspondence with the actual fatty acid composition of the sample, since probably some fractionation occurred due to the use of the chloroform-acetone mixture (see also paragraph 2.3.6.3.1).

Apart from free glycerols and fatty acids, mono-esters of triglycerol and tetraglycerol were expected to be present as well in the more polar fractions originating from the tri- and polyglycerol esters. However, because they were not observed in the isolated fractions, an even more polar solvent (methanol) was selected for further elution. Thus total striping of the column was probably achieved since for all samples, the different glycerols, which are the most polar constituents in the samples, were present in this supplementary fraction. In addition, palmitic and stearic acid were detected as well in all samples. For the diglycerol esters, especially the compounds present in fraction 4 were detected.

For the triglycerol esters however, dominating presence of the peaks eluting at 19-21 min could be revealed (Figure 23). As this fifth fraction seemed to contain a major compound, which was not isolated yet, a supplementary column chromatographic separation was achieved for this fraction after precipitation of the fatty acids by cooling (5.25g silica, 5% inactivated, 1.2 cm ID, ethyl acetate-isopropanol-water 5:2:1 (v:v:v)). The dominating peaks present in fraction 5 of sample 134 (similar as those present in sample 138, Figure 23 and 23, 19-21 min), were purely present in the volume fraction 10-35 mL (chromatogram not shown). Again polyglycerol analysis revealed the presence of triglycerol and its branched isomers (not shown). These chromatographic data support the hypothesis that the isolated substances are the mono-esters of triglycerol. This could be confirmed again by the LC-MS data obtained (Table 26).

Similarly to the other esters already discussed, a clear separation in couples of peaks can be observed depending on the fatty acid present in the ester. Separating these mono-esters on a longer capillary column to gather more information about the presence of particular isomers, was not achievable because of the low peak intensity, probably due to the high molecular weight of the compounds considered. Despite this fact and the high number of possible isomers it can be assumed, based on the identification of the mono-esters of diglycerol presented above, that the peaks with the highest intensity are the esters of α,α,α-triglycerol 14, esterified on the α position.

Probably the mono-esters of tetraglycerol should be present as well in this last fraction, but they
could not be detected in the gas chromatogram, probably due to their low concentration. Presence of these esters in this last fraction was confirmed by fractionation of sample 134 (Table 26). Attempts to isolate the pure mono-esters of tetruglycerol using a similar column chromatographic separation as described above failed.

2.3.6.4. Conclusion: gas chromatographic analysis of polyglycerol samples

From the above-described column chromatographic separation, the isolation of pure mono- and di-esters of respectively di- and triglycerol is possible. This is an essential step in the development of a quantitative analytical method for the analysis of polyglycerol esters as illustrated for the quantitative analysis of polyglycerols. Although mono-esters of tetruglycerol could not be isolated as such, their localisation in the chromatograms from samples 138 and 147 was possible because all the other peaks could be identified as shown in Figure 32.

The broad and not intense peaks eluting at the end of the chromatogram, are probably due to the di-esters of tetruglycerol. From this chromatogram and the earlier discussed results, it becomes clear that the following esters co-eluted during the gas chromatographic experiments: di-esters of diglycerol and di-esters of triglycerol, di-acyl glycerols and mono-esters of di- and triglycerol, di-esters of diglycerol and mono-esters of tetruglycerol. This co-eluting behaviour would even be more emphasised if other fatty acids would be used (in a higher extend) in the manufacture of the polyglycerol fatty acid esters (e.g. C14:0 and C20:0). Thus it can be concluded that a single chromatographic analysis of a polyglycerol sample is not sufficient to get complete compositional information. Despite these disadvantages, the method presented allows to have a clear idea about the concentration of polyglycerol esters of major importance.
Figure 32. Chromatogram of the derivatised polyglycerol fatty acid ester identifying the various compounds present in the sample.
2.3.7. Proposed analytical scheme for the analysis of polyglycerol fatty acid esters

Since the gas chromatographic analysis is not sufficient to gather complete analytical data about the composition of polyglycerol esters, essentially because of co-elution of several compounds, a prefractionation scheme as outlined in Figure 33 can be proposed. In a first fraction, eluted with chloroform-acetone 94:4 (v:v), apolar compounds up to di-esters of diglycerol can be isolated (e.g. di-acyl glycerols, tri-acyl glycerols). Afterwards, acetone elution isolates di-esters of triglycerol (partially) and mono-esters of diglycerol (completely). Finally a methanol elution allows isolation of other mono-esters and the remaining di-esters of triglycerol. These isolated fractions can subsequently be analysed gas chromatographically without any risk of interfering peaks. In combination with the results of a polyglycerol analysis and some additional chemical analyses, a total analytical scheme for the characterisation of polyglycerol fatty acid esters is proposed.

![Figure 33. Proposed analytical scheme for the analysis of polyglycerol fatty acid esters](image-url)
3. Bisphenol A antigen and antibody production

3.1. Introduction

3.1.1. Selected research strategy

As can be concluded from the general review presented in the first chapter, the traditional analytical methods to study migration are quite instrumental. One of the goals of the presented research was to evaluate the usefulness of immunochemical methods to study migration. Immunochemical methods have some major advantages compared to instrumental analytical techniques as already mentioned before (paragraph 1.4.2): no need for expensive chromatographic and spectrometric equipment, high sample throughput, avoidance or restriction of sample clean-up, high sensitivity. Therefore the use of immunochemical methods in food analysis is so well documented. Immunochemical techniques are based on the molecular recognition between the analyte and an antibody. Therefore, such methods could have particular potential in specific migration analysis. Bisphenol A was selected as a model compound. This selection was mainly based on the following criteria. First of all, bisphenol A is an important monomer for the production of high quality food contact materials as already emphasised before (see paragraph 1.2.6.5.2). In addition, bisphenol A has an interesting toxicity profile, which will be shortly presented in paragraph 3.1.2.

Development of immunochemical methods for bisphenol A, necessitates the production of bisphenol A specific antibodies. The most convenient way to achieve this is to induce the production of polyclonal antibodies in a host animal. Therefore, its immune system should be exposed to so-called immunogens or immunizing antigens. An antigen is traditionally regarded as a molecule evoking an immune response, like for example antibody production. On the other hand, the term antigen is also used to denote molecules to which antibodies bind, in for example an immunoassay. A molecule with intrinsic structural complexity, such as a protein or a polysaccharide, is considered to be highly immunogenic if its molecular weight exceeds 10 000 (Catty, 1988). Smaller molecules are in general non immunogenic. However, by coupling these haptnes to a macromolecule (the carrier), they can be made immunogenic. This phenomenon allowed the development of immunoassays to quantify different kinds of low molecular weight molecules (Mäkela and Seppälä, 1986). Because bisphenol A is not immunogenic, it needs to be coupled to a carrier to generate bisphenol A specific antibodies in a host animal. This may require
derivatisation of the bisphenol A molecule into an intermediate which is more appropriate for coupling. Some strategies to achieve such a coupling reaction will be presented in paragraph 3.1.3.

For the host animal, the use of mammals like mice, rabbits or sheep is well documented. In this research however, the use of chickens was preferred. Chickens have some distinct advantages compared to mammals to produce antibodies. These will be highlighted in paragraph 3.1.4, apart from some other general aspects about chicken immunoglobulins.

3.1.2. Toxicological relevance of bisphenol A

One of the reasons why bisphenol A was selected as a model compound in this study, is its toxicity due to which its presence in foods represents a safety issue. It is not the intention to fully review the toxicity data on bisphenol A here. Some major aspects, illustrated with a number of selected references are presented instead.

Bisphenol A is particularly known due its estrogentic character. Its xeno-estrogenic activity has been reported already many years ago (Dodds and Lawson, 1936). Recent research however revealed that this estrogentic activity is a multi-cause phenomenon. First of all, it has been proved in many experimental models that bisphenol A binds to the so-called estrogen receptors, but in another way and to a minor extent compared to the endogenous estrogen 17-β-estradiol (Gaido et al., 1997; Gould et al., 1998; Bergeron et al., 1999; Yoan et al., 2000). Bisphenol A also enhances cell proliferation in general (Kleinman et al., 1995; Strawn et al., 1995) and the cell proliferation of the female sexual organs in particular (Dodge et al., 1996; Goloubkova et al., 2000; Diel et al., 2000). Furthermore, bisphenol A is reported to induce prolactine secretion (Chu and Gorski, 2000; Goloubkova et al., 2000) and seems to invoke an anti-androgenous activity by blocking the androgenic receptor (Sohoni and Sumpter, 1998).

Because of these reported estrogentic effects, the influence of bisphenol A on all stages of development and in particular the development of the male sexual organs has been studied in detail. For rats and mices, no influence of bisphenol A exposure to pregnant animals on the number of male infants was reported (Morrissey et al., 1987). For fish on the other hand, a significant influence of bisphenol A exposure on the ratio of male and female fish was observed (Yokota et al., 1999). Furthermore, a negative influence on the sperm production was reported in mice as well (Vom Saal et al., 1994). It should be noted that the latter results could not be confirmed (Cagen et al., 1999; Ashby et al., 1999). Apart from the prenatal exposure, also prepuberal and puberal exposure in mice have been reported to negatively affect the sperm quality (Takao et al., 1999b).
A final aspect of importance which should be stressed is that the estrogenic effect of xeno-estrogenic substances is highly concentration dependent. At low levels of exposure, estrogenic effects dominate, while at higher exposure levels other toxicological effects are observed (Sheehan, 2000). Therefore, it can be concluded that the detection of low amounts of xeno-estrogenic substances in the diet is of particular importance.

In addition to the xeno-estrogenic effect the possible carcinogenicity of bisphenol A has been studied. Although bisphenol A is not a mutagen (Andersen et al., 1978) and although reports linking bisphenol A exposure to cancer incidence are limited (Ashby and Tennants, 1988 & 1991), a number of observations illustrate the possible carcinogenic character of the compound. Bisphenol A is reported to affect the mitosis (Metzler and Pfeiffer, 1995; Pfeiffer et al., 1997) and to induce aneuploidy (Pfeiffer et al., 1997, Tsutsui et al., 1998; Tsutsui et al., 2000). In addition, bisphenol A metabolites have shown to react with DNA inducing the production of DNA adducts (Atkinson and Roy, 1995a-b; Tsutsui et al., 1998). As already indicated, bisphenol A may induce cell proliferation as well.

Finally, it should be mentioned that bisphenol A exhibits an acute toxicity in both animals (LD₅₀ 150 mg.kg⁻¹, intraperitoneal injection in mice; Sax and Lewis, 1988) and humans (dermatitis; Fregert, 1981, Morrissey et al., 1987).

3.1.3. Bisphenol A hapten synthesis strategies

Generally haptens are coupled to proteins (Catty and Raykundalia, 1989). Although this coupling can be spontaneous when the hapten is a reactive molecule, in most cases, a suitable hapten is not available and needs to be synthetised (Mäkela and Seppälä, 1986).

For bisphenol A, the major functional group present is the phenolic hydroxyl group. Therefore the major coupling reactions of hydroxyl carrying haptens will be reviewed. Especially, possible applications of the described reactions in the synthesis of bisphenol A protein conjugates will be highlighted. Basically two major cases can be considered. The first involves the use of the hydroxyl group for coupling after activation of bisphenol A or the carrier. The second group of coupling reactions proceeds via the haptenation of bisphenol A into intermediates containing a carboxylic acid group, which can be coupled to the protein. It should be realised that most of the reactions shown, have not been applied yet for the production of suitable bisphenol A haptens.
3.1.3.1. Direct coupling of bisphenol A to a carrier

Most of the methods described for coupling hydroxyl containing haptens to carrier proteins relate to carbohydrates and are based on reactions involving carboxy groups or vicinal hydroxyl groups (Mäkela and Seppälä, 1986). For bisphenol A however, these methods are not appropriate. Activation of the hydroxyl group by cyanogen bromide as described by Axén and Ernback (1971) might be a valuable option (Figure 34). If an excess of bisphenol A 1 is reacted with cyanogen bromide, an intermediate cyanate structure 28 would be formed, reacting instantaneously with bisphenol A 1 to form the reactive imidocarbonate 29. The coupling reaction with a protein would give rise to the N-substituted imidocarbamate 30, which would partially be turned into the N-substituted carbamate 31 under influence of water. On the other hand, the isourea derivative 32 would be formed as well. Thus a mixture of three different adducts would be generated from one single hapten. It should be noted that the conjugates are reported to be relatively unstable (Cuatrecasas and Parikh, 1972).

Figure 34. Haptenation via activation of bisphenol A with cyanogen bromide (Prot-NH₂ represents a protein molecule)
Activation of the hydroxyl function by the introduction of a di acid chloride as described by Morgan et al. (1986a) for sterigmatocystin might be a second possibility. Thus from bisphenol A, intermediates 33 and 34 would be formed (Figure 35) reacting further with a protein to form conjugates 35 and 36.

Similarly, reaction with phosgene as described by Erlanger et al. (1957) would produce the reactive chlorocarbonates 37 and 38, giving finally rise to conjugates 31 and 39 (Figure 36).

Apart from the hapten, the carrier can be activated as well enabling the production of bisphenol A protein adducts. Lommen et al. (1995) and Skerritt et al. (2000) described the use of 1,4-butanediol.
diglycidylether as a coupling agent for hydroxylated compounds to a carrier protein. In a first step, the coupling agent is reacted with the protein, whose nucleophilic groups easily attack the epoxy groups of the former. Due to the secondary structure of the protein, the second epoxy group of intermediate 40 would not significantly react intra- and intermolecularly (Lommen et al., 1995). If in the second stage, bisphenol A would be added, adduct 41 would obtained (Figure 37).

Figure 37. Haptenation via carrier activation using 1,4-butanediodiglycidyl ether

Houen and Jensen (1995) and Skerritt et al. (2000) described the preactivation of proteins with divinyl sulfone (DVS) into the activated intermediate 42, without substantial side reactions. Subsequently coupling to bisphenol A would produce adduct 43 (Figure 38).

Figure 38. Haptenation via carrier activation using divinylsulfone

For the sake of completeness it should be mentioned that sometimes in order to improve the immunogenicity of an adduct by increasing the number of conjugated haptens, the protein is sometimes modified as described by Chu et al. (1982). In this method, the carboxylic groups of the carrier protein are modified by ethylenediamine. Thus supplementary amino groups become available for conjugation to a hapten by using one of the discussed methods.

3.1.3.2. Coupling of bisphenol A via carboxylic acid intermediates

A carboxylic acid group may be introduced in bisphenol A by one of the methods shown in Figure 39. Inman (1975) described the so-called carboxymethylation reaction, involving the binding of a
halogen acetate to the hydroxyl group creating a stable ether linkage. Thus intermediates 44 and 45 would be produced (Figure 39.a) for bisphenol A. The esterification with dicarboxylic acid anhydrides such as succinic and glutaric anhydride is frequently described in literature (e.g. Abouzied et al., 1993; Chu et al., 1984a-b; Robins et al., 1984; Forlani et al., 1992). Similarly for bisphenol A the hemisuccinates 46 and 48 and hemiglutarates 47 and 49 would be obtained (Figure 39.b). In fact, this reaction was recently applied by Kodeira et al. (2000) for the production of glutaric acid derivatives of bisphenol A. The introduction of a carboxylic acid group in phenolic structures via a reaction with diazophenylacetic acid is described as well (Mäkela and Seppälä, 1986). Thus derivatives 50 and 51 would be obtained from bisphenol A (Figure 39.c).

Figure 39. Introduction of carboxylic acid groups into bisphenol A
Once the carboxylic group is introduced, again various methods are described to couple these intermediates to a protein (Figure 40). For the sake of clarity these reactions are illustrated for one particular component, substance 46.

The conversion into acylazides involves the use of thionylchloride giving rise to an intermediate acid chloride which would then be converted into derivative 52 for bisphenol A (Figure 40.a). The current use of the method is rather restricted (Mäkela and Seppälä, 1986).

Bauminger and Wilchel (1980) described the carbodiimide mediated coupling. Although the reaction mechanism is not yet fully understood, it is postulated that an intermediate 53 would be formed which would react either with the protein to give the conjugate 54 or rearrange to the acyl urea 55 (Figure 40.b). This method is frequently applied (e.g. Abad et al., 1997; Fan et al., 1984; Kemp et al., 1986; Lau et al., 1981; Morgan et al., 1986b; Yeung et al., 1996).

In the mixed anhydride reaction, originally described by Erlanger et al. (1957), the carboxylic acid modified hapten is reacted with isobutylchlorocarbonate. For bisphenol A, the reactive intermediate 56 would be obtained (Figure 40.c). Subsequently, reaction with a carrier protein would give conjugate 54. This method has been used extensively as well (e.g. Briand et al., 1985; Cairoli et al., 1996; Chu et al., 1976; Garden and Sporns, 1994; Gendolf et al., 1984; Thouvenot and Morfin, 1983).

The conversion of a carboxyl group into a reactive N-hydroxy succinimide ester was first described by Cuatrecasas and Parikh (1972) for the activation of modified agarose. Since then however the method has been extensively used for haptenation (e.g. Abad, 1997 et al.; Cairoli, 1996 et al.; Feng, 1994 et al.; Hill et al., 1993; Kitagawa et al., 1981; Usleber et al., 1993). For bisphenol A, the reaction would involve the preparation of the activated ester 57 in the presence of dicyclohexylcarbodiimide (DCC) (Figure 40.d). The N-succinimide group would be removed upon the addition of a protein giving the bisphenol A-protein conjugate 54.

3.1.3.3. Conclusion: overall strategy for bisphenol A antigen and immunogen synthesis

The goal of this research is to synthetize bisphenol A antigens which can be applied in immunoassays. Although it is very difficult or even impossible to predict the specificity and affinity of antibodies towards a particular antigen, it is clear that these characteristics are largely influenced by the extent of uniformity and density of hapten conjugation. Furthermore, hapten orientation on the adducts is considered of importance as well. Therefore, it can be concluded from the previous review that the choice of the coupling reaction and the reactive centre(s) of the hapten involved, will highly influence the antibody specificity and affinity (Catty and Raykundalia, 1989). Since it is considered essential that the obtained adducts are as uniform as possible, a single bisphenol A
hapten should be obtained, which preferably is coupled to a protein via a single reactive centre. Therefore, the use of cyanogen bromide (Figure 34) for example is not considered as useful for this particular research. Because bisphenol A is a bifunctional molecule however, it should be realised that activation or derivatisation reactions involving the hydroxyl groups, will always induce the production of at least two haptens. Therefore it was considered essential to protect one of these hydroxyl groups before the other hydroxyl group is used in haptentation reactions. Thus intermediate 58 would be obtained (Figure 41).

Figure 40. Activation routes of carboxyl group end capped intermediates for haptenation

As the use of haptens containing a carboxylic acid group is so frequently described in literature, it is
proposed to derivatise bisphenol A in such an intermediate. If the reactions with dicarboxylic acid anhydrides are considered, a supplementary advantage arises because of the presence of an extended carbon spacer arm in intermediate 59, probably favouring the bisphenol A exposure during immunologic reactions.

Before intermediate 59 is linked to a protein, it is proposed to remove the protecting group to obtain hapten 60. In such a way, the contact between the conjugate and various chemicals can be minimized. Finally this intermediate 60 will be coupled to a carrier protein using one of the methods described, giving rise to conjugate 61.

![Figure 41. Strategy for the synthesis for bisphenol A-protein adducts (*PG : protecting group)](image)

### 3.1.4. Chicken immunoglobulins

Egg yolk proteins are distributed in two particular parts: the granules and the plasma in which the former are suspended. Granule proteins are composed of α- and β-lipovitellines (70 %), phosvitine (16 %) and low-density lipoproteins (12 %) (Burley and Cook, 1961). Some of these proteins are very important because of their functional characteristics (Baldwin, 1986). The plasma proteins consist of the α-, β- and γ-livetins and low density proteins (McCully et al., 1962). The α- and β-livetins were identified as chicken serum albumin and α2-glycoprotein respectively (Hatta et al., 1990). The γ-livetins are the chicken immunoglobulins, which are secreted from the blood plasma into the ripening egg follicle (Lösch et al., 1986).

In fact egg yolk immunoglobulins correspond to the blood serum IgG immunoglobulins and are
known as IgY (Leslie and Clem, 1969). The other blood serum immunoglobulins, IgM and IgA, are found dominantly in egg white (Rose et al., 1974) but were found in the yolk as well in very low concentrations due to a possible protein diffusion from the white into the yolk sac (Lösch et al., 1986). Quantitatively spoken only IgY is relevant in eggs since IgA and IgM concentrations in the egg white are considerably lower than the IgY concentration in the yolk (10 mg.mL$^{-1}$ yolk versus <0.1 mg.mL$^{-1}$ egg white, Otake et al., 1991, Lösch et al., 1986).

The interest for IgY isolation arises from the possible applications of these immunoglobulins in diagnostics and therapeutics. Moreover they have immunoprophylactic potential (Schade and Hlinak, 1993).

Therapeutic and prophylactic applications may be possible in animal production and in the treatment or prevention of human intestinal diseases (Lösch et al, 1986, Akita and Nakai, 1992). Oral administration of specific egg yolk antibodies towards gastrointestinal infections by *Escherichia coli* (Ikemori et al., 1992; O’Farrelly et al., 1992), *Salmonella enteriditis* (Peralta et al., 1994) and murine rotavirus (Bartz et al., 1980) in animal models is already described. Similarly the passive immunization of infants by supplementing food with specific antibodies from the colostrum of immunized cows against *E. coli* (Hilpert et al., 1987; Tacket et al., 1988), rotavirus (Brussow et al., 1987; Ebina et al., 1985) or *Shegella flexneri* (Tacket et al., 1992) is known. Similarly egg yolk immunoglobulins of immunized hens can be applied in the fortification of infant foods as suggested by Akita and Nakai (1992) and in special cases of foods as well (Lösch et al., 1986).

Apart from their use as a kind of functional food or feed ingredient, polyclonal antibodies can be applied in almost all immunologically based diagnostic methods such as enzyme immunoassay (Khil’ko et al., 1989, Bar-Joseph and Malkinson, 1980) and radioimmunoassay (Viera et al., 1986), immunoprecipitation (Song et al., 1985), immunofluorescence (Doller et al., 1987), etc. Most of the applications refer to their use in enzyme immunoassays for microbiological (Ricke and Schaeffer, 1990, Rose and Mockett, 1983) or chemical (Fertel et al., 1981) analysis. In Table 27., the more recent applications of chicken immunoglobulins in chemical analysis are summarized.

Despite of the fact that chicken egg yolk immunoglobulins are currently not used at their full potential, they possess a large number of advantages compared to their mammal analogues. The use of chickens for specific immunoglobulin production is more convenient compared to the use of mammals, because the antibodies are delivered in an egg and consequently no invasive techniques are necessary to harvest them. No bleeding of the animal is necessary which is beneficial for animal
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welfare (Hassl & Aspöck, 1988; Svendsen et al., 1995; Polson et al., 1980). Poultry have a lower phylogenetic status than mammals and it is therefore desirable to use birds instead of mammals (Svendsen et al., 1995). Consequently a better compatibility with modern animal protection regulations is assured (Akita and Nakai, 1992). Within this respect it is worthwhile noticing that the ECVAM report recommends the use of chicken antibodies to mammalian antibodies for ethical purposes (Schade et al., 1996). Antibody production is more economical (Polson et al., 1980; Hassl et al., 1987; Svendsen et al., 1995) because of the higher immunoglobulin production, cheaper housing, the chickens lower susceptibility for diseases and because the production could proceed in commercial egg production units. A supplementary advantage is the evolutionary distance from mammals, which offer the possibility to produce specific antibodies towards for example mammalian antigens (Jensenius et al., 1981). It should also be stressed that chicken antibodies have some supplementary advantages to mammalian antibodies because they lack reactivity with Fc receptors, complement and rheumatoid factors and human anti-mouse IgG antibodies. Consequently well known interferences in immunoassays can be avoided (Kricka, 1999; Larsson et al., 1993).

Table 27. Recent applications of chicken immunoglobulins in chemical analysis

<table>
<thead>
<tr>
<th>Type of component</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>proteins</td>
<td></td>
</tr>
<tr>
<td>- lactoferrin</td>
<td>Meisel (1990)</td>
</tr>
<tr>
<td>- peanut protein</td>
<td>Blais and Philippe (2000)</td>
</tr>
<tr>
<td>- soy bean glycinine</td>
<td>Meisel (1993)</td>
</tr>
<tr>
<td>mycotoxins</td>
<td></td>
</tr>
<tr>
<td>- deoxynivalenol</td>
<td>Schneider et al. (2000)</td>
</tr>
<tr>
<td>- zearalenone</td>
<td>Pichler et al. (1998)</td>
</tr>
<tr>
<td>- T-2 toxin</td>
<td>Kierek-Jaszczuk et al. (1997)</td>
</tr>
<tr>
<td>spiramycin</td>
<td>Albrecht et al. (1996)</td>
</tr>
<tr>
<td>herbicides</td>
<td>Welzig et al. (2000)</td>
</tr>
</tbody>
</table>

The reason for their restricted use probably is the problem of IgY isolation and purification from the complex egg yolk matrix. A recent review (De Meulenaer and Huyghebaert, 2001b) indicates that convenient isolation and purification methods applicable in the laboratory or even in an industrial environment are currently available. Therefore and because of the advantages of chicken
immunoglobulins, the chicken was selected as the host animal for antibody production.

3.2. Materials and Methods

3.2.1. Reagents and buffers

In addition to some of the reagents already mentioned (paragraph 2.2.1), the following were used as well.

Sodium hydrogencarbonate and sodium hydroxide were obtained from Chem-Lab, Belgium. Anhydrous disodium carbonate, sodium chloride, hydrochloric acid 25%, dimethylformamide (DMF), tetrahydrofuran (THF) and diethylether were purchased from UCB, Belgium. Bisphenol A 97%, potassium dihydrogenphosphate, disodium hydrogenphosphate dodecahydrate, imidazole, tert-butyl(chloro)dimethylsilane (tBCDS), anhydrous sodium sulphate, N-hydroxy succinimide, 4-dimethylaminopyridine, glutaric anhydride, succinic anhydride, N,N,N-tributyl-1-butylammonium fluoride (TBAF), N,N'-dicyclohexylcarbodiimide (DCC), acetic acid, potassium carbonate, ammonium chloride, trimethylsilylchloride (tMCS) sodium tetraborate decahydrate, citric acid and ammoniumsulphate were from Acros Organics, USA. Bovine serum albumin (BSA, fraction V, 96%), ovalbumin (OVA, Grade III, +97%), Freund’s incomplete adjuvans, Freund’s complete adjuvans, 2,4,6-trinitrobenzenesulfonic acid 95% (TNBS) and Tween 20 were from Sigma Chemical, USA. Hydrogen peroxide 30%, orthophenylenediamine (OPD), dry pyridine and potassium chloride were from Merck, Germany. Horseradish peroxidase conjugated rabbit anti chicken IgG was from ICN Biomedicals Inc, USA. Potassium caseinate and the skimmed milk powder were gifts of Rovita, Germany and Belgomilk, Belgium respectively. Sephadex G25 was purchased from Pharmacia and was equilibrated for at least 16h in an excess of phosphate buffered saline prior to use.

All reagents were of analytical grade unless otherwise mentioned.

Dried THF was obtained by continues reflux of THF over sodium using benzophenon as an indicator.

Phosphate buffered saline (PBS) was prepared by making up a mixture of 8.0 g NaCl, 0.2 g KH₂PO₄, 2.8 g Na₂HPO₄.12H₂O and 0.2 g KCl up to one litre with distilled water. Coating buffer (pH 9.6) consisted of 1.59 g Na₂CO₃ and 2.93 g NaHCO₃ diluted till one litre with distilled water. Dilution buffer (PBS-Tween 20) consisted of PBS with 0.05% (v:v) Tween 20. Wash solution was 0.9% (w:v) NaCl with 0.05% (v:v) Tween 20. Blocking solution was PBS with 3% (w:v) K-caseinate. Substrate buffer (pH 5.0) consisted of 7.3 g citric acid and 11.86 g Na₂HPO₄.2H₂O made up to 1 litre with water. Substrate solution was a 40 mg OPD in 100 mL of substrate buffer to which just before use 5 mL of 0.03% (v:v) H₂O₂ was added. Stop solution was 2.5 M HCl.
3.2.2. Hapten synthesis

Synthesis of 4-1-(4-[(tert-butyl(dimethyl)silyl)oxy]phenyl)methylethyl]phenol \(64\) (Figure 43)

To a solution of 2.28 g (10 mmol) bisphenol A 1 in 50 mL DMF, 1.70 g (25 mmol) imidazole was added. Subsequently, a solution of 1.51 g (10 mmol) tBCDS in 20 mL DMF was added drop wise at room temperature (RT). The reaction mixture was stirred for 1 h at RT and pored out in 100 mL of water. The mixture was extracted with hexane (2×50 mL and 2×25 mL, thus avoiding the co-extraction of unreacted bisphenol A. The organic phase was dried over sodium sulphate and evaporated to dryness under reduced pressure. The white residue was dissolved in 5 mL of hexane-ethyl acetate 6:1 (v:v) and further purified by column chromatography. Therefore a glass column (32 mm internal diameter) was filled with 60 g of silica gel using hexane-ethyl acetate 6:1 (v:v) as a mobile phase. Elution was accomplished by gravity. From the fraction eluting between 160-350 mL 1.11 g (32 %) of pure product could be obtained after evaporation to dryness under reduced pressure.

Synthesis of 5-1-(4-[(tert-butyl(dimethyl)silyl)oxy]phenyl)-1-methylethyl]phenoxy]-5-oxopentanoic acid \(67\) (Figure 45)

To a solution of 1368 mg (4 mmol) 64 in 30 mL dry tetrahydrofuran (THF), 13.68 g (120 mmol) glutaric anhydride and 489 mg 4-dimethylaminopyridine (4 mmol) was added. The mixture was heated to reflux for 2 h, pored out in 50 mL of water, acidified with 10 N HCl to pH<2 and extracted with chloroform (2×50 mL and 2×25 mL). The organic phase was dried over sodium sulphate and evaporated to dryness under reduced pressure. The residue was dissolved in 20 mL of diethyl ether and cooled for 2h at –18°C to precipitate part of the glutaric anhydride in excess, which was removed by filtration. After evaporation to dryness under reduced pressure, the residue was dissolved in 2 mL hexane-ethyl acetate 4:1 (v:v) and further purified using column chromatography. Therefore, a glass column (10 mm internal diameter) was filled with 5 g silica gel and hexane. Elution was accomplished with 40 mL hexane-ethyl acetate (4:1) (v:v). After evaporation to dryness under reduced pressure the residue was dissolved in 2 mL hexane-ethyl acetate 4:1 (v:v) and again purified using a similar chromatographic set-up. From the fraction eluting between 5-55 mL 967 mg (53%) of pure product could be obtained after evaporation to dryness under reduced pressure.

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out in 30 mL of water, acidified with 10 N HCl to pH < 2 and extracted with chloroform (3×20 mL). The combined organic fractions are washed with 25% HCl (10×10 mL), dried over sodium sulphate and evaporated to dryness under reduced pressure, yielding 633 mg of pure hapten \(47\) (74%).

### 3.2.3. Preparation and characterization of immunizing and coating conjugates.

Haptens were covalently attached to bovine serum albumin (BSA) and ovalbumin (OVA) respectively. For the synthesis of the immunizing conjugates, 102 mg hapten \(47\) (0.3 mmol) in 3 mL DMF was mixed with 48 mg (0.4 mmol) N-hydroxy succinimide and 62 mg (0.3 mmol) DCC. The solution was left for 16 h at RT and the crystals formed were removed by decantation. The solution was added drop wise to 10 mL of a protein solution (15 mg BSA.mL\(^{-1}\) in 50 mM sodium carbonate at pH 9.6). This solution was stirred for 4 h at RT and finally the conjugates were purified by gel filtration on Sephadex G 25 using PBS as eluant.

Three different coating conjugates were produced using OVA, with a varying bisphenol A to protein ratio as indicated in paragraph 3.3.2. Similar reaction conditions as for the production of BSA-bisphenol conjugates were used, except for the volume of the protein solution, since the protein concentration in the carbonate buffer was kept constant at 15 mg OVA.mL\(^{-1}\).

The extent of coupling for each conjugate was determined using the trinitrobenzosulfonic acid method as described by Fields (1972). Keeping into account the number of available amino groups in BSA and OVA as reported by Habeeb (1966), the bisphenol A load of the immunizing and coating antigen were estimated.

### 3.2.4. Chicken immunization and immunoglobulin isolation

In a first series of immunization experiments, three Isa Brown chickens (numbered 1-3) of 40 weeks old were injected intramuscularly with 1 mL of a 1:1 (v:v) mixture of Freund complete adjuvant and PBS containing 500 µg of immunizing antigen. After three weeks a supplementary injection of 1 mL of a 1:1 (v:v) mixture of Freund incomplete adjuvant and PBS containing 500 µg of immunizing antigen was given. Afterwards booster injections of 500 µg of immunizing conjugate in PBS were repeated every three weeks during a 70 day period, after which the immunization procedure was stopped. A similar protocol was followed in a second series of immunization experiments in which the hens (numbered 4-6) were younger at the start of the immunization procedure (20 weeks). Eggs were collected daily and individually identified. The immunoglobulins were isolated from the individual egg yolks using a modified aqueous dilution method described by Akita and Nakai
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(1992). Briefly, ‘v’ mL of egg yolk was separated from the egg and diluted with $8 \times v$ mL of water and pH was set with 1N HCl between 5.0 and 5.2. After 16 h incubation at 4°C and centrifugation (10 000 g, 1h, 4°C), the supernatant was filtered. After addition of 72 g of ammonium sulphate and 170 mL of water, 1h incubation at RT and centrifugation (10 000 g, 20 min, RT), the residue was dissolved in a 19% (w:v) sodium sulphate solution. After 20 min of incubation at RT and centrifugation (2 000 g, 20 min, RT) the residue was dissolved in a 14% (w:v) sodium sulphate solution. After 20 min of incubation at RT and centrifugation (2 000 g, 20 min, RT) the residue was dissolved in $v/6$ mL PBS and stored in small aliquots at −18°C. For further use they were diluted from this final solution.

3.2.5. Indirect ELISA

Ninety-six well F 96 Maxisorp Nunc immuno plates from Nunc (Denmark) were coated with coating antigen A solution (12.5 µg.mL$^{-1}$ in coating buffer; 100 µL/well) by overnight incubation at 4°C in the dark. Plates were washed three times (200 µL wash solution/well) and blocked (200 µL blocking solution/well) for 2h at RT, in the dark. Afterwards the plates were washed twice as previously. The respective primary antibody dilutions were added (100 µL/well in dilution buffer) and the plates were incubated for 1 h at 37°C. Afterwards the plates were washed three times as previously. For the detection reaction, HRP-conjugated secondary antibody was added (100 µL/well, 3.4 µg.mL$^{-1}$ in dilution buffer). After 1 h incubation at 37°C and washing of the plates (three times), 100 µL/well of substrate solution was added, followed by an additional incubation at 37°C for 1h. Finally 25 µL/well of the stop solution was added before measuring the absorbance at 492 nm. Absorbances were corrected for blanc readings obtained by using immunoglobulins isolated from the eggs of non immunized chickens. No differences between blanc readings using these immunoglobulins or those isolated out of immunized chickens, prior to immunization, was observed. Measurements were performed in quadruple.

3.2.6. Instruments

The Titertek multiskan plus MK II (USA) was used throughout this research. NMR spectra were obtained using a JEOL PMX 270 SI (270 MHz) instrument, using tetramethylsilane as a reference. For the mass spectra a Varian MAT 112 mass spectrometer (USA) (70 eV) was used which was coupled with a Varian aerograph 2700 gas chromatograph (USA). The gas chromatograph was equipped with a CPSil 5CB column (Chrompack, the Netherlands) (internal diameter 0.32 mm, film thickness 0.25 µm, 5m length). A Perkin-Elmer model 1310 infrared spectrometer was used to obtain IR spectra.
For the evaluation of the reaction mixtures obtained during hapten synthesis, GLC analysis was used as well. Therefore a Perkin Elmer GC 8700 was used equipped with a CPSil 5CB column (Chrompack, the Netherlands) (internal diameter 0.32 mm, film thickness 0.25 µm, 5 m length) and a FID detector. Gas flows were as follows: He at 1 mL.min\(^{-1}\), \(\text{H}_2\) at 30 mL.min\(^{-1}\) and \(\text{O}_2\) at 300 mL.min\(^{-1}\). Detector temperature was set at 340°C and the samples were injected on column (1 µL). Temperature programming was as follows: 80°C, 1 min; 10°C.min\(^{-1}\) to 250°C, 10 min.

### 3.3. Results and discussion

#### 3.3.1. Hapten synthesis

#### 3.3.1.1. Protection of a hydroxyl group of bisphenol A

Various methods are described to introduce protecting groups for hydroxyl functions in phenolic structures. As silyl ethers are relatively easily removed (Corey and Venkateswarlu, 1971), the possibilities of this option were evaluated. Because of the experience with the synthesis of trimethylsilyl ethers for mono-and disaccharide analysis, the reaction between bisphenol A \(1\) and trimethylsilylchloride was considered (Figure 42). As trimethylsilyl ethers are very susceptible to solvolysis in protic media (Corey and Venkateswarlu, 1971), this seemed an appropriate choice, since removal of the protecting trimethylsilyl group would be easily achieved in further stages of the hapten synthesis. A mixture of the mono and di silylated bisphenol A derivatives \(62\) and \(63\) and unreacted bisphenol A \(1\) was obtained, as revealed by GLC analysis. Although separation of this mixture using TLC seemed to be possible, a column chromatographic fractionation on silica gel was not possible due to hydrolysis of the silyl ethers. Therefore compound \(62\) could not be isolated in a pure form.

![Figure 42. Reaction of bisphenol A \(1\) with trimethylsilylchloride (tMCS)](image)
Consequently, another silyl ether was considered by reacting bisphenol A 1 with tert-butyl(chloro)dimethylsilane (tBCDS). The tert-butyl(dimethyl)siloxy group is reported to be 10^4 times more stable compared to the trimethylsiloxy group (Corey and Venkateswarlu, 1971) and was therefore more promising in the isolation of a more stable mono-protected bisphenol A derivative 64.

Reaction conditions were adapted from Corey and Venkateswarlu (1971) and Sinha et al. (1995) (Figure 43.) The addition of imidazol as a catalyst and dimethylformamide as solvent proved to be very effective. It is assumed that the reaction proceeds via N-dimethyl-tert-butyl-silylimidazole of which can be expected to be a very reactive silylating agent (Corey and Venkateswarlu, 1971).

![Figure 43. Reaction of bisphenol A 1 with tert-butyl(chloro)dimethylsilane (tBCDS)](image)

In the preliminary experiments, reactions on a 1 mmol bisphenol A 1 were performed to optimise reaction conditions and extraction. Reaction mixtures were essentially evaluated using TLC and GLC techniques. Initially, the reaction mixture was extracted using ethyl acetate as described by Sinha et al (1995) for the production of a deoxynivalenol derivative. In our experiments however emulsion formation, which could be reduced only partially by the addition of sodium chloride, complicated the extraction. Moreover, it seemed that ethyl acetate co-extracted unreacted bisphenol A 1. If the ethyl acetate extract was redissolved in hexane, the mono and diderivative of bisphenol A 64 and 65 were isolated almost free from bisphenol A, which remained insoluble. Therefore it was decided to extract the reaction mixture with hexane instead, thus preventing the emulsion formation and co-extraction of bisphenol A 1. The latter was considered as a supplementary advantage because bisphenol A 1 could not interfere in the column chromatographic clean-up as described. Scaling up of the reaction to a 10 mmol bisphenol A 1 level, increased the use of the extraction solvent. Petroleum ether p.a. was therefore considered as an alternative to the more expensive hexane, but a too high variation in the yield of the mono-derivative 64 was observed (20-30%).

Reaction conditions were varied to optimise the production of intermediate 64. Increasing the
amounts of tBCDS in order to reduce the presence of bisphenol A 1 in the reaction mixture, increased the yields of dierivative 65 (approximately 8, 13 and 44% for respectively 1, 2 and 3 eq tBCDS), without affecting the yield of the monoderivative 64 in a similar manner (30-35%). As in the chromatographic clean-up the dierivative 65 eluted first, a low concentration in the crude reaction mixture was preferred. Therefore only 1 eq tBCDS was used in subsequent experiments. The amounts of imidazole added to the reaction mixture did not influence the yields of both reaction products in the range studied (2.5 eq up to 12.5 eq).

Extending the reaction time from 1 up to 3 hours did not alter the reaction mixture as well. Neither an increase of the reaction temperature to 60°C instead of room temperature did. These observations are in contrast with the more severe reaction conditions used previously by Sinha et al. (1995) or Corey and Venkateswarlu (1971). This illustrates that bisphenol A 1 reacts readily with tBCDS, most probably because the available hydroxyl groups are not sterically shielded and weakly acidic.

For the chromatographic clean-up a compromise between yield, purity and solvent consumption needed to be found. As soon as most of the dierivative 65 was eluted, collection of the monoderivative 64 started to a total volume of 350 mL. Further elution with the more polar ethyl acetate and finally methanol revealed the presence of monoderivative 64, bisphenol A 1 and some other unknown low molecular weight impurities. As maximally 150 mg of residual material could be isolated, this fraction was not considered for isolation of derivative 64.

Based on gas chromatographic analysis and spectral data, the isolated 4-[1-(4-[[tert-butyl(dimethyl)silyl]oxy]phenyl)methylethyl]phenol 64 was considered to be pure (>95%) enough for further use. Spectral data of compounds 64 and 65 are summarized in Tables 28 and 29.
Table 28. Spectral data of 4-[1-(4-[tert-butyl(dimethyl)silyloxy]phenyl)methylethyl]phenol 64

| 1H NMR (CDCl₃) | δ: 0.08 (s, 6H, SiCH₃), 0.86 (s, 9H, tBu), 1.49 (s, 6H, CH₃), 6.56 (d, 2H, aromatic), 6.63 (d, 2H, aromatic), 6.95 (d, 4H, aromatic). |
| 13C NMR (CDCl₃) | δ: -4.26 (2×CH₃-Si), 18.29 (C_quat, tBu), 25.82 (tBu), 31.20 (2×CH₃), 41.80 (C-C₃H₅), 114.82 (2×CH, aromatic), 119.42 (2×CH, aromatic), 127.80 and 128.05 (4×CH, aromatic), 143.50 and 143.77 (2×C aromatic), 153.20 and 153.30 (C_quat-OH and C_quat-OSi). |
| MS: | m/z (%): 343 (41); 329 (27); 328 (80); 286 (21); 136 (18); 135 (100); 107 (11); 73 (20). |
| IR (cm⁻¹) | ν_max: 3272 (OH); 1511 (Ph); 1252 (Si(CH₃)₂) |

Table 29. Spectral data of tert-butyl{4-[1-(4-[tert-butyl(dimethyl)silyloxy]phenyl)-1-methylethyl]fenoxyl}dimethylsilane 65

| 1H NMR (CDCl₃) | δ: 0.08 (s, 12H, SiCH₃); 0.86 (s, 18H, tBu); 1.51 (s, 6H, CH₃); 6.60 (d, 4H, aromatic); 6.96 (d, 4H, aromatic) |
| 13C NMR (CDCl₃) | δ: -4.21 (4×CH₃-Si); 18.31 (2×C_quat, tBu); 25.72 and 25.84 (tBu); 31.23 (2×CH₃); 41.95 (C-C₃H₅); 119.20 and 119.31 (4×CH, aromatic); 127.71 and 127.81 (4×CH, aromatic); 143.81 (2×C aromatic); 153.37 (2×C_quat-OSi) |
| MS: | m/z (%): no M⁺; 427 (26); 207 (37); 171.2 (12); 86 (13); 84 (32); 73 (16); 51 (15); 49 (81); 47 (100) |
| IR (cm⁻¹) | ν_max: 1607, 1509, 1473 (Ph); 1237 (Si(CH₃)₂) |

3.3.1.2. **Introduction of a carboxy ended spacer arm**

For the introduction of a carboxy group containing spacer arm in the bisphenol A molecule, the reaction with dicarboxylic acid anhydrides was evaluated. Therefore, both succinic and glutaric anhydride were used.

Preliminary experiments using succinic anhydride and similar experimental conditions as described by Abouzied et al. (1993) and Robins et al. (1984) were unsuccessful (100°C, 2-16h in pyridine, 3-6 eq). Alternative reaction conditions adapted from Chu et al. (1984a-b) (24h reflux in THF, 30 eq) did not allow the isolation of the pure derivative 66 (Figure 44), because the initial reaction product was still partially present in addition to some unidentified compounds. Therefore further attempts to couple succinic anhydride to bisphenol A were not undertaken.
Reaction with glutaric anhydride was more successful. Adapting the reaction conditions described by Chu et al. (1984a-b), derivative 67 could be obtained in acceptable yields (Figure 45). Because of the high amounts of glutaric anhydride used, problems during sample clean-up occurred. Lowering the amount of anhydride was not possible because of incomplete reaction, even at longer reaction times. Therefore, attempts to isolate the pure derivative 67 concentrated on column chromatographic techniques and selective precipitation of the excess of anhydride. The latter could be achieved using diethyl ether at low temperature. The crude derivative 67 was further purified using a double column chromatographic clean-up. Spectral data of this compound are presented in Table 30.
Table 30. Spectral data of 5-[4-[1-(4-[[tert-butyl(dimethyl)silyl]oxy]phenyl)-1-methylethyl]phenoxy]-5-oxopentanoic acid 67

<table>
<thead>
<tr>
<th>Spectral Data</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1H NMR (CDCl3)</strong></td>
<td>δ: 0.08 (s, 6H, SiCH₃), 0.86 (s, 9H, tBu), 1.54 (s, 6H, CH₃), 1.96 (quint, 2H, CH₂CH₂COOH), 2.41 (t, 2H, CH₃COOH), 2.55 (t, 2H, CH₃COOPh), 6.62 (d, 2H, aromatic), 6.85 (d, 2H, aromatic); 6.96 and 7.08 (d, 2×2H, aromatic).</td>
</tr>
<tr>
<td><strong>13C NMR (CDCl3)</strong></td>
<td>δ: -4.23 (2×CH₃-Si), 18.08 (CH₂CH₂COOH), 19.65 (C quat, tBu), 25.59 (tBu), 30.91 (2×CH₃), 32.64 (CH₂COOPh), 33.11 (CH₂COOH), 42.02 (C-CH₃), 119.23 (2×CH aromatic), 120.62 (2×CH aromatic), 127.62 and 127.71 (4×CH aromatic), 142.88 and 148.17 (2×C aromatic), 148.57 (C quat-OsSi), 153.34 (C quat-OCO), 171.43 (COOPh), 178.80 (COOH).</td>
</tr>
<tr>
<td>MS</td>
<td>m/z (%): 88 (24); 86 (90); 84 (100); 49 (26); 47 (30).</td>
</tr>
<tr>
<td>IR (cm⁻¹)</td>
<td>νmax: 3416 (OH); 1749 (COOPh); 1704 (COOH); 1510 (Ph); 1226 (Si(CH₃)₂)</td>
</tr>
</tbody>
</table>

### 3.3.1.3. Removal of the protecting group

The last step in the synthesis of a suitable bisphenol A hapten, consisted of the removal of the protecting group from derivative 67. This step is quite delicate because the hydrolysis of the ester bound should be avoided. Based on Corley and Venkateswarlu (1971), the following experimental combinations were evaluated respectively: acetic acid/water (1:1, v:v) for 16 h at RT or 4 h reflux, MeOH saturated with K₂CO₃ for 3 h at RT, 1 M NH₄Cl in water/THF (1:1) (v:v) for 4 h at RT. None of these were successful because of incomplete reactions or side reactions (e.g. hydrolysis of ester bound). Therefore the use of TBAF, as reported previously by Sinha et al. (1995), was evaluated. Initial experiments at room temperature resulted in a complex reaction mixture. By lowering the temperature however, side reactions could be minimized, enabling the isolation of hapten 47 (Figure 46). Again problems were encountered however during sample clean-up because of the presence of tributylamine in the reaction mixture. Initial attempts to remove this major impurity using TLC as reported by Sinha et al. (1995) failed. An intensive extraction with concentrated hydrochloric acid on the other hand was able to remove the side products present, without inducing hydrolysis of the ester bound. The spectral data of hapten 47 are summarized in Table 31.
Figure 46. Removal of the protecting group from compound 64

Table 31. Spectral data of 5-{4-[1-(4-hydroxyphenyl)-1-methylethyl]phenoxy}-5-oxopentanoic acid 47

<table>
<thead>
<tr>
<th>Spectral Method</th>
<th>Chemical Shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1H NMR (CDCl₃)</strong></td>
<td>δ: 1.63 (s, 6H, CH₃), 2.06 (quint, 2H, CH₂CH₂COOH), 2.49 (t, 2H, CH₂COOH), 2.67 (t, 2H, CH₂COOPh), 6.75 (d, 2H, aromatic), 6.95 (d, 2H, aromatic), 7.07 (d, 2H, aromatic).</td>
</tr>
<tr>
<td><strong>13C NMR (CDCl₃)</strong></td>
<td>δ: 20.36 (CH₃COOH), 31.58 (C-CH₃), 33.43 (CH₂COOPh), 34.08 (CH₂COOH), 42.65 (C-CH₃), 115.39 (2×CH aromatic); 121.33 (2×CH aromatic); 128.46 and 128.52 (CH aromatic), 143.36 (C aromatic), 149.00 (C aromatic), 149.33 (Cquat-OH); 154.22 (Cquat-OCO); 172.65 (COOPh); 179.43 (COOH).</td>
</tr>
<tr>
<td><strong>MS</strong></td>
<td>m/z (%): 228 (37); 213 (100); 86 (23,77); 84 (35).</td>
</tr>
<tr>
<td><strong>IR (cm⁻¹)</strong></td>
<td>νmax: 3416 (OH); 1749 (COOPh); 1704 (COOH); 1510 (pH); 1226 (Si(CH₃)₂)</td>
</tr>
</tbody>
</table>

### 3.3.2. Synthesis and characterization of hapten-protein conjugates

For the coupling of hapten 47, a suitable protein needed to be selected. Based on literature data, bovine serum albumin was selected as a protein to produce the immunizing conjugates while for the coating conjugates, ovalbumin was selected. This latter choice introduced a supplementary advantage because in such a way non specific binding of the chicken immunoglobulins to the coating conjugates would be minimized.

For the coupling, hapten 47 was converted into the reactive N-hydroxy succinimide ester 68, which was not isolated as such. Because of the release of water, a dicarboiimide was added (Figure 47). Finally the activated hapten 68 was coupled to the two proteins. The bisphenol A load of the conjugate could be modulated by changing the ratios of hapten 47/protein during the coupling reaction. The extend of coupling was studied by the relative number of free aminogroups present in the protein as described by Fields (1972). Thus, one immunizing conjugate and three coating conjugates were obtained as presented in Table 32.
Figure 47. Coupling of hapten 47 to proteins

Table 32. Immunizing and coating bisphenol A antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Bisphenol A/protein ratio during synthesis (mol.mol⁻¹)</th>
<th>Actual Bisphenol A/protein ratio (mol.mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>immunizing antigen</td>
<td>138</td>
<td>26</td>
</tr>
<tr>
<td>coating antigen A</td>
<td>90</td>
<td>4.86</td>
</tr>
<tr>
<td>coating antigen B</td>
<td>45</td>
<td>3.80</td>
</tr>
<tr>
<td>coating antigen C</td>
<td>22.5</td>
<td>2.95</td>
</tr>
</tbody>
</table>

### 3.3.3. Chicken immunization

Chickens were immunized as described and the eggs were identified and collected daily. For the isolation of the immunoglobulins, basically the aqueous dilution technique as described by Akita and Nakai (1992) was followed with some modifications. SDS-PAGE analysis revealed that the isolated immunoglobulins were indeed sufficiently purified (not shown). Response of the chickens towards the immunization procedure was evaluated by using an indirect ELISA as described.

In the first series of immunization experiments, only two of the immunized chickens reacted towards the applied immunization procedure. Because one of these chickens stopped egg production 35 days after the start of the immunization procedure, the amount of useful collected eggs was too small. Therefore, only the eggs from one single chicken were used throughout further experiments. As can be seen from Figure 48., appreciable response was observed about 1 month
after the immunization procedure started. In addition, this response could be maintained till at least 70 days, after which the immunization experiments were aborted because of the large number of useful eggs already collected.

![Figure 48. Response of chicken 2, immunized with bisphenol A-BSA conjugate at various days after the start of the immunization (● = 7 days; ■ = 28 days; ▲ = 42 days, ◆ = 45 days, × = 70 days) (response B, was normalized to the highest absorbance level observed, B₀, which was maximally 1.3)](image)

In the second series of immunization experiments, all chickens reacted quite well to the immunization with the bisphenol A-BSA conjugate, in contrast to the first immunization series. Possibly, the younger age of the chickens could be a cause of this. Again, immune response could be maintained during a long period. In contrast to the first immunization experiment however, lower titers were obtained, as can be observed from Figure 49. Possibly again the age of the chickens could be an explanation for these observations, although it should be noted that the immunized population is far too small to come to firm conclusions in this regard. Although recently a limited amount of reports on the production of bisphenol A specific antibodies were published (Kodeira et al., 2000; Nishii et al., 2000; Ohkuma et al., 2002), this is the first reported successful chicken immunization with plastic monomer-protein conjugates in general and with bisphenol A-protein conjugates in particular. It should be noted as well, that due to the daily egg production and the
high concentration of immunoglobulins in the egg yolk, large quantities of useful antibodies could be collected throughout this research.

**Figure 49.** Response of chickens 4-6, immunized with bisphenol A-BSA conjugate at various days after the start of the immunization (■ = chicken 4, day 58; ▲ = chicken 5, day 45; ◇ = chicken 6, day 45) (response was normalized to the highest absorbance level observed B₀, which was maximally 1.2)

### 3.4. Conclusions

In this chapter, the synthesis of a suitable bisphenol A hapten was presented. Furthermore, the hapten was coupled to BSA in order to obtain a immunizing conjugate, which was injected in chickens. Immunoglobulins were isolated from the egg yolks. Apart from coupling the hapten to BSA, it was also coupled to OVA obtaining a coating conjugate which could be used in an indirect ELISA. This indirect ELISA was used to evaluate the reactivity of the isolated antibodies towards bisphenol A. Two series of chickens could be successfully immunized, but differences in response between the two series and within the series were observed.
4. Use of Bisphenol A antibodies in enzyme-linked immunosorbent assays

4.1. Introduction

As already emphasised in Chapter 3, antibodies can be applied for various purposes. One of the more important analytical applications are the so-called enzyme-linked immunosorbent assays (ELISA’s). Such assays are based on the chemical conjugation of an enzyme to either an antigen or an antibody (‘enzyme-linked’), which allows the detection of immuno complexes formed on a solid phase (‘immunosorbent’). This is because the fixed enzyme, once the free reagents present in excess are washed away, can yield a coloured product upon the addition of a substrate and a suitable chromogen. This general principle can be applied in various formats. In this introduction, only the assay examined in the reported experimental work is briefly presented.

In the previous chapter (Chapter 3), the indirect non-competitive ELISA has been used for the detection of bisphenol A- specific antibodies in the IgY isolate from immunized chickens. A similar format, but in a competitive mode, was used intensively to evaluate the usefulness of the isolated antibodies for the quantification of bisphenol A. As indicated in Figure 50, after coating of the multi-well plates with coating antigen and blocking the remaining available binding places, both the antibodies and the sample containing the analyte are added to the wells. Consequently a competition arises between the free and bound antigen to bind to the antibodies. After removal of the excess of primary antibodies, a tracer is added. This tracer consists of a secondary anti IgY antibody linked to an enzyme. Again, the tracer in excess is removed after incubation. Subsequently the substrate of the enzyme together with a chromogen are added. After the enzymatic reaction, the tracer can be quantified due to the colour change of the chromogen. Thus the amount of bound primary antibody (bisphenol A antibody) is determined indirectly.

The main objective of this research was to investigate whether the isolated antibodies could be used in such an enzyme-linked immunosorbent assay for the quantification of bisphenol A in relevant matrices. Therefore, the influence of several parameters on the assay performance was investigated. Subsequently, the specificity of the assay was studied. Finally, the assays applicability to analyse bisphenol A in real food matrices was explored.
4.2. Materials and Methods

4.2.1. Reagents and buffers

In addition to the reagents mentioned in paragraph 3.2.1, the following reagents and buffers were used, unless otherwise mentioned.

- 4, 4’-Dihydroxybenzophenon 99%, 4, 4’-ethyldenedebisphenol 99%, 4-cumylphenol 99%, bis-(4-hydroxyphenyl)-methane 98%, p-cresol 99%, m-cresol 99%, 4-hydroxydiphenylmethane 99%, 4,4’-cyclohexylidenebisphenol 98%, 2,2-bis-(4-hydroxyphenyl)-perfluoropropane 97%, bis-(4-hydroxyphenyl)-sulphone 98%, 4,4’-(1,4-phenylene-diisopropylidene)-bisphenol 98%, 4,4’-isopropylidene bis(2,6-dimethylphenol) 98%, 3,4’-isopropylidene-diphenol 98%, 4,4’-(1,3-phenylenediisopropylidene)bisphenol 99%, 1,4-dihydroxybenzene, 4,4’-dihydroxybiphenyl 97%, butyl benzyl phthalate, 4-butylphenol and 4,4’-(1-phenylethylidene) bisphenol 99% were from Aldrich Chemical Company, USA. Benzoic acid pa was obtained from Chem-Lab, Belgium. Butylhydroxyanisol was from Koch-light laboratories, England. BADGE was a generous gift from Ciba Specialty Chemicals, Belgium. 1,3-dihydroxybenzene, potassium thiocyanate and sodium bromide were purchased from UCB, Belgium. Phenol 99%, 4-nonylphenol (mixture of isomers) 99%,
di-n-butyl phthalate, benzyl alcohol, sodium perchlororate, EDTA, di-sodium EDTA and potassium iodide were from Acros Organics, USA. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) 98 % (ABTS) was from Sigma Chemical, USA. Commercial sunflower oil was obtained from Vandemoortele (Belgium).

All reagents used were of analytical grade or better, unless otherwise mentioned.

If ABTS was used as a detection reagent, substrate buffer (pH 4.0) consisted of 0.05 M tri-sodium citrate in distilled water. Substrate solution consisted of 30 mg ABTS in 100 mL of substrate buffer to which just before use 5 mL of 6 % (v:v) H₂O₂ was added. Stop solution was a 0.1 M HF, 0.008 M NaOH and 0.001 M Na₂EDTA solution.

Aqueous solutions of bisphenol A and the substances for which cross reactivity was evaluated were prepared as follows. One gram of substance was dissolved in 50 mL of methanol and this solution was diluted in water up to the desired concentration. Methanol concentration in the final solutions was considered to be negligible (constant concentration 1.5% v:v). For aqueous methanolic bisphenol A solutions, in which higher concentrations of methanol were used, methanol was added additionally till the desired concentration was reached.

4.2.2. Initial experiments

4.2.2.1. Enzymatic reactions

For all enzymatic reactions, the secondary antibody–horseradish peroxidase conjugate was diluted up to a concentration of 1.35 µg.mL⁻¹ in substrate buffer. Of this solution, 25 µL was added to each well of a 96-well plate (polystyrene, Corning Flat bottom) together with 100 µL of the appropriate substrate solution (in triplicate). After 30 minutes of incubation at 37°C, 25 µL of the appropriate stop solution was added to each well and absorbance was measured within five minutes after the addition of the stop solution at the specified wavelengths (for OPD 492 nm; for ABTS 405 nm) on a Titertek multiskan plus MK II (USA). Composition of substrate buffers and solutions are given elsewhere (paragraphs 3.2.1 and 4.2.1). Blanc experiments refer to experiments in which the addition of enzyme solution was replaced by the addition of the same volume substrate buffer. These conditions were used unless otherwise mentioned.

4.2.2.2. Signal reading and plate studies

Three plate readers were evaluated: Organon technika (The Netherlands), Titertek multiskan (USA) and the Titertek multiskan plus MK II (USA).

To evaluate the repeatability of the readers, 200 µL of a diluted OPD solution was added to each
well of a 96-well plate (polystyrene, Corning Flat bottom). The OPD solution was prepared by mixing 1 mL of a secondary antibody–horseradish peroxidase conjugate dilution as obtained previously (paragraph 4.2.2.1) with 4 mL substrate solution (paragraph 4.2.2.1) and by subsequent incubation of this mixture for 60 minutes at 37°C. After the addition of the stop solution (1 mL), the mixture was diluted with substrate buffer until the absorbance amounted approximately 1.0.

For the evaluation of the plates, the ELISA protocol described in paragraph 3.2.5 was followed with the following modification. Instead of adding different IgY dilutions in each well, the same (1/4000) IgY dilution of one particular egg (chicken 2, day 42) was added to each well, obtaining an overall absorbance of about 1.0.

### 4.2.2.3. Blocking solution studies for the indirect ELISA

For the evaluation of the blocking solutions, the ELISA protocol described in paragraph 3.2.5 was followed, with exception of the blocking solution described. Blanc experiments refer to experiments in which primary antibodies were isolated from the eggs of non immunized chickens.

### 4.2.3. Immunosorbent assays

#### 4.2.3.1. Indirect competitive ELISA

##### 4.2.3.1.1. Assay optimisation experiments

**General conditions of the immunoassay**

Ninety-six well F 96 Maxisorp Nunc immuno plates from Nunc (Denmark) or Greiner plates (Microlon® 600, flat bottom, extra high binding capacity, Germany) were coated with coating antigen A solution (12.5 µg.mL⁻¹ coating buffer, 100 µL/well) by overnight incubation at 4°C in the dark. Plates were washed three times (200 µL wash solution/well) and blocked (200 µL blocking solution/well) for 2 h at RT, in the dark. Afterwards the plates were washed twice as previously. For the competition step, 50 µL of the appropriate bisphenol A-dilution and 50 µL of the primary antibody solution were added to each well. Primary antibodies were diluted as follows: 20 µL of the original primary antibody solution in PBS was further diluted to 7.24 mL with PBS. Subsequently, 26.64 mL PBS containing 0.3 % (w:v) BSA, 520 µL NaOH 0.1N and 5.6 mL of a 4M NaCl solution were added, obtaining a final dilution of the primary antibody of 1/2000, a pH of 8.0, a BSA concentration of 0.2 % (w:v) and a calculated ionic strength of 700 mM. This dilution is referred to as the competition buffer. The plates were incubated for 1 h at 37°C. Afterwards, the plates are washed as described above (three times). For the detection reaction, the HRP-conjugated
secondary antibody was added (100 µL/well, 3.4 µg.mL⁻¹ dilution buffer). After 1 h incubation at 37°C and washing of the plates (three times), 100 µL/well of substrate solution was added, followed by an additional incubation for 1h at 37°C. Finally 25 µL/well of the appropriate stop solution was added before measuring the absorbance at the appropriate wavelength within 5 minutes (492 nm). Absorbances were corrected for blanc readings obtained by using immunoglobulins isolated from the eggs of non immunized chickens. As detection reagent, OPD was used. These conditions were followed unless otherwise stated.

**Ionic strength studies**

The general assay conditions were applied except for the amount of 4 M NaCl added to the competition buffer, which was adjusted to vary its ionic strength. The amount of PBS was reduced accordingly, keeping the primary antibody concentration constant for all experiments. If necessary the addition of NaCl solution was replaced by the addition of deionised water. The ionic strength was calculated using the following formula

$$I = \frac{1}{2} \sum_{i} d_i f_i^2$$  \[41\]

where I is the ionic strength, d is the concentration of each ion and f is its charge. Reported ionic strengths refer to those of the diluted IgY solution before it is applied in the assay. BSA itself was not present in the competition buffer for the reported experiment.

**Surface active component studies**

The influence of the following surface active agents in the competition buffer was evaluated using the general assay format, as a function of the ionic strength and their concentration: bovine serum albumin (0.2 % w:v), Tween 20 (0-0.4 % v:v) and potassium caseinate (0.2 % w:v).

**pH studies**

The amount of NaOH (0.1 N) or HCl (0.1 N) added to the competition buffer was adjusted together with the amount 4 M NaCl and PBS in such a way that the desired pH was reached, keeping the ionic strength constant. Initially these studies were performed using Tween 20, but apart from this surfactant also potassium caseinate (0.2% w:v) and BSA (0.2% w:v) were used respectively. Otherwise the general assay conditions were used.

**Chaotropic ions**

The optimised assay format was used in these experiments, but the amount of 4M NaCl added to the competition buffer was adjusted in such a way that the addition of the chaotropic ions at the indicated concentrations did not influence the final ionic strength of the competition buffer.

**Coating antigen studies**
Three different coating antigens, as indicated in Table 32, were used at varying concentrations (0.4-12.5 µg.mL⁻¹) during the coating of the multi-well plates. Otherwise the general assay format was followed.

**Chromogen**

The optimised assay format was followed, except that if OPD was used as a substrate, conditions of the enzymatic reactions were adjusted accordingly.

For all other experiments, the general assay format was used, except for the specified parameter which was varied as indicated.

### 4.2.3.1.2. Assay specificity

Competitive assays using coating antigen C (0.8 µg.mL⁻¹ in coating buffer) were performed according to the general assay format, using various structural bisphenol A analogues to determine their respective I₅₀ values (µM). I₅₀ is the concentration of the analyte at which half of the maximal signal intensity is reached. Cross reactivity was calculated as (e.g. Abad and Montaya, 1997)

\[
\text{Cross reactivity (\%) = } \frac{I_{50, \text{bisphenol A}}}{I_{50, \text{compound}}} \times 100
\]

### 4.2.3.1.3. Application of the indirect competitive ELISA for dairy emulsions

Competitive immunoassays according to the optimised assay format were accomplished using milk samples which were spiked with bisphenol A at the appropriate concentration. Reconstituted milk was prepared as follows: 10 g of skimmed milk powder is dissolved in 60 mL of distilled water. For the addition of bisphenol A, the appropriate aqueous solution was added at this stage as well. If necessary, sunflower oil at the appropriate concentration is emulsified in the dispersion using an Ultraturrax mixer at moderate speed. Afterwards, the mixture is diluted till a final volume of 100 mL is reached. Pasteurized milk samples, packed in PET bottles at various fat contents were obtained from retail shops. These samples were spiked with a concentrated methanolic bisphenol A solution, keeping the methanol concentration constant at 1.5 % (v:v).

### 4.2.3.1.4. Application of the indirect competitive ELISA for fatty foods

Competitive immunoassays according to the optimised assay format were accomplished using aqueous methanolic bisphenol A solutions at the appropriate methanol and bisphenol A concentration.

*Chemical interactions between packaging materials and foodstuffs*
4.2.3.1.5. Optimised format

Ninety-six well F 96 Maxisorp Nunc immuno plates from Nunc (Denmark) or Greiner plates (Microlon ® 600, flat bottom, extra high binding capacity, Germany) were coated with coating antigen C solution (0.8 µg.mL⁻¹ coating buffer, 100 µL/well) by overnight incubation at 4°C in the dark. Plates were washed three times (200 µL wash solution/well) and blocked (200 µL blocking solution/well) for 2 h at RT, in the dark. Afterwards the plates were washed twice as previously. For the competition step, 50 µL of the appropriate bisphenol A-dilution and 50 µL of the primary antibody solution were added to each well. Primary antibodies were diluted as follows: 20 µL of the original primary antibody solution in PBS was further diluted to 7.24 mL with PBS. Subsequently, 26.64 mL PBS containing 0.3 % (w:v) BSA, 520 µL NaOH 0.1N and 5.6 mL of a 4M NaCl solution were added, obtaining a final dilution of the primary antibody of 1/2000, a pH of 8.0, a BSA concentration of 0.2 % (w:v) and a calculated ionic strength of 700 mM. This dilution is referred to as the competition buffer. The plates were incubated for 1 h at 37°C. Afterwards, the plates are washed as described above (three times). For the detection reaction, the HRP-conjugated secondary antibody was added (100 µL/well, 3.4 µg.mL⁻¹ dilution buffer). After 1 h incubation at 37°C and washing of the plates (three times), 100 µL/well of substrate solution containing ABTS as a chromogen was added followed by an additional incubation at 37°C for 1 h. Finally 25 µL/well of the appropriate stop solution was added before measuring the absorbance at the appropriate wavelength (405 nm). Absorbances were corrected for blanc readings obtained by using immunoglobulins isolated from the eggs of non immunized chickens.

All reagents were warmed-up till incubation temperatures, plates were stacked per couple in between two empty plates and covered during incubation with a protective film.

If necessary, concentrations of the primary antibodies needed to be adjusted in such a way that the maximal absorbance in absence of analyte amounted about 1.3.

4.2.3.2. Data processing

Competition curves were obtained in quadruplicate. For the statistical evaluation a 95 % confidence interval was applied. The obtained competition curves were fitted to the four parameter logistic function corresponding to the equation [43] (Englebienne, 2000) using a commercial software package (SPSS 10.0).
As indicated in Figure 51, $B_0$ is the maximal absorbance, obtained in the absence of the analyte ($x = 0 \mu M$). $S$ is the lower asymptote to the competition curve. The $I_{50}$ value ($\mu M$) is equal to the concentration of the analyte at which the absorbance equals half of the maximal absorbance. Consequently, it is related to the assay sensitivity. It is obvious from Figure 51 that the assay sensitivity is also determined by the factor $p$. This factor is the so-called Hill slope. In most of the assays performed, essentially the $I_{50}$ value was of prime importance however with regard to the assay sensitivity, since most competition curves were characterised with comparable $p$ factors (0.65-0.75). Therefore the estimated $I_{50}$ value was used to evaluate the sensitivity of the assay, as moreover is usually done by other researchers as well (e.g. Abad and Montaya, 1997).

![Figure 51. Some theoretical competition curves based on equation [43] to illustrate the influence of the equation constants on the assay performance ($S$ is equal to zero in all cases; $\bigcirc$ : $I_{50} = 1 \mu M$; $p = 1$; $\blacksquare$ : $I_{50} = 0.01 \mu M$; $p = 1$; no symbol, plain line : $I_{50} = 1 \mu M$; $p = 0.5$)](image)

When required, curves were normalised by expressing the experimental absorbance levels ($B$) as $B/B_{0,max}$, where $B_{0,max}$ is the maximal absorbance in absence of analyte for the group of competition...
4.3. Results and discussion

4.3.1. Initial experiments

As illustrated in the introduction to this chapter (paragraph 4.1), an enzyme-linked immunosorbent assay consists of a number of consecutive steps of which for all the experiments the following are comparable: blocking, the enzymatic reaction, and the reading of the signal. In addition, the used plates will be comparable as well. Because these aspects had an influence during all the experiments performed, initial attention was attributed to their optimisation.

4.3.1.1. Enzymatic reactions

For the enzymatic reactions, initially orthophenylenediamine (OPD) was selected as a chromogen, while in later experiments also the use of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was considered. The following parameters of the enzymatic reactions, using the indicated chromogens between the brackets, were selected for further evaluation: chromogen concentration (OPD, ABTS), substrate concentration (OPD, ABTS), the factor time (OPD, ABTS), the citrate concentration and pH (ABTS) and the stop solution (ABTS).

Various chromogen concentrations were tested (0-1 mg OPD.mL⁻¹; 0-0.5 mg ABTS.mL⁻¹). As could be expected, a linear relationship between the chromogen concentration and the signal intensity was observed (for OPD : 0-100 µg.mL⁻¹; for ABTS : 0-150 µg.mL⁻¹). No influence of the chromogen concentration on the blanc readings (without enzyme) could be observed within these concentration ranges. Final chromogen concentrations were selected in such a way that they became not a limiting factor in the final assays used (OPD : 400 µg.mL⁻¹; ABTS 300 µg.mL⁻¹). This was confirmed in assays in which double concentrations of chromogen were used during the enzymatic reactions, without influence on the final absorbance signal.

Another critical parameter, especially if OPD was used as a chromogen, was the substrate (hydrogen peroxide) concentration. As illustrated in Figure 52, the net absorbance (corrected for the blanc reading), was maximal at a particular peroxide concentration (± 0.0015 %, v:v). This was quite surprising since Catty and Raykundalia (1989) and Portsmann et al. (1981), advised to use much higher substrate concentrations (e.g. 1.5 %, v:v). At these concentrations however, net absorbance levels became very low (Figure 52). In addition, high blanc readings (without enzyme) were
observed, which of course is undesirable. Low concentrations (e.g. 0.00015 %, v:v; Figure 52) resulted also in low absorbance levels. Since additional experiments in the concentration range 0.0006-0.0015 % (v:v) revealed that only minor differences in the net absorbance levels were obtained, a final substrate concentration of 0.0015 % (v:v) was selected if OPD was used as a chromogen.

![Figure 52. Influence of substrate concentration (hydrogen peroxide) on the net absorbance of OPD solutions in the presence of peroxydase](image)

For ABTS a continuous increase in signal intensity was observed until the substrate concentration amounted 0.05 % (v:v). In contrast to OPD however, no decrease in signal intensity was observed if higher substrate concentrations were applied (up to 0.6 % v:v, not shown). No effect of the substrate concentration on the blanc reading (without enzyme) could be observed either, in the range tested. The low absorbance observed for both chromogens, if low substrate concentrations are present, can be explained by the restricted reagent concentration. At high substrate concentrations, the enzymatic reaction will proceed at a rate which becomes independent upon the substrate concentration, explaining the results observed for ABTS. The deviating behaviour for OPD can be partially explained by the high blanc readings which were observed using the high substrate concentrations reported. Since the overall absorbance levels (not corrected for the blanc) in these experimental conditions were also lower then those observed with the optimal substrate concentrations.
concentration, this could not be the only explanation. Possibly, the chromogen dependent formation of an inactive substrate-enzyme complex at these high substrate concentrations could be the cause (Porstmann et al., 1981). Because of the chromogen dependent character of this inactivation, the observed difference between OPD and ABTS could be explained as well.

As could be expected, incubation time was positively correlated with signal intensity (only tested for OPD, 10-60 min, not shown). Doubling the incubation time from 30 min, as used in initial immunoassays, to 60 minutes, increased signal intensity with 50% without changing the blanco (without enzyme).

Another aspect of practical importance with regard to the time, was the influence of the time gap (0-20 min) between the mixing of all the reagents at room temperature and the incubation at 37°C (for 30 min; only tested for OPD). If the time gap was restricted up to 5 min, which is practically easy to be realised, no significant differences in absorbance levels were observed. It should be noted however that if the time gap exceeded 20 min, the difference in absorbance level was only slightly different from the reference (time gap 0 min) : $1.102 \pm 0.010$ for the reference and $1.222 \pm 0.028$ for the time gap of 20 min. So the modest enzymatic activity at room temperature is overrun almost completely during the incubation at 37°C. Since an incubation time of 60 minutes at 37°C was respected during the assays performed later, it can be expected that the time gap between the mixing of all the reagents at room temperature and the incubation of 37°C is not a crucial factor influencing assay performance.

The influence of the time (5-20 min) between the end of the enzymatic reactions (addition of the stop solution) and the measurement on the absorbance level recorded was evaluated as well, because of practical concerns. Both for OPD and ABTS no significant difference in the net absorbance levels could be observed in the time frame tested. For OPD however an increase in the overall absorbance level was observed together with a similar increase in the blanco signal (without enzyme) at the highest substrate concentrations (1.5 %, v:v) tested. Again this observation illustrates the inappropriate use of the high substrate concentrations as recommended by some authors. For ABTS, it should be noted that the observations reported were only valid for selected stop solutions, as explained further on.

Additional attention to the citrate concentration and the pH was given if ABTS was used as a chromogen because several concentrations were recommended in literature (Catty and Raykundalia, 1989 : 0.1 M, pH 6.0; Saunders, 1979 : 0.05 M, pH 4). The lower the citrate concentration, the higher the absorbance level observed, irrespective of the pH of the buffer used.
Better results were also obtained if the more acidic pH level was selected. A further decrease of the citrate concentration (at pH 4) resulted in even higher absorbance levels. In addition however, blanc signal (without enzyme) increased as well. Presumably, the citrate prevents direct chromogen oxidation.

A final aspect of importance if ABTS was used as a substrate was the stop solution used. Again various possibilities were found in literature (Table 33). Since Saunders (1979) already stressed the importance of a carefully prepared stopping reagent, these reagents were compared for further use.

Table 33. Various solutions to stop the hydrogen peroxide mediated enzymatic oxidation of ABTS

<table>
<thead>
<tr>
<th>Solution code</th>
<th>Composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1 mM NaN₃</td>
<td>Porstmann et al., 1981</td>
</tr>
<tr>
<td>2</td>
<td>0.3 M NaF</td>
<td>Catty and Raykundalia, 1989</td>
</tr>
<tr>
<td>3</td>
<td>0.1 M HF, 0.01 M NaOH, 1 mM EDTA</td>
<td>Saunders, 1979</td>
</tr>
<tr>
<td>4</td>
<td>0.1 M HF, 0.008 M NaOH, 1mM Na₂EDTA</td>
<td>Saunders, 1979</td>
</tr>
</tbody>
</table>

Solution 1 was immediately rejected because very low absorbance levels were observed. Solution 2 was not retained as well, because the final absorbance level observed seemed to be less stable as a function of time compared to stop solutions 3 and 4, which were stable in the time frame tested, as reported previously. No differences between these two latter solutions were observed with respect to the signal intensity and stability. Since EDTA is difficult to dissolve however, preference for further use was given to solution 4.

### 4.3.1.2. Signal reading and plates

During the initial immunosorbent assays, it was quickly revealed that enormous problems with assay repeatability existed. Two important causes could be identified: the spectrophotometers and the plates used.

For the spectrophotometers three different instruments were evaluated. All 96 wells of a multiwell plate were filled identically with a solution containing the oxidised chromogen (OPD) and the absorbance was recorded in quadruplate for each instrument. The relative standard deviation for each well and the average relative standard deviation over all the wells were calculated and compared (Table 34).

As can be observed, one instrument clearly caused serious repeatability problems. Consequently for...
Use of Bisphenol A antibodies in enzyme-linked immunosorbent assays

further measurements, the Titertek multiskan plus MK II instrument was preferred.

Table 34. Results of the repeatability experiments concerning the spectrophotometer (average absorbance level amounted approximately 1.0)

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Maximal relative standard deviation (%)</th>
<th>Average relative standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organon technika</td>
<td>14.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Titertek multiskan</td>
<td>5.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Titertek multiskan plus MK II</td>
<td>1.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Since for the plates especially the homogeneity of the coating of the reactants is of importance, all the 96 wells of a plate were treated identically starting from the initial coating step. Three plates of each tested brand were evaluated. The relative standard deviation on the absorption levels of all 96 wells was calculated for each plate individually. In addition, for each brand the relative standard deviation on the average absorbance level of each individual plate was compared (Table 35).

Table 35. Results of the repeatability experiments concerning the plates (average absorbance level amounted approximately 1.0)

<table>
<thead>
<tr>
<th>Plate brand</th>
<th>Relative standard deviation per plate</th>
<th>Relative standard deviation on the plate averages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linbro</td>
<td>7.6</td>
<td>32.4</td>
</tr>
<tr>
<td>Greiner, 96 well plate</td>
<td>16.0</td>
<td>26.2</td>
</tr>
<tr>
<td>Corning</td>
<td>12.8</td>
<td>20.6</td>
</tr>
<tr>
<td>Maxisorp-platen, Nunc</td>
<td>10.2</td>
<td>10.5</td>
</tr>
<tr>
<td>Maxisorp-platen, Nunc, without</td>
<td>6.2</td>
<td>4.0</td>
</tr>
<tr>
<td>edges</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As can be observed, relative standard deviations within one plate were extremely high for some brands, indicating that within these individual plates inhomogeneous coating occurred. Also the variability between the plates of each single brand was in some cases rather high, demonstrating the large variability between several plates of one single brand. The plates of Nunc seemed to have the best intra- and interplate homogeneity. It should be noted in this respect that some plates tested, were not typically immunoplates (e.g. Greiner, ordinary 96-well plates), which could explain some
of the bad results observed. Several immuno-plates of an other producer (Greiner) were evaluated as well and it was striking that only one type behaved as good as the Maxisorp plates of Nunc (not shown). All these results illustrate the importance of quality control and proper selection of the immuno-plates prior to the development of an enzyme-linked immunosorbent assay.

From the results in Table 35, another interesting observation can be made. If the wells on the edges are not taken into account, it is obvious that even better results are obtained. This can be explained by the so-called edge effect for which temperature differences between the inner and outer wells of a plate are reported to be the main cause (Burt et al., 1979; Oliver et al., 1981). Temperature gradients negatively influence the homogeneity of the results, especially if short incubation times and refrigerated reagent solutions are applied and plates are stacked on each other. A possible solution could be to avoid the use of these outer wells, thus reducing the number of available wells from 96 to 60, which is on the other hand a major disadvantage. Warming-up of the reagents till incubation temperatures, stacking the plates per couple in between two empty plates and covering the plates during incubation with a protective film are tools reported to reduce the edge effect (Esser, 2000a). This could be confirmed with experimental data obtained in the immunoassays further performed.

### 4.3.1.3. Composition of the blocking solutions for indirect assays

After coating of the immuno plates, the remaining free binding places should be occupied by the blocking agents to reduce non-specific binding of reagents later on during the assay. In such a manner high blanc readings and consequently false positive results can be avoided. In the available literature, several blocking solutions have been described as indicated in Table 36. In addition to those mentioned, it should be stressed that also solutions of BSA are frequently used for blocking purposes. Since the chickens were immunized with bisphenol A-BSA conjugates, this option was not considered.

Wells which were only coated with these blocking solutions did not show significant binding of chicken immunoglobulins, indicating that they all exhibit low affinity for these antibodies. The absorbance levels of the blanc readings (IgY from non immunized chickens) were taken into account for evaluation, using the levels obtained in the assays with blocking solution [1] as a reference. Results reported relate only to the absorbance for the wells containing the 1/2000 IgY dilution. Similar conclusions could be drawn from the absorbance levels recorded for the other wells containing a different IgY dilution. In Figure 53 the ratio of the blanc signal in an assay,
performed with the respective blocking solutions, to the reference blanc signal is shown.

Table 36. Overview of the blocking solutions tested

| Code | Composition                                      | References                                                      |
|------|--------------------------------------------------|                                                                |
| [1]  | 0.1 % gelatine, 0.05 % Tween 20 in 0.9 % NaCl    | Pichler et al. (1998), Woychik et al. (1984)                    |
| [2]  | 2 % gelatine, 0.05 % Tween 20 in 0.9 % NaCl      |                                                                 |
| [3]  | 3 % gelatine, 0.05 % Tween 20 in 0.9 % NaCl      |                                                                 |
| [4]  | 0.1 % gelatine in PBS                            | Giraudi et al. (1999), Cairoli et al. (1996),                   |
| [5]  | 2 % gelatine in PBS                              | Forlani et al. (1992), Morissette et al.                       |
| [7]  | 3% caseinate in PBS                              | Usleber et al. (1994)                                          |
| [8]  | 5% skimmed milk powder in PBS                    | Feng et al. (1994), Joyeux et al. (1996)                       |
| [9]  | 3% ovalbumin in PBS                              | Abouzied et al. (1993), Azcona Oliviera et al. (1992), Liu et al. (1985) |

Figure 53. Relative absorbance of blanc signals for the various blocking agents. (Relative absorbance was equal to : Blanc reading for blocking agent [n]/Blanc reading for blocking agent [1]; Reference absorbance amounted on average 0.44; values are averages of at least triplicate measurements)
Blanc readings for the reference blocking solution was maximal (Figure 53). Increasing the gelatine concentration in these solutions decreased the blanc signal. If Tween 20 was omitted from the gelatine solution, even lower blanc readings were obtained. Probably, Tween 20 competed in the former blocking solutions with the gelatine as suggested by Esser (2000b). Ovalbumin was considered to be inappropriate as well. Dairy protein solutions (casein and skimmed milk powder) were even more effective compared to the gelatine solutions in reducing the blanc absorbance signal. Because skimmed milk powder is however more complex in composition compared to the caseinate used, preference was given to the later protein isolate to use it as a blocking agent in further experiments.

### 4.3.2. Indirect Competitive ELISA

For the evaluation of their usefulness, the isolated antibodies from the bisphenol A immunized chickens were applied in an indirect competitive ELISA. To do so, the influence of several assay parameters on the assay performance were evaluated.

#### 4.3.2.1. Assay optimization

##### 4.3.2.1.1. Ionic strength of the competition buffer

Figure 54 shows some typical competition curves obtained from the assay for various ionic strengths of the competition buffer. In Table 37, the estimated $B_0$ and $I_{50}$ values for some of the competition curves are summarized. The estimated values for the lower ionic strengths are omitted because of the large variability on the estimated values. The ionic strength of the competition buffer revealed to be very important with regard to the assay performance. At low ionic strengths, the $I_{50}$ values became unacceptability high. The values were not significantly influenced anymore by the ionic strength starting approximately from 800 mM. A further increase of the ionic strength however resulted in a dramatic reduction of the maximal absorbance (Table 37). Therefore the range of 400-800 mM was considered as optimal taking into account the achievable sensitivity range. Finally a 700 mM ionic strength was selected for further use.

Similar findings were observed previously in immunoassays for other organic compounds using mammal antibodies (Harrison et al., 1989; Li et al., 1991; Marco et al., 1993; Lee et al., 1995; Abad and Montaya, 1997). According to Abad and Montaya (1997) these observations indicate that the interaction between antibodies and hydrophobic compounds is influenced by the polarity of the buffers used. It should be noted in this respect that in a parallel research performed with regard to
the application of chicken immunoglobulins for the detection of peanut proteins, a similar but less intense effect was observed (De Meulenaer et al., 2002), although such finding were not reported using antibodies from other animals. Therefore it can not be excluded that the observed results in the presented bisphenol A assay are partially attributable to the fact that chicken antibodies were used.

![Figure 54. Competition curves with a varying ionic strength of the competition buffer (■ = 200 mM; ▲ = 300 mM; x = 400 mM; ○ = 800 mM; ♦ = 3000 mM)](image)

**Table 37. Estimated B₀ and I₅₀ values of competition curves as a function of the ionic strength of the competition buffer**

<table>
<thead>
<tr>
<th>Ionic strength (mM)</th>
<th>400</th>
<th>800</th>
<th>1600</th>
<th>2400</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₀</td>
<td>0.96 ± 0.01</td>
<td>0.72 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.47 ± 0.03</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>I₅₀ (µM)</td>
<td>13.67 ± 1.34</td>
<td>3.13 ± 0.35</td>
<td>3.94 ± 0.59</td>
<td>4.72 ± 0.52</td>
<td>3.47 ± 0.37</td>
</tr>
</tbody>
</table>

As a conclusion, special attention to the ionic strength of the competition solution should be given. In the presented experiment, bisphenol A was dissolved in distilled water. If real samples would be analysed however, it should be realised that these may contain minerals as well. Consequently the
ionic strength of the competition buffer should be adjusted (e.g. by changing the amount of 4M NaCl added) if necessary. In order to have an idea about the ionic strength of the dissolved sample, a conductivity measurement could be useful since a linear relationship between the ionic strength (0-1 M; conductivity ranging between 0-100 mS) and the conductivity of phosphor buffered saline solutions was found (not shown). For more complex matrices however, as for example milk, this methodology was not sufficient as presented elsewhere (paragraph 4.3.2.3). For such samples, ionic strength should be adjusted until the optimal level is obtained and subsequently spiked samples should be used for calibration.

From Figure 54 and Table 37, it was revealed as well that the sensitivity of the assay was lower than expected (about 500 ppb), since immunoassays are known to be very sensitive. Therefore, further attempts to improve assay sensitivity were performed.

4.3.2.1.2. Surface active agents in the competition buffer

Surface active compounds such as Tween 20 are frequently applied in immunoassays to reduce non-specific interactions. Therefore the influence of the Tween 20 concentration on the assay performance was evaluated. As can be seen from Figure 55, I₅₀ values increased drastically due to the presence of Tween 20 at several ionic strengths tested. Consequently, Tween 20 concentration should be as low as possible to achieve better assay sensitivity. If no Tween 20 is present however, reproducibility of the competition curves was very low. Similar observations were made previously in a number of other immunoassays for organic compounds (Stanker et al., 1989; Chiu et al., 1995; Abad and Montaya, 1997).

Apart from the effects on assay sensitivity, Tween 20 affected the maximum absorbance B₀ as well: B₀ values became maximal at relative low Tween 20 concentrations (0.025 % v:v) at all ionic strengths tested. These observations are not in complete agreement with those previously reported by Abad and Montaya (1997) who found a constant decrease of the maximal absorbance as function of the Tween 20 concentration. For the sake of completeness, it should be noted that at lower ionic strengths then those reported in Figure 55, even higher maximal absorbance levels were observed, which was in correspondence with the data shown in Figure 54 and Table 37.

Apart from Tween 20, also the use of Tween 60 was considered, but all competition curves were characterized with higher I₅₀ values at the same ionic strength and the same surfactant concentration (0.01-0.015 % v:v). The use of potassium caseinate (0.1 % w:v) in the competition
buffer resulted in better $I_{50}$ values, but overall absorbance levels became too low. In contrast to earlier observations of Abad and Montaya (1997), the use of BSA (0.1 % w:v) did not result in better assay characteristics compared to the use of Tween 20 (0.01 % v:v). The use of BSA however was interesting because of other reasons as explained in the following paragraph (paragraph 4.3.2.1.3).

![Graph showing influence of Tween 20 concentration at various ionic strengths on the $I_{50}$ (filled symbols) and $B_0$ (open symbols) values](image)

**Figure 55.** Influence of Tween 20 concentration at various ionic strengths on the $I_{50}$ (filled symbols) and $B_0$ (open symbols) values (●,$\phi = 700$ mM; ▲, $\Lambda = 1600$ mM; ■, □ = 2000 mM)

### 4.3.2.1.3. pH of the competition buffer

Because bisphenol A can be considered as a weak organic acid, the pH of the competition buffer was tested together with the use of various surface active agents. Generally, at pH levels lower then five and higher then ten, very low maximal absorbance levels were obtained (<0.2). In addition, absorbance levels did not vary significantly as a function of the bisphenol A concentration. Probably due to the denaturation of the immunoglobulins.

Within the range in which acceptable results were obtained (pH 6-10), the influence of the pH on the assay performance was not significant for all surfactants tested (not shown). This was again surprising since pH dependence of both signal intensity and sensitivity of ELISA’s have been reported (Abad and Montaya, 1997; Jung et al., 1991; Lee et al., 1995). Remarkably however, it was observed that assay reproducibility increased if BSA was used instead of Tween 20, in a pH
dependent manner (starting from pH 8 to pH 10). No explanation could be given for these phenomena.

4.3.2.1.4. Dilution of the primary antibody

In order to obtain good assay characteristics of a competitive ELISA, limiting concentrations of immunoreagents are required. Therefore, the influence of the primary antibody dilution on the assay performance was evaluated. From Figure 56 and Table 38, it could be concluded that by lowering the concentration of the primary antibody assay sensitivity increased significantly. At the same time however, maximal absorbance decreased as well, which could be expected. Therefore a compromise should be found (in this particular case a dilution of 1/2000 in the competition buffer was accepted). Similar results were obtained for other eggs, but for the sake of clarity they are not included in Figure 56.

![Figure 56. Influence of primary antibody dilution on the assay performance](image)

In Figure 56 a competition curve using the antibodies isolated from an egg of chicken 5 was included as well. As can be observed, the curve was very similar to the other ones. It should be noted though, that a much higher concentration of the immunoglobulins was applied. This is in
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correspondence with the results obtained previously with regard to the titers of these respective eggs (Figures 47-48). So despite the fact that these immunoglobulins were not as reactive compared to the immunoglobulins obtained from the eggs of chicken 2, they can be applied in immunoassays, if their concentration is adjusted properly.

Table 38. Estimated B0 and I50 values of competition curves as a function of the dilution of the primary antibody (all antibodies were from chicken 2, day 42, see also Figure 56)

<table>
<thead>
<tr>
<th>Dilution of primary antibody</th>
<th>1/1000</th>
<th>1/2000</th>
<th>1/3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0</td>
<td>1.00 ± 0.01</td>
<td>0.81 ± 0.01</td>
<td>0.65 ± 0.01</td>
</tr>
<tr>
<td>I50 (µM)</td>
<td>128.85 ± 22.28</td>
<td>52.82 ± 7.19</td>
<td>37.40 ± 4.73</td>
</tr>
</tbody>
</table>

Considering the dilution of the antibodies used, the volume of the isolated IgY solution obtained from one egg (typically 2-3 mL) and the daily egg production, it becomes clear that a chicken offers an almost infinite source of immunoglobulins. This is, as already mentioned in paragraph 3.1.4., one of the major advantages of using chickens as host animals for antibody production.

4.3.2.1.5. Presence of chaotropic ions during the competition step

The use of so-called chaotropic ions, like thiocyanate, to improve assay characteristics has been reported (Amano et al., 1995). Apart from thiocyanate also other ions are known to be chaotropic such as perchlorate and iodide (Amano et al., 1995; Joyeux et al., 1996). Therefore their use was considered as well in this study in order to evaluate their effect on the assay performance. Bromide, which is also an halogen like iodide, was included as well for comparison purposes.

The results reported (Figure 57), indicate that the presence of the chaotropic ions resulted in a concentration dependent decrease of the maximal absorbance (B0) for all ions studies, except for bromide. The effect on assay sensitivity (I50) was especially pronounced if low concentrations of the ion were present, except for thiocyanate. Again no significant effect for bromide could be observed in the concentration range tested. It should be noted however that the effect on the assay sensitivity was rather moderate for all the other ions studied.

Similar concentration dependent effects of chaotropic ions on the assay performance have been reported (Nawa, 1992), although they were more pronounced. A possible explanation for the improved assay characteristics is probably due to a lowering of the non specific interactions between antigens and immunoglobulins because chaotropic ions are known to inhibit the formation
of immune complexes (Nawa, 1992; Amano et al., 1995; Ferreira and Katzin, 1995; Joyeux et al.,
1996). Therefore the weaker non specific interactions will be inhibited in the first place. Of course, at
higher concentrations, also the specific interactions will be affected, resulting in the observed lower
maximal absorbance levels. Curiously, although bromide is a halogen like iodide, even with a
higher atomic weight, it is not a chaotropic ion.
Because of the restricted effect on assay performance, the use of chaotropic ions was not further
considered.

Figure 57. Effect of various chaotropic ions on I_{50}(filled symbols) and B_0 (open symbols) values

4.3.2.1.6. Incubation time during the competition step
Another important parameter with regard to the competition step, was the time. Apart from the
practical importance with regard to the length of the total assay, time could be important as well with regard to its performance. As can be observed from Figure 58, lowering the incubation time resulted in lower $I_{50}$ values, while the reduction in the maximal absorbance level remained restricted if an incubation time of at least 60 min was respected. At lower incubation times, maximal absorbance levels decreased as well but the reduction of the $I_{50}$ values became less pronounced. Weller et al. (1992) observed similar phenomena for both assay sensitivity and maximal absorbance. These observations could be due to a heterogeneity in polyclonal antibody population, causing some subpopulations to react faster with the antigen then others. Weller et al. (1992) suggested however that merely a kinetic effect is responsible for the observed phenomena. This implies that no equilibrium is reached between the antigens and the immunoglobulins, which taking into account the long incubation time applied (2h) would be quite surprising. However, no further attempts were undertaken to better understand the observed phenomena.

Nevertheless it is clear that by shortening the incubation time during the competition reaction, improved assay sensitivity can be obtained. Precautions should be taken however to ensure that maximal absorbance levels remain high enough and that by shortening the incubation time assay variability would not increase. Therefore an incubation time of 60 min was respected in the experiments.

![Figure 58](image.png)

Figure 58. Influence of the time of the competition step on $I_{50}$ (filled symbols) and $B_0$ (open symbols) values
4.3.2.1.7. **Coating antigens**

The influence of the coating antigen on the assay performance was evaluated as well. Using coating antigen A (Table 32), it was observed that by increasing its concentration, higher I$_{50}$ levels were obtained (Figure 59). This seems logical because due to the competition reaction, higher amounts of bisphenol A are necessary to prevent 50% of the immunoglobulins from binding onto the increased amount of coating antigen. The maximal absorbance increased as well until a plateau was reached, indicating that all immunoglobulins were bound to the plate upon a particular antigen concentration ($\pm 3\mu g.mL^{-1}$) in absence of bisphenol A. Of course, at even higher coating antigen concentrations, I$_{50}$ values increased further on, because of the competition effect.

![Figure 59. Influence of the coating antigen A concentration on I$_{50}$ (filled symbols) and B$_0$ (open symbols) values.](image)

Because of these observations, other coating antigens were produced with a lower bisphenol A load and similar experiments were performed. As can be seen from Figure 60, a similar trend as the one observed in the previous experiments was obtained. It should be noted however that for the antigen with the lowest bisphenol A load tested (coating antigen C), the decrease in I$_{50}$ values was less pronounced compared to the other antigens. Despite the bisphenol A load of the coating antigen B was lower compared to coating antigen A, higher I$_{50}$ values were observed at all concentrations...
levels tested. This was surprising because of the earlier observations shown in Figure 59. Presumably, apart from the number of bisphenol A molecules per coating antigen molecule, some other factors are important as well. Possibly, for example the orientation of the bisphenol A molecules on the coating antigen could change as well by changing its hapten load.

4.3.2.1.8. Influence of tracer concentration

In final step of the ELISA before the enzymatic reaction, the secondary antibody enzyme conjugate is added to detect the bound primary antibodies. Especially an effect on the maximal absorbance level was observed (Figure 61). Although increasing $I_{50}$ levels were obtained upon an increasing the tracer concentration, the effect was not as intense in comparison to the other parameters already studied.

4.3.2.1.9. Influence of tracer incubation time

In previous experiments (paragraph 4.3.2.1.6.), it was revealed that a decrease of the incubation time during the competition reaction could improve assay sensitivity. Therefore the effects of the tracer
incubation time on the assay performance was evaluated as well. From Figure 62, it is obvious that
the observed effects are in correspondence with those reported previously. A decrease of the
incubation time resulted in an increased assay sensitivity, which on the other hand is counter-acted
via a decrease in maximal absorbance level.

![Graph showing the influence of secondary antibody-enzyme conjugate concentration on I50 and B0 values.](image)

Figure 61. Influence of the concentration of the secondary antibody-enzyme conjugate on the I50
(filled symbols) and B0 (open symbols) values
4.3.2.1.10. Influence of the chromogen

Since OPD is frequently applied as a chromogen in peroxidase-based immunoassays, this chromogen was selected initially to conduct the experiments reported. Apart from OPD however, also other chromogens are used, such as ABTS. Saunders (1979) intensively studied the applicability of this chromogen and in fact preferred its use above OPD. Therefore its use was considered as well in the present study. In Figure 63, two competition curves are represented using respectively OPD and ABTS as a chromogen.

It is obvious that maximal absorbance levels using ABTS were considerably lower (mind the difference in two absorbance scales !). On the other hand, lower blanc readings were observed as well. Therefore, the lower maximal absorbance level was not considered as a major problem if ABTS was used as a substrate. It should be noted as well that in other experiments, using another batch of ABTS, higher maximal absorbance levels (up to 0.6) were observed, without any effect on the blanc readings.

As can be observed, the competition curve obtained using ABTS as a chromogen is slightly shifted to the right compared to OPD. This is reflected as well in the estimated $I_{50}$ values: $2.40 \mu M \pm 0.19$ for ABTS and $11.36 \mu M \pm 0.67$ for OPD (95% confidence level). Possibly, the observed improvement in
assay sensitivity is due to the lower blanc absorbance levels if ABTS is used as a chromogen. Also in other experiments in which higher maximal absorbances were obtained, significant improvement in assay sensitivity was obtained if ABTS was used as a chromogen instead of OPD. Therefore, OPD was replaced by ABTS during the course of this study as a chromogen.

![Figure 63. Influence of the chromogen on the assay performance. (■, □: OPD, left absorbance scale; ◆, ◆: ABTS, right absorbance scale; filled symbols refer to net absorbance readings, open symbols refer to blanc absorbance readings)](image)

**4.3.2.2. Specificity of the indirect competitive ELISA**

The specificity of the assay was evaluated for a number of compounds. Structural analogues, of which several are allowed to be used within the EU for the production of plastic food contact materials, were included. In addition some other phenolic compounds of which some are known to be xeno-estrogenic (nonylphenol 82, butylhydroxyanisol 92) were studied. Apart from these phenolic compounds however, also some other well known xeno-estrogenic compounds (dibenzyl phthalate 2, butyl benzyl phthalate 3, BADGE 4) present in food contact materials were studied. Results obtained for immunoglobulins isolated from one particular egg (chicken 2, day 42) are summarized in Table 39.
As can be observed from this table, closely structurally related compounds show cross-reactivity: Cumylphenol 73 and 3,4'-isopropylidene-diphenol 77 showed respectively about 40 and 30% of cross-reactivity. Taking into account that only minor structural differences between these compounds and the target molecule exist (removal of one hydroxyl group in the case of cumylphenol 73 and the different position of one hydroxyl group on the phenolic ring structure for 3,4'-isopropylidene-diphenol 77), it is rather surprising to see that such small molecular differences are detected by the immunoglobulins. Probably the selection of the hapten, used for the production of the immunizing conjugate, could be the reason for the fairly high cross-reactivity observed for cumylphenol 73. It is surprising in this regard that for 4-hydroxydiphenylmethane 72 (replacement of one hydroxyl group with one methyl group with respect to bisphenol A) a strong reduction in cross-reaction was detected. Possibly the higher apolar character of this compound could be an explanation for this phenomenon. It should be noted in this respect that the cross-reactivity observed could be an advantage as well. Probably, molecules with a strong structural similarity as bisphenol A 1 could have a similar toxicological profile. Therefore, the detection of these analogues could be of interest from a food safety point of view as well.

As can be observed, removal of one of the central methyl groups (4, 4'-ethylidenebisphenol 71), reduced cross-reactivity drastically up to almost 20%, while total removal of these central methyl groups (bis-(4-hydroxyphenyl)-methane 70) resulted in even lower molecular recognition. Replacement of the central methyl groups with other functional groups gave similar results (2,2-bis-(4-hydroxyphenyl)-perfluoropropane 74; 4,4'-(1-phenylethylidene)bisphenol 75; 4,4'-cyclohexylidenebisphenol 85; 4,4'-dihydroxybiphenyl 88; 4, 4'-dihydroxybenzophenon 89; bis-(4-hydroxyphenyl)-sulphone 90). This indicates that the central methyl groups play an important role in the immunological recognition reaction. This is an important observation since the hapten used for antigen synthesis was selected in such a way that these central methyl groups were both present.

Bisphenol A analogues in which the two phenolic groups are separated with a supplementary aromatic ring (4,4'-(1,4-phenylene-diisopropylidene)-bisphenol 86; 4,4'-(1,3-phenylene-diisopropylidene)bisphenol 87), did show some limited cross-reactivity. This could not be due to the recognition of one single phenolic group, since cross-reactivity versus phenol 78 could not be observed. If on the other hand other functionalities are introduced in the phenolic structure, some molecular recognition seemed to occur, taking into account the results observed for 4-butylphenol 81. Presumably, the presence of an apolar group in combination with the phenolic moiety enhances immunological reactivity. If this apolar group became more voluminous, like in the case of...
nonylphenol 82, or smaller, like in the case of the cresols 79 and 80, cross reactivity was reduced again. Therefore, probably butylhydroxyanisol 92 showed some unexpected appreciable cross reactivity. It should be noted in this respect as well that both nonylphenol 82 and butylhydroxyanisol 92 are reported to be xeno-estrogenic compounds, like bisphenol A. Replacement of the apolar moiety with a supplementary hydroxyl group (dihydroxy benzenes 83 and 84) resulted in low cross reactivity as well. Replacement of the phenolic functionality as such by other groups (benzylalcohol 91, benzoic acid 93) gave similar results. Other xeno-estrogenic compounds, that can be present in plastic food contact materials, such as the studied phthalates could not be recognized by the immunoglobulins. This clearly indicates that no link between the xeno-estrogenic character and the immunological recognition is present. This is in correspondence with the data observed for another xeno-estrogenic compound, BADGE, which shows some structural similarities with bisphenol A.

Cross reactivity for a selected number of compounds (70, 71, 78, 88, 89, 92) was also studied for eggs from the same chicken isolated on other days. No significant differences could be observed with the data reported in Table 39 (not shown). This does not seem surprising since the immunoglobulins originated from the same chicken. Therefore the same compounds were evaluated with respect to the immunoglobulins isolated from an egg of chicken 5 (day 45). Again, comparable levels of cross reactivity were obtained: bis-(4-hydroxyphenyl)-methane 70 (2 %); 4, 4’-ethylidenebisphenol 71 (19 %); butylhydroxyanisol 92 (14 %).

As a conclusion, the presented assay can be considered as very specific, although for some structural analogues important cross reactivity was observed. Moreover, it seems that comparable levels of cross reactivity were observed irrespective of the data of egg production or of the chicken producing the egg.
Table 39. Cross reactivities of the immunoglobulins isolated from the egg of day 45, from chicken 2

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Name</th>
<th>Cross reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2,R3 = CH3, R1,R4 = OH</td>
<td>bisphenol A 1</td>
<td>100</td>
</tr>
<tr>
<td>R2,R3 = H, R1,R4 = OH</td>
<td>bis-(4-hydroxyphenyl)-methane 70</td>
<td>5</td>
</tr>
<tr>
<td>R2 = CH3, R3 = H, R1,R4 = OH</td>
<td>4, 4'-ethylidenebisphenol 71</td>
<td>18</td>
</tr>
<tr>
<td>R1,R2,R3 = H, R4 = OH</td>
<td>4-hydroxydiphenylmethane 72</td>
<td>3</td>
</tr>
<tr>
<td>R1=H, R2,R3 = CH3, R4 = OH</td>
<td>4-cumylphenol 73</td>
<td>43</td>
</tr>
<tr>
<td>R2,R2 = CF3, R1,R4 = OH</td>
<td>2,2-bis-(4-hydroxyphenyl)-perfluoropropane 74</td>
<td>3</td>
</tr>
<tr>
<td>R2= CH3, R3 = C6H5, R1,R4 = OH</td>
<td>4,4'-isopropylidenebisphenol 75</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>R1, R3,R5,R7 = CH3, R2,R6 = OH, R4 = H</td>
<td>4,4'-isopropylidene bis(2,6-dimethylphenol) 76</td>
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<td>R1 = C4H9, R2 = C6H5</td>
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</tr>
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<td>butyl benzyl phthalate 3</td>
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</tr>
<tr>
<td>Chemical structure</td>
<td>Name</td>
<td>Cross reactivity (%)</td>
</tr>
<tr>
<td>--------------------</td>
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<td>----------------------</td>
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<tr>
<td>R₁, R₂, R₃ = H</td>
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<tr>
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<td>m-cresol 80</td>
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Table 39. continued

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<td>bisphenol A diglycidyl ether (BADGE) 4</td>
<td>2</td>
</tr>
</tbody>
</table>

*Chemical interactions between packaging materials and foodstuffs*
4.3.2.3. Application of the indirect competitive ELISA for dairy emulsions

Because polycarbonate bottles can be re-used, their application for the packaging of pasteurised milk increases. The use of polycarbonate baby-bottles becomes also more popular then the use of the traditional glass bottles, because of their superior physical properties. However, due to the washing of the bottles, a partial degradation of the polymer can not be excluded. As indicated in the first chapter, an accelerated migration of bisphenol A from the bottle could occur in these circumstances. Therefore it would be interesting to have a tool which is able to check the migration immediately in the food of interest, which in this particular case is milk. Current analytical methods which are available for bisphenol A analysis would require extensive extraction and clean-up steps to tackle this analytical problem as shown in chapter 1. Since milk is an aqueous liquid, it would be possible to apply it directly in the presented assay. This would be a major advantage compared to the traditional analytical techniques.

Initial experiments on whole reconstituted milk revealed a strong reduction in assay quality (increased I_{50} and reduced B_{0}). Because comparable results were obtained for skimmed reconstituted milk, the reduced assay performance could not solely be due to an absorption of bisphenol A by the fat globules rendering it unavailable for immunochemical reactions (results not shown).

Since milk contains an appreciable amount of salts, the ionic strength of the competition buffer was adjusted because of the earlier observations with regard to the influence of ionic strength on the assay performance. From Figure 64, it is obvious that at the ionic strengths of the competition buffer, which are comparable to those normally used if aqueous bisphenol A solutions are analysed, reduced B_{0} and increased I_{50} levels were obtained, confirming the results mentioned above. By lowering the ionic strength of the competition buffer, competition curves are gradually shifted towards the competition curve observed for aqueous bisphenol A solutions. Consequently, the assay performance could be influenced in such a way that the inhibition curves obtained in milk were almost similar to those obtained in water.

In a supplementary experiment, the difference between milk samples with a varying fat content was evaluated (skimmed, semi-skimmed, whole milk). From Figure 65 it is clear that no differences between the semi-skimmed and whole milk could be observed, while the differences with the skimmed milk were very small. In fact, no significant differences in I_{50} levels were observed for all three samples (26.04± 1.84 µM; 23.92±1.32 µM; 26.60± 1.38 µM; for skimmed, semi-skimmed and whole milk respectively). Consequently it can be concluded that the reduced assay sensitivity compared to the assay in water can not be due to the absorption of the bisphenol A by the fat globules. Since bisphenol A is an apolar compound it can be expected it is indeed partially absorbed.
by the fat. Therefore the immunoglobulins seems able to interact at the water-oil interface inducing
immunochemical reaction the bisphenol A molecules.

Figure 64. Competition curves obtained in skimmed milk at selected ionic strengths of the
competition buffer (aqueous bisphenol A solution: ◇ = 700 mM; bisphenol A in milk: ● = 140 mM; ◆
= 220 mM; △ = 300 mM; ▲ = 400 mM; ■ = 600 mM)

The observed differences between the competition curves obtained in milk or in water can be
attributed to the complex sample matrix. Proteins or specific interactions with minerals like iodine,
as illustrated in a previous paragraph (4.3.2.1.5), could be responsible for the observed differences.

It can be concluded that the assay can be applied for the direct analysis of milk samples, without
any need of sample pre-treatment. Because of the observed matrix effects however, it should be
realised that a calibration with a sample of similar composition is necessary. Moreover it is clear that
due to these matrix effects, assays sensitivity decreases.
4.3.2.4. Application of the indirect competitive ELISA for fatty foods

From the first chapter it could be concluded that migration of bisphenol A is especially important in fatty food matrices. Therefore the applicability of the assay for the quantification of bisphenol A in lipids was investigated. Of course, the lipid extract or the oil can not be used as such in the assay. A draft CEN procedure was prepared for the quantification of bisphenol A in oil. This method is based on an extraction of bisphenol A from the oil using an aqueous methanol solution (50% v:v) and subsequent HPLC-UV analysis (Franz and Rijk, 1996). If the aqueous methanolic extract could be applied in the assay as such, the instrumental analytical procedure in the CEN method could be avoided. Therefore the assay characteristics were evaluated as a function of the methanol concentration in the competition buffer (Figure 66).

The presence of methanol during the competition step resulted in a significant concentration dependent decrease in the B₀ and I₅₀ levels. At the highest methanol concentration tested, high assay variability was observed as well. This is in correspondence with the results obtained by Abad and Montaya (1997) who investigated the influence of several organic solvents on the characteristics of a carbaryl assay. Probably the denaturing effect of the alcohol on proteins is responsible for these effects.
Despite of the observed effects, the presence of about 10 % of methanol resulted in an $I_{50}$ value which is still lower compared to those obtained in optimal conditions in milk. Therefore such methanol concentrations are considered to be acceptable and moreover, the dilution necessary to reach this level remains restricted. Taking into account the sensitivity loss due to the dilution of the aqueous methanolic extract and the sensitivity loss due to the presence of methanol, even higher concentrations could be tolerated. Consequently, the presented assay could be applicable for the analysis of lipidic matrices, again taking into account that the overall sensitivity of the assay is rather restricted.

### 4.3.3. Conclusions

An indirect competitive enzyme-linked immunosorbent assay could be developed using the antibodies isolated from the eggs of BSA-bisphenol A immunized chickens. The sensitivity of the assay was however lower then could be expected from other immunoassays in general and from assays recently developed for bisphenol A analysis (Kodeira et al., 2000; Nishii et al., 2000; Ohkuma et al., 2002). A step-wise approach was followed to increase the assay sensitivity by varying several assay parameters. Only a slight improvement of the assay sensitivity compared to the first experiments could be obtained. From these experiments, it seemed that care should be taken to

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Figure 66. Influence on methanol during the competition on the $I_{50}$(filled symbols) and $B_0$ (open symbols).
avoid matrix dependent effects on assay performance (e.g. salt effects, surface active agents).

Despite the fact that assay sensitivity was lower than expected, the specificity of the assay to various bisphenol A like molecules, which could migrate from food contact materials was evaluated. In addition, also some other phenolic compounds and xeno-estrogenic compounds were investigated. Generally, the assay could be considered to be very specific.

Finally, the applicability of the assay for the analysis of relevant food samples was investigated. Only a slight decrease in assay sensitivity was observed if the assay was applied for the direct analysis of milk. For fatty foods, a simple extraction with an aqueous methanol solution followed by a restricted dilution of the extract to reduce the methanol concentration, similar results could be obtained. Both examples illustrate the potency of the developed assay.
4. Use of Bisphenol A antibodies in enzyme-linked immunosorbent assays

4.1. Introduction

As already emphasised in Chapter 3, antibodies can be applied for various purposes. One of the more important analytical applications are the so-called enzyme-linked immunosorbent assays (ELISA’s). Such assays are based on the chemical conjugation of an enzyme to either an antigen or an antibody (‘enzyme-linked’), which allows the detection of immuno complexes formed on a solid phase (‘immunosorbent’). This is because the fixed enzyme, once the free reagents present in excess are washed away, can yield a coloured product upon the addition of a substrate and a suitable chromogen. This general principle can be applied in various formats. In this introduction, only the assay examined in the reported experimental work is briefly presented.

In the previous chapter (Chapter 3), the indirect non-competitive ELISA has been used for the detection of bisphenol A-specific antibodies in the IgY isolate from immunized chickens. A similar format, but in a competitive mode, was used intensively to evaluate the usefulness of the isolated antibodies for the quantification of bisphenol A. As indicated in Figure 50, after coating of the multiwell plates with coating antigen and blocking the remaining available binding places, both the antibodies and the sample containing the analyte are added to the wells. Consequently a competition arises between the free and bound antigen to bind to the antibodies. After removal of the excess of primary antibodies, a tracer is added. This tracer consists of a secondary anti IgY antibody linked to an enzyme. Again, the tracer in excess is removed after incubation. Subsequently the substrate of the enzyme together with a chromogen are added. After the enzymatic reaction, the tracer can be quantified due to the colour change of the chromogen. Thus the amount of bound primary antibody (bisphenol A antibody) is determined indirectly.

The main objective of this research was to investigate whether the isolated antibodies could be used in such an enzyme-linked immunosorbent assay for the quantification of bisphenol A in relevant matrices. Therefore, the influence of several parameters on the assay performance was investigated. Subsequently, the specificity of the assay was studied. Finally, the assays applicability to analyse bisphenol A in real food matrices was explored.
4.2. Materials and Methods

4.2.1. Reagents and buffers

In addition to the reagents mentioned in paragraph 3.2.1, the following reagents and buffers were used, unless otherwise mentioned.

4, 4’-Dihydroxybenzophenon 99%, 4, 4’-ethylidenebisphenol 99%, 4-cumylphenol 99%, bis-(4-hydroxyphenyl)-methane 98%, p-cresol 99%, m-cresol 99%, 4-hydroxydiphenylmethane 99%, 4,4’-cyclohexylidenebisphenol 98%, 2,2-bis-(4-hydroxyphenyl)-perfluoropropane 97%, bis-(4-hydroxyphenyl)-sulphone 98%, 4,4’-(1,4-phenylene-diisopropylidene)-bisphenol 98%, 4,4’-isopropylidene bis(2,6-dimethylphenol) 98%, 3,4’-isopropylidene-diphenol 98%, 4,4’-(1,3-phenylenediisopropylidene)bisphenol 99%, 1,4-dihydroxybenzene, 4,4’-dihydroxybiphenyl 97%, butyl benzyl phthalate, 4-butylphenol and 4,4’-(1-phenylethylidene) bisphenol 99% were from Aldrich Chemical Company, USA. Benzoic acid pa was obtained from Chem-Lab, Belgium. Butylhydroxyanisol was from Koch-light laboratories, England. BADGE was a generous gift from Ciba Specialty Chemicals, Belgium. 1,3-dihydroxybenzene, potassium thiocyanate and sodium bromide were purchased from UCB, Belgium. Phenol 99%, 4-nonylphenol (mixture of isomers) 99%,
di-n-butyl phthalate, benzyl alcohol, sodium perchlororate, EDTA, di-sodium EDTA and potassium iodide were from Acros Organics, USA. 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) 98 % (ABTS) was from Sigma Chemical, USA. Commercial sunflower oil was obtained from Vandemoortele (Belgium).

All reagents used were of analytical grade or better, unless otherwise mentioned.

If ABTS was used as a detection reagent, substrate buffer (pH 4.0) consisted of 0.05 M tri-sodium citrate in distilled water. Substrate solution consisted of 30 mg ABTS in 100 mL of substrate buffer to which just before use 5 mL of 6 % (v:v) H₂O₂ was added. Stop solution was a 0.1 M HF, 0.008 M NaOH and 0.001 M Na₂EDTA solution.

Aqueous solutions of bisphenol A and the substances for which cross reactivity was evaluated were prepared as follows. One gram of substance was dissolved in 50 mL of methanol and this solution was diluted in water up to the desired concentration. Methanol concentration in the final solutions was considered to be negligible (constant concentration 1.5% v:v). For aqueous methanolic bisphenol A solutions, in which higher concentrations of methanol were used, methanol was added additionally till the desired concentration was reached.

### 4.2.2. Initial experiments

#### 4.2.2.1. Enzymatic reactions

For all enzymatic reactions, the secondary antibody–horseradish peroxidase conjugate was diluted up to a concentration of 1.35 µg.mL⁻¹ in substrate buffer. Of this solution, 25 µL was added to each well of a 96-well plate (polystyrene, Corning Flat bottom) together with 100 µL of the appropriate substrate solution (in triplicate). After 30 minutes of incubation at 37°C, 25 µL of the appropriate stop solution was added to each well and absorbance was measured within five minutes after the addition of the stop solution at the specified wavelengths (for OPD 492 nm; for ABTS 405 nm) on a Titertek multiskan plus MK II (USA). Composition of substrate buffers and solutions are given elsewhere (paragraphs 3.2.1 and 4.2.1). Blanc experiments refer to experiments in which the addition of enzyme solution was replaced by the addition of the same volume substrate buffer. These conditions were used unless otherwise mentioned.

#### 4.2.2.2. Signal reading and plate studies

Three plate readers were evaluated: Organon technika (The Netherlands), Titertek multiskan (USA) and the Titertek multiskan plus MK II (USA).

To evaluate the repeatability of the readers, 200 µL of a diluted OPD solution was added to each
well of a 96-well plate (polystyrene, Corning Flat bottom). The OPD solution was prepared by mixing 1 mL of a secondary antibody–horseradish peroxidase conjugate dilution as obtained previously (paragraph 4.2.2.1) with 4 mL substrate solution (paragraph 4.2.2.1) and by subsequent incubation of this mixture for 60 minutes at 37°C. After the addition of the stop solution (1 mL), the mixture was diluted with substrate buffer until the absorbance amounted approximately 1.0.

For the evaluation of the plates, the ELISA protocol described in paragraph 3.2.5 was followed with the following modification. Instead of adding different IgY dilutions in each well, the same (1/4000) IgY dilution of one particular egg (chicken 2, day 42) was added to each well, obtaining an overall absorbance of about 1.0.

4.2.2.3. **Blocking solution studies for the indirect ELISA**

For the evaluation of the blocking solutions, the ELISA protocol described in paragraph 3.2.5 was followed, with exception of the blocking solution described. Blank experiments refer to experiments in which primary antibodies were isolated from the eggs of non immunized chickens.

4.2.3. **Immunosorbent assays**

4.2.3.1. **Indirect competitive ELISA**

4.2.3.1.1. **Assay optimisation experiments**

**General conditions of the immunoassay**

Ninety-six well F 96 Maxisorp Nunc immuno plates from Nunc (Denmark) or Greiner plates (Microlon ® 600, flat bottom, extra high binding capacity, Germany) were coated with coating antigen A solution (12.5 µg.mL⁻¹ coating buffer, 100 µL/well) by overnight incubation at 4°C in the dark. Plates were washed three times (200 µL wash solution/well) and blocked (200 µL blocking solution/well) for 2 h at RT, in the dark. Afterwards the plates were washed twice as previously. For the competition step, 50 µL of the appropriate bisphenol A-dilution and 50 µL of the primary antibody solution were added to each well. Primary antibodies were diluted as follows: 20 µL of the original primary antibody solution in PBS was further diluted to 7.24 mL with PBS. Subsequently, 26.64 mL PBS containing 0.3 % (w:v) BSA, 520 µL NaOH 0.1N and 5.6 mL of a 4M NaCl solution were added, obtaining a final dilution of the primary antibody of 1/2000, a pH of 8.0, a BSA concentration of 0.2 % (w:v) and a calculated ionic strength of 700 mM. This dilution is referred to as the competition buffer. The plates were incubated for 1 h at 37°C. Afterwards, the plates are washed as described above (three times). For the detection reaction, the HRP-conjugated
secondary antibody was added (100 µL/well, 3.4 µg.mL⁻¹ dilution buffer). After 1 h incubation at 37°C and washing of the plates (three times), 100 µL/well of substrate solution was added, followed by an additional incubation for 1 h at 37°C. Finally 25 µL/well of the appropriate stop solution was added before measuring the absorbance at the appropriate wavelength within 5 minutes (492 nm). Absorbances were corrected for blank readings obtained by using immunoglobulins isolated from the eggs of non immunized chickens. As detection reagent, OPD was used. These conditions were followed unless otherwise stated.

**Ionic strength studies**

The general assay conditions were applied except for the amount of 4 M NaCl added to the competition buffer, which was adjusted to vary its ionic strength. The amount of PBS was reduced accordingly, keeping the primary antibody concentration constant for all experiments. If necessary the addition of NaCl solution was replaced by the addition of deionised water. The ionic strength was calculated using the following formula

\[ I = \frac{1}{2} \sum_{i} d_i f_i^2 \]  

where I is the ionic strength, d is the concentration of each ion and f is its charge. Reported ionic strengths refer to those of the diluted IgY solution before it is applied in the assay. BSA itself was not present in the competition buffer for the reported experiment.

**Surface active component studies**

The influence of the following surface active agents in the competition buffer was evaluated using the general assay format, as a function of the ionic strength and their concentration: bovine serum albumin (0.2 % w:v), Tween 20 (0-0.4 % v:v) and potassium caseinate (0.2 % w:v).

**pH studies**

The amount of NaOH (0.1 N) or HCl (0.1 N) added to the competition buffer was adjusted together with the amount 4 M NaCl and PBS in such a way that the desired pH was reached, keeping the ionic strength constant. Initially these studies were performed using Tween 20, but apart from this surfactant also potassium caseinate (0.2% w:v) and BSA (0.2% w:v) were used respectively. Otherwise the general assay conditions were used.

**Chaotropic ions**

The optimised assay format was used in these experiments, but the amount of 4M NaCl added to the competition buffer was adjusted in such a way that the addition of the chaotropic ions at the indicated concentrations did not influence the final ionic strength of the competition buffer.

**Coating antigen studies**

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*Chemical interactions between packaging materials and foodstuffs*
Three different coating antigens, as indicated in Table 32, were used at varying concentrations (0.4-12.5 µg.mL⁻¹) during the coating of the multi-well plates. Otherwise the general assay format was followed.

**Chromogen**

The optimised assay format was followed, except that if OPD was used as a substrate, conditions of the enzymatic reactions were adjusted accordingly.

For all other experiments, the general assay format was used, except for the specified parameter which was varied as indicated.

### 4.2.3.1.2. Assay specificity

Competitive assays using coating antigen C (0.8 µg.mL⁻¹ in coating buffer) were performed according to the general assay format, using various structural bisphenol A analogues to determine their respective I₅₀ values (µM). I₅₀ is the concentration of the analyte at which half of the maximal signal intensity is reached. Cross reactivity was calculated as (e.g. Abad and Montaya, 1997)

\[
\text{Cross reactivity (\%) } = \frac{I_{50, \text{bisphenol A}}}{I_{50, \text{compound}}} \times 100
\]

### 4.2.3.1.3. Application of the indirect competitive ELISA for dairy emulsions

Competitive immunoassays according to the optimised assay format were accomplished using milk samples which were spiked with bisphenol A at the appropriate concentration. Reconstituted milk was prepared as follows: 10 g of skimmed milk powder is dissolved in 60 mL of distilled water. For the addition of bisphenol A, the appropriate aqueous solution was added at this stage as well. If necessary, sunflower oil at the appropriate concentration is emulsified in the dispersion using an Ultraturrax mixer at moderate speed. Afterwards, the mixture is diluted till a final volume of 100 mL is reached. Pasteurized milk samples, packed in PET bottles at various fat contents were obtained from retail shops. These samples were spiked with a concentrated methanolic bisphenol A solution, keeping the methanol concentration constant at 1.5 % (v:v).

### 4.2.3.1.4. Application of the indirect competitive ELISA for fatty foods

Competitive immunoassays according to the optimised assay format were accomplished using aqueous methanolic bisphenol A solutions at the appropriate methanol and bisphenol A concentration.
4.2.3.1.5. **Optimised format**

Ninety-six well F 96 Maxisorp Nunc immuno plates from Nunc (Denmark) or Greiner plates (Microlon® 600, flat bottom, extra high binding capacity, Germany) were coated with coating antigen C solution (0.8 µg·mL⁻¹ coating buffer, 100 µL/well) by overnight incubation at 4°C in the dark. Plates were washed three times (200 µL wash solution/well) and blocked (200 µL blocking solution/well) for 2 h at RT, in the dark. Afterwards the plates were washed twice as previously. For the competition step, 50 µL of the appropriate bisphenol A-dilution and 50 µL of the primary antibody solution were added to each well. Primary antibodies were diluted as follows: 20 µL of the original primary antibody solution in PBS was further diluted to 7.24 mL with PBS. Subsequently, 26.64 mL PBS containing 0.3 % (w:v) BSA, 520 µL NaOH 0.1N and 5.6 mL of a 4M NaCl solution were added, obtaining a final dilution of the primary antibody of 1/2000, a pH of 8.0, a BSA concentration of 0.2 % (w:v) and a calculated ionic strength of 700 mM. This dilution is referred to as the competition buffer. The plates were incubated for 1 h at 37°C. Afterwards, the plates are washed as described above (three times). For the detection reaction, the HRP-conjugated secondary antibody was added (100 µL/well, 3.4 µg·mL⁻¹ dilution buffer). After 1 h incubation at 37°C and washing of the plates (three times), 100 µL/well of substrate solution containing ABTS as a chromogen was added followed by an additional incubation at 37°C for 1 h. Finally 25 µL/well of the appropriate stop solution was added before measuring the absorbance at the appropriate wavelength (405 nm). Absorbances were corrected for blanc readings obtained by using immunoglobulins isolated from the eggs of non immunized chickens.

All reagents were warmed-up till incubation temperatures, plates were stacked per couple in between two empty plates and covered during incubation with a protective film.

If necessary, concentrations of the primary antibodies needed to be adjusted in such a way that the maximal absorbance in absence of analyte amounted about 1.3.

4.2.3.2. **Data processing**

Competition curves were obtained in quadruplate. For the statistical evaluation a 95 % confidence interval was applied. The obtained competition curves were fitted to the four parameter logistic function corresponding to the equation [43] (Englebienne, 2000) using a commercial software package (SPSS 10.0).
As indicated in Figure 51, B₀ is the maximal absorbance, obtained in the absence of the analyte (x = 0 µM). S is the lower asymptote to the competition curve. The I₅₀ value (µM) is equal to the concentration of the analyte at which the absorbance equals half of the maximal absorbance. Consequently, it is related to the assay sensitivity. It is obvious from Figure 51 that the assay sensitivity is also determined by the factor p. This factor is the so-called Hill slope. In most of the assays performed, essentially the I₅₀ value was of prime importance however with regard to the assay sensitivity, since most competition curves were characterised with comparable p factors (0.65-0.75). Therefore the estimated I₅₀ value was used to evaluate the sensitivity of the assay, as moreover is usually done by other researchers as well (e.g. Abad and Montaya, 1997).

Figure 51. Some theoretical competition curves based on equation [43] to illustrate the influence of the equation constants on the assay performance (S is equal to zero in all cases; ○ : I₅₀ = 1 µM; p = 1; ■ : I₅₀ = 0.01 µM; p = 1; no symbol, plain line : I₅₀ = 1 µM; p = 0.5)

When required, curves were normalised by expressing the experimental absorbance levels (B) as (B/B₀ₘₐₓ), where B₀ₘₐₓ is the maximal absorbance in absence of analyte for the group of competition
curves considered.

4.3. **Results and discussion**

4.3.1. **Initial experiments**

As illustrated in the introduction to this chapter (paragraph 4.1), an enzyme-linked immunosorbent assay consists of a number of consecutive steps of which for all the experiments the following are comparable: blocking, the enzymatic reaction, and the reading of the signal. In addition, the used plates will be comparable as well. Because these aspects had an influence during all the experiments performed, initial attention was attributed to their optimisation.

4.3.1.1. **Enzymatic reactions**

For the enzymatic reactions, initially orthophenylenediamine (OPD) was selected as a chromogen, while in later experiments also the use of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was considered. The following parameters of the enzymatic reactions, using the indicated chromogens between the brackets, were selected for further evaluation: chromogen concentration (OPD, ABTS), substrate concentration (OPD, ABTS), the factor time (OPD, ABTS), the citrate concentration and pH (ABTS) and the stop solution (ABTS).

Various chromogen concentrations were tested (0-1 mg OPD.mL⁻¹; 0-0.5 mg ABTS.mL⁻¹). As could be expected, a linear relationship between the chromogen concentration and the signal intensity was observed (for OPD: 0-100 µg.mL⁻¹; for ABTS: 0-150 µg.mL⁻¹). No influence of the chromogen concentration on the blank readings (without enzyme) could be observed within these concentration ranges. Final chromogen concentrations were selected in such a way that they became not a limiting factor in the final assays used (OPD: 400 µg.mL⁻¹; ABTS 300 µg.mL⁻¹). This was confirmed in assays in which double concentrations of chromogen were used during the enzymatic reactions, without influence on the final absorbance signal.

Another critical parameter, especially if OPD was used as a chromogen, was the substrate (hydrogen peroxide) concentration. As illustrated in Figure 52, the net absorbance (corrected for the blank reading), was maximal at a particular peroxide concentration (± 0.0015 %, v:v). This was quite surprising since Catty and Raykundalia (1989) and Portsmann et al. (1981), advised to use much higher substrate concentrations (e.g. 1.5 %, v:v). At these concentrations however, net absorbance levels became very low (Figure 52). In addition, high blank readings (without enzyme) were
observed, which of course is undesirable. Low concentrations (e.g. 0.00015 %, v:v; Figure 52) resulted also in low absorbance levels. Since additional experiments in the concentration range 0.0006-0.0015 % (v:v) revealed that only minor differences in the net absorbance levels were obtained, a final substrate concentration of 0.0015 % (v:v) was selected if OPD was used as a chromogen.

![Figure 52. Influence of substrate concentration (hydrogen peroxide) on the net absorbance of OPD solutions in the presence of peroxydase](image)

For ABTS a continuous increase in signal intensity was observed until the substrate concentration amounted 0.05 % (v:v). In contrast to OPD however, no decrease in signal intensity was observed if higher substrate concentrations were applied (up to 0.6 % v:v, not shown). No effect of the substrate concentration on the blanc reading (without enzyme) could be observed either, in the range tested. The low absorbance observed for both chromogens, if low substrate concentrations are present, can be explained by the restricted reagent concentration. At high substrate concentrations, the enzymatic reaction will proceed at a rate which becomes independent upon the substrate concentration, explaining the results observed for ABTS. The deviating behaviour for OPD can be partially explained by the high blanc readings which were observed using the high substrate concentrations reported. Since the overall absorbance levels (not corrected for the blanc) in these experimental conditions were also lower then those observed with the optimal substrate concentration.
concentration, this could not be the only explanation. Possibly, the chromogen dependent formation of an inactive substrate-enzyme complex at these high substrate concentrations could be the cause (Porstmann et al., 1981). Because of the chromogen dependent character of this inactivation, the observed difference between OPD and ABTS could be explained as well.

As could be expected, incubation time was positively correlated with signal intensity (only tested for OPD, 10-60 min, not shown). Doubling the incubation time from 30 min, as used in initial immunoassays, to 60 minutes, increased signal intensity with 50% without changing the blanc readings (without enzyme).

Another aspect of practical importance with regard to the time, was the influence of the time gap (0-20 min) between the mixing of all the reagents at room temperature and the incubation at 37°C (for 30 min; only tested for OPD). If the time gap was restricted up to 5 min, which is practically easy to be realised, no significant differences in absorbance levels were observed. It should be noted however that if the time gap exceeded 20 min, the difference in absorbance level was only slightly different from the reference (time gap 0 min): 1.102 ±0.010 for the reference and 1.222 ± 0.028 for the time gap of 20 min. So the modest enzymatic activity at room temperature is overrun almost completely during the incubation at 37°C. Since an incubation time of 60 minutes at 37°C was respected during the assays performed later, it can be expected that the time gap between the mixing of all the reagents at room temperature and the incubation of 37°C is not a crucial factor influencing assay performance.

The influence of the time (5-20 min) between the end of the enzymatic reactions (addition of the stop solution) and the measurement on the absorbance level recorded was evaluated as well, because of practical concerns. Both for OPD and ABTS no significant difference in the net absorbance levels could be observed in the time frame tested. For OPD however an increase in the overall absorbance level was observed together with a similar increase in the blanc signal (without enzyme) at the highest substrate concentrations (1.5 %, v:v) tested. Again this observation illustrates the inappropriate use of the high substrate concentrations as recommended by some authors. For ABTS, it should be noted that the observations reported were only valid for selected stop solutions, as explained further on.

Additional attention to the citrate concentration and the pH was given if ABTS was used as a chromogen because several concentrations were recommended in literature (Catty and Raykundalia, 1989 : 0.1 M, pH 6.0; Saunders, 1979 : 0.05 M, pH 4). The lower the citrate concentration, the higher the absorbance level observed, irrespective of the pH of the buffer used.
Better results were also obtained if the more acidic pH level was selected. A further decrease of the citrate concentration (at pH 4) resulted in even higher absorbance levels. In addition however, blanc signal (without enzyme) increased as well. Presumably, the citrate prevents direct chromogen oxidation.

A final aspect of importance if ABTS was used as a substrate was the stop solution used. Again various possibilities were found in literature (Table 33). Since Saunders (1979) already stressed the importance of a carefully prepared stopping reagent, these reagents were compared for further use.

Table 33. Various solutions to stop the hydrogen peroxide mediated enzymatic oxidation of ABTS

<table>
<thead>
<tr>
<th>Solution code</th>
<th>Composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1 mM NaN₃</td>
<td>Porstmann et al., 1981</td>
</tr>
<tr>
<td>2</td>
<td>0.3 M NaF</td>
<td>Catty and Raykundalia, 1989</td>
</tr>
<tr>
<td>3</td>
<td>0.1 M HF, 0.01 M NaOH, 1 mM EDTA</td>
<td>Saunders, 1979</td>
</tr>
<tr>
<td>4</td>
<td>0.1 M HF, 0.008 M NaOH, 1 mM Na₂EDTA</td>
<td>Saunders, 1979</td>
</tr>
</tbody>
</table>

Solution 1 was immediately rejected because very low absorbance levels were observed. Solution 2 was not retained as well, because the final absorbance level observed seemed to be less stable as a function of time compared to stop solutions 3 and 4, which were stable in the time frame tested, as reported previously. No differences between these two latter solutions were observed with respect to the signal intensity and stability. Since EDTA is difficult to dissolve however, preference for further use was given to solution 4.

4.3.1.2. Signal reading and plates

During the initial immunosorbent assays, it was quickly revealed that enormous problems with assay repeatability existed. Two important causes could be identified: the spectrophotometers and the plates used.

For the spectrophotometers three different instruments were evaluated. All 96 wells of a multiwell plate were filled identically with a solution containing the oxidised chromogen (OPD) and the absorbance was recorded in quadruplicate for each instrument. The relative standard deviation for each well and the average relative standard deviation over all the wells were calculated and compared (Table 34).

As can be observed, one instrument clearly caused serious repeatability problems. Consequently for
further measurements, the Titertek multiskan plus MK II instrument was preferred.

Table 34. Results of the repeatability experiments concerning the spectrophotometer (average absorbance level amounted approximately 1.0)

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Maximal relative standard deviation (%)</th>
<th>Average relative standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organon technika</td>
<td>14.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Titertek multiskan</td>
<td>5.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Titertek multiskan plus MK II</td>
<td>1.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Since for the plates especially the homogeneity of the coating of the reactants is of importance, all the 96 wells of a plate were treated identically starting from the initial coating step. Three plates of each tested brand were evaluated. The relative standard deviation on the absorption levels of all 96 wells was calculated for each plate individually. In addition, for each brand the relative standard deviation on the average absorbance level of each individual plate was compared (Table 35).

Table 35. Results of the repeatability experiments concerning the plates (average absorbance level amounted approximately 1.0)

<table>
<thead>
<tr>
<th>Plate brand</th>
<th>Relative standard deviation per plate</th>
<th>Relative standard deviation on the plate averages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linbro</td>
<td>7.6</td>
<td>32.4</td>
</tr>
<tr>
<td>Greiner, 96 well plate</td>
<td>16.0</td>
<td>26.2</td>
</tr>
<tr>
<td>Corning</td>
<td>12.8</td>
<td>20.6</td>
</tr>
<tr>
<td>Maxisorp-platen, Nunc</td>
<td>10.2</td>
<td>10.5</td>
</tr>
<tr>
<td>Maxisorp-platen, Nunc, without edges</td>
<td>6.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

As can be observed, relative standard deviations within one plate were extremely high for some brands, indicating that within these individual plates inhomogeneous coating occurred. Also the variability between the plates of each single brand was in some cases rather high, demonstrating the large variability between several plates of one single brand. The plates of Nunc seemed to have the best intra- and interplate homogeneity. It should be noted in this respect that some plates tested, were not typically immunoplates (e.g. Greiner, ordinary 96-well plates), which could explain some
of the bad results observed. Several immuno-plates of an other producer (Greiner) were evaluated as well and it was striking that only one type behaved as good as the Maxisorp plates of Nunc (not shown). All these results illustrate the importance of quality control and proper selection of the immuno-plates prior to the development of an enzyme-linked immunosorbent assay.

From the results in Table 35, an another interesting observation can be made. If the wells on the edges are not taken into account, it is obvious that even better results are obtained. This can be explained by the so-called edge effect for which temperature differences between the inner and outer wells of a plate are reported to be the main cause (Burt et al., 1979; Oliver et al., 1981). Temperature gradients negatively influence the homogeneity of the results, especially if short incubation times and refrigerated reagent solutions are applied and plates are stacked on each other. A possible solution could be to avoid the use of these outer wells, thus reducing the number of available wells from 96 to 60, which is on the other hand a major disadvantage. Warming-up of the reagents till incubation temperatures, stacking the plates per couple in between two empty plates and covering the plates during incubation with a protective film are tools reported to reduce the edge effect (Esser, 2000a). This could be confirmed with experimental data obtained in the immunoassays further performed.

### 4.3.1.3. Composition of the blocking solutions for indirect assays

After coating of the immuno plates, the remaining free binding places should be occupied by the blocking agents to reduce non-specific binding of reagents later on during the assay. In such a manner high blanc readings and consequently false positive results can be avoided. In the available literature, several blocking solutions have been described as indicated in Table 36. In addition to those mentioned, it should be stressed that also solutions of BSA are frequently used for blocking purposes. Since the chickens were immunized with bisphenol A-BSA conjugates, this option was not considered.

Wells which were only coated with these blocking solutions did not show significant binding of chicken immunoglobulins, indicating that they all exhibit low affinity for these antibodies.

The absorbance levels of the blanc readings (IgY from non immunized chickens) were taken into account for evaluation, using the levels obtained in the assays with blocking solution [1] as a reference. Results reported relate only to the absorbance for the wells containing the 1/2000 IgY dilution. Similar conclusions could be drawn from the absorbance levels recorded for the other wells containing a different IgY dilution. In Figure 53 the ratio of the blanc signal in an assay,
performed with the respective blocking solutions, to the reference blanc signal is shown.

Table 36. Overview of the blocking solutions tested

<table>
<thead>
<tr>
<th>Code</th>
<th>Composition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1]</td>
<td>0.1 % gelatine, 0.05 % Tween 20 in 0.9 % NaCl</td>
<td>Pichler et al. (1998), Woychik et al. (1984)</td>
</tr>
<tr>
<td>[2]</td>
<td>2 % gelatine, 0.05 % Tween 20 in 0.9 % NaCl</td>
<td></td>
</tr>
<tr>
<td>[3]</td>
<td>3 % gelatine, 0.05 % Tween 20 in 0.9 % NaCl</td>
<td></td>
</tr>
<tr>
<td>[4]</td>
<td>0.1 % gelatine in PBS</td>
<td>Giraudi et al. (1999), Cairoli et al. (1996),</td>
</tr>
<tr>
<td>[5]</td>
<td>2 % gelatine in PBS</td>
<td>Forlani et al. (1992), Morissette et al.</td>
</tr>
<tr>
<td>[7]</td>
<td>3% caseinate in PBS</td>
<td>Usleber et al. (1994)</td>
</tr>
<tr>
<td>[8]</td>
<td>5% skimmed milk powder in PBS</td>
<td>Feng et al. (1994), Joyeux et al. (1996)</td>
</tr>
<tr>
<td>[9]</td>
<td>3% ovalbumin in PBS</td>
<td>Abouzied et al. (1993), Azcona Oliviera et al.</td>
</tr>
</tbody>
</table>

Figure 53. Relative absorbance of blanc signals for the various blocking agents. (Relative absorbance was equal to: Blanc reading for blocking agent [n]/Blanc reading for blocking agent [1]; Reference absorbance amounted on average 0.44; values are averages of at least triplicate measurements)
Blanc readings for the reference blocking solution was maximal (Figure 53). Increasing the gelatine concentration in these solutions decreased the blanc signal. If Tween 20 was omitted from the gelatine solution, even lower blanc readings were obtained. Probably, Tween 20 competed in the former blocking solutions with the gelatine as suggested by Esser (2000b). Ovalbumin was considered to be inappropriate as well. Dairy protein solutions (casein and skimmed milk powder) were even more effective compared to the gelatine solutions in reducing the blanc absorbance signal. Because skimmed milk powder is however more complex in composition compared to the caseinate used, preference was given to the later protein isolate to use it as a blocking agent in further experiments.

### 4.3.2. Indirect Competitive ELISA

For the evaluation of their usefulness, the isolated antibodies from the bisphenol A immunized chickens were applied in an indirect competitive ELISA. To do so, the influence of several assay parameters on the assay performance were evaluated.

#### 4.3.2.1. Assay optimization

##### 4.3.2.1.1. Ionic strength of the competition buffer

Figure 54 shows some typical competition curves obtained from the assay for various ionic strengths of the competition buffer. In Table 37, the estimated $B_0$ and $I_{50}$ values for some of the competition curves are summarized. The estimated values for the lower ionic strengths are omitted because of the large variability on the estimated values. The ionic strength of the competition buffer revealed to be very important with regard to the assay performance. At low ionic strengths, the $I_{50}$ values became unacceptability high. The values were not significantly influenced anymore by the ionic strength starting approximately from 800 mM. A further increase of the ionic strength however resulted in a dramatic reduction of the maximal absorbance (Table 37). Therefore the range of 400-800 mM was considered as optimal taking into account the achievable sensitivity range. Finally a 700 mM ionic strength was selected for further use.

Similar findings were observed previously in immunoassays for other organic compounds using mammal antibodies (Harrison et al., 1989; Li et al., 1991; Marco et al., 1993; Lee et al., 1995; Abad and Montaya, 1997). According to Abad and Montaya (1997) these observations indicate that the interaction between antibodies and hydrophobic compounds is influenced by the polarity of the buffers used. It should be noted in this respect that in a parallel research performed with regard to
the application of chicken immunoglobulins for the detection of peanut proteins, a similar but less intense effect was observed (De Meulenaer et al., 2002), although such finding were not reported using antibodies from other animals. Therefore it can not be excluded that the observed results in the presented bisphenol A assay are partially attributable to the fact that chicken antibodies were used.

Figure 54. Competition curves with a varying ionic strength of the competition buffer (■ = 200 mM; ▲ = 300 mM; × = 400 mM; ○ = 800 mM; ◆ = 3000 mM)

Table 37. Estimated $B_0$ and $I_{50}$ values of competition curves as a function of the ionic strength of the competition buffer

<table>
<thead>
<tr>
<th>Ionic strength (mM)</th>
<th>400</th>
<th>800</th>
<th>1600</th>
<th>2400</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_0$</td>
<td>0.96 ± 0.01</td>
<td>0.72 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.47 ± 0.03</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>$I_{50}$ (µM)</td>
<td>13.67 ± 1.34</td>
<td>3.13 ± 0.35</td>
<td>3.94 ± 0.59</td>
<td>4.72 ± 0.52</td>
<td>3.47 ± 0.37</td>
</tr>
</tbody>
</table>

As a conclusion, special attention to the ionic strength of the competition solution should be given.

In the presented experiment, bisphenol A was dissolved in distilled water. If real samples would be analysed however, it should be realised that these may contain minerals as well. Consequently the
ionic strength of the competition buffer should be adjusted (e.g. by changing the amount of 4M NaCl added) if necessary. In order to have an idea about the ionic strength of the dissolved sample, a conductivity measurement could be useful since a linear relationship between the ionic strength (0-1 M; conductivity ranging between 0-100 mS) and the conductivity of phosphor buffered saline solutions was found (not shown). For more complex matrices however, as for example milk, this methodology was not sufficient as presented elsewhere (paragraph 4.3.2.3). For such samples, ionic strength should be adjusted until the optimal level is obtained and subsequently spiked samples should be used for calibration.

From Figure 54 and Table 37, it was revealed as well that the sensitivity of the assay was lower than expected (about 500 ppb), since immunoassays are known to be very sensitive. Therefore, further attempts to improve assay sensitivity were performed.

4.3.2.1.2. Surface active agents in the competition buffer

Surface active compounds such as Tween 20 are frequently applied in immunoassays to reduce non-specific interactions. Therefore the influence of the Tween 20 concentration on the assay performance was evaluated. As can be seen from Figure 55, I50 values increased drastically due to the presence of Tween 20 at several ionic strengths tested. Consequently, Tween 20 concentration should be as low as possible to achieve better assay sensitivity. If no Tween 20 is present however, reproducibility of the competition curves was very low. Similar observations were made previously in a number of other immunoassays for organic compounds (Stanker et al., 1989; Chiu et al., 1995; Abad and Montaya, 1997).

Apart from the effects on assay sensitivity, Tween 20 affected the maximum absorbance B0 as well: B0 values became maximal at relative low Tween 20 concentrations (0.025 % v:v) at all ionic strengths tested. These observations are not in complete agreement with those previously reported by Abad and Montaya (1997) who found a constant decrease of the maximal absorbance as function of the Tween 20 concentration. For the sake of completeness, it should be noted that at lower ionic strengths then those reported in Figure 55, even higher maximal absorbance levels were observed, which was in correspondence with the data shown in Figure 54 and Table 37.

Apart from Tween 20, also the use of Tween 60 was considered, but all competition curves were characterized with higher I50 values at the same ionic strength and the same surfactant concentration (0.01-0.015 % v:v). The use of potassium caseinate (0.1 % w:v) in the competition
buffer resulted in better I₅₀ values, but overall absorbance levels became too low. In contrast to earlier observations of Abad and Montaya (1997), the use of BSA (0.1 % w:v) did not result in better assay characteristics compared to the use of Tween 20 (0.01 % v:v). The use of BSA however was interesting because of other reasons as explained in the following paragraph (paragraph 4.3.2.1.3)

![Graph](image)

Figure 55. Influence of Tween 20 concentration at various ionic strengths on the I₅₀ (filled symbols) and B₀ (open symbols) values (•,◊ = 700 mM; ▲,▲ = 1600 mM; ■,□ = 2000 mM)

### 4.3.2.1.3. pH of the competition buffer

Because bisphenol A can be considered as a weak organic acid, the pH of the competition buffer was tested together with the use of various surface active agents. Generally, at pH levels lower then five and higher then ten, very low maximal absorbance levels were obtained (<0.2). In addition, absorbance levels did not vary significantly as a function of the bisphenol A concentration. Probably due to the denaturation of the immunoglobulins.

Within the range in which acceptable results were obtained (pH 6-10), the influence of the pH on the assay performance was not significant for all surfactants tested (not shown). This was again surprising since pH dependence of both signal intensity and sensitivity of ELISA’s have been reported (Abad and Montaya, 1997; Jung et al., 1991; Lee et al., 1995). Remarkably however, it was observed that assay reproducibility increased if BSA was used instead of Tween 20, in a pH
dependent manner (starting from pH 8 to pH 10). No explanation could be given for these phenomena.

4.3.2.1.4. Dilution of the primary antibody

In order to obtain good assay characteristics of a competitive ELISA, limiting concentrations of immunoreagents are required. Therefore, the influence of the primary antibody dilution on the assay performance was evaluated. From Figure 56 and Table 38, it could be concluded that by lowering the concentration of the primary antibody assay sensitivity increased significantly. At the same time however, maximal absorbance decreased as well, which could be expected. Therefore a compromise should be found (in this particular case a dilution of 1/2000 in the competition buffer was accepted). Similar results were obtained for other eggs, but for the sake of clarity they are not included in Figure 56.

Figure 56. Influence of primary antibody dilution on the assay performance ( ■ = chicken 2, day 42, dilution 1/1000; × = chicken 2, day 42, dilution 1/2000; ● = chicken 2, day 42, dilution 1/3000; ○ = chicken 5, day 45, dilution 1/200; dilutions refer to the dilution in the competition buffer).

In Figure 56 a competition curve using the antibodies isolated from an egg of chicken 5 was included as well. As can be observed, the curve was very similar to the other ones. It should be noted though, that a much higher concentration of the immunoglobulins was applied. This is in
Use of Bisphenol A antibodies in enzyme-linked immunosorbent assays

...correspondence with the results obtained previously with regard to the titers of these respective eggs (Figures 47-48). So despite the fact that these immunoglobulins were not as reactive compared to the immunoglobulins obtained from the eggs of chicken 2, they can be applied in immunoassays, if their concentration is adjusted properly.

Table 38. Estimated B₀ and I₅₀ values of competition curves as a function of the dilution of the primary antibody (all antibodies were from chicken 2, day 42, see also Figure 56)

<table>
<thead>
<tr>
<th>Dilution of primary antibody</th>
<th>1/1000</th>
<th>1/2000</th>
<th>1/3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₀</td>
<td>1.00 ± 0.01</td>
<td>0.81 ± 0.01</td>
<td>0.65 ± 0.01</td>
</tr>
<tr>
<td>I₅₀ (µM)</td>
<td>128.85 ± 22.28</td>
<td>52.82 ± 7.19</td>
<td>37.40 ± 4.73</td>
</tr>
</tbody>
</table>

Considering the dilution of the antibodies used, the volume of the isolated IgY solution obtained from one egg (typically 2-3 mL) and the daily egg production, it becomes clear that a chicken offers an almost infinite source of immunoglobulins. This is, as already mentioned in paragraph 3.1.4., one of the major advantages of using chickens as host animals for antibody production.

4.3.2.1.5. Presence of chaotropic ions during the competition step

The use of so-called chaotropic ions, like thiocyanate, to improve assay characteristics has been reported (Amano et al., 1995). Apart from thiocyanate also other ions are known to be chaotropic such as perchlorate and iodide (Amano et al., 1995; Joyeux et al., 1996). Therefore their use was considered as well in this study in order to evaluate their effect on the assay performance. Bromide, which is also an halogen like iodide, was included as well for comparison purposes.

The results reported (Figure 57), indicate that the presence of the chaotropic ions resulted in a concentration dependent decrease of the maximal absorbance (B₀) for all ions studies, except for bromide. The effect on assay sensitivity (I₅₀) was especially pronounced if low concentrations of the ion were present, except for thiocyanate. Again no significant effect for bromide could be observed in the concentration range tested. It should be noted however that the effect on the assay sensitivity was rather moderate for all the other ions studied.

Similar concentration dependent effects of chaotropic ions on the assay performance have been reported (Nawa, 1992), although they were more pronounced. A possible explanation for the improved assay characteristics is probably due to a lowering of the non specific interactions between antigens and immunoglobulins because chaotropic ions are known to inhibit the formation...
of immune complexes (Nawa, 1992; Amano et al., 1995; Ferreira and Katzin, 1995; Joyeux et al., 1996). Therefore the weaker non specific interactions will be inhibited in the first place. Of course, at higher concentrations, also the specific interactions will be affected, resulting in the observed lower maximal absorbance levels. Curiously, although bromide is a halogen like iodide, even with a higher atomic weight, it is not a chaotropic ion. Because of the restricted effect on assay performance, the use of chaotropic ions was not further considered.

Figure 57. Effect of various chaotropic ions on $I_{50}$ (filled symbols) and $B_0$ (open symbols) values

4.3.2.1.6. Incubation time during the competition step

Another important parameter with regard to the competition step, was the time. Apart from the
practical importance with regard to the length of the total assay, time could be important as well with regard to its performance. As can be observed from Figure 58, lowering the incubation time resulted in lower $I_{50}$ values, while the reduction in the maximal absorbance level remained restricted if an incubation time of at least 60 min was respected. At lower incubation times, maximal absorbance levels decreased as well but the reduction of the $I_{50}$ values became less pronounced. Weller et al. (1992) observed similar phenomena for both assay sensitivity and maximal absorbance. These observations could be due to a heterogeneity in polyclonal antibody population, causing some subpopulations to react faster with the antigen then others. Weller et al. (1992) suggested however that merely a kinetic effect is responsible for the observed phenomena. This implies that no equilibrium is reached between the antigens and the immunoglobulins, which taking into account the long incubation time applied (2h) would be quite surprising. However, no further attempts were undertaken to better understand the observed phenomena.

Nevertheless it is clear that by shortening the incubation time during the competition reaction, improved assay sensitivity can be obtained. Precautions should be taken however to ensure that maximal absorbance levels remain high enough and that by shortening the incubation time assay variability would not increase. Therefore an incubation time of 60 min was respected in the experiments.

Figure 58. Influence of the time of the competition step on $I_{50}$ (filled symbols) and $B_0$ (open symbols) values
4.3.2.1.7. Coating antigens

The influence of the coating antigen on the assay performance was evaluated as well. Using coating antigen A (Table 32), it was observed that by increasing its concentration, higher \( I_{50} \) levels were obtained (Figure 59). This seems logical because due to the competition reaction, higher amounts of bisphenol A are necessary to prevent 50% of the immunoglobulins from binding onto the increased amount of coating antigen. The maximal absorbance increased as well until a plateau was reached, indicating that all immunoglobulins were bound to the plate upon a particular antigen concentration (± 3\( \mu \)g mL\(^{-1} \)) in absence of bisphenol A. Of course, at even higher coating antigen concentrations, \( I_{50} \) values increased further on, because of the competition effect.

![Figure 59. Influence of the coating antigen A concentration on \( I_{50} \) (filled symbols) and \( B_0 \) (open symbols) values.](image)

Because of these observations, other coating antigens were produced with a lower bisphenol A load and similar experiments were performed. As can be seen from Figure 60, a similar trend as the one observed in the previous experiments was obtained. It should be noted however that for the antigen with the lowest bisphenol A load tested (coating antigen C), the decrease in \( I_{50} \) values was less pronounced compared to the other antigens. Despite the bisphenol A load of the coating antigen B was lower compared to coating antigen A, higher \( I_{50} \) values were observed at all concentrations.
levels tested. This was surprising because of the earlier observations shown in Figure 59. Presumably, apart from the number of bisphenol A molecules per coating antigen molecule, some other factors are important as well. Possibly, for example the orientation of the bisphenol A molecules on the coating antigen could change as well by changing its hapten load.

Figure 60. Influence of the kind of coating antigen and its concentration on $I_{50}$ (filled symbols) and $B_0$ (open symbols) values (♮,◊ = coating antigen A; ■,□ = coating antigen B; ▲,▲ coating antigen = C; for the characteristics of these coating antigens: see Table 32)

4.3.2.1.8. Influence of tracer concentration

In final step of the ELISA before the enzymatic reaction, the secondary antibody enzyme conjugate is added to detect the bound primary antibodies. Especially an effect on the maximal absorbance level was observed (Figure 61). Although increasing $I_{50}$ levels were obtained upon an increasing the tracer concentration, the effect was not as intense in comparison to the other parameters already studied.

4.3.2.1.9. Influence of tracer incubation time

In previous experiments (paragraph 4.3.2.1.6.), it was revealed that a decrease of the incubation time during the competition reaction could improve assay sensitivity. Therefore the effects of the tracer
incubation time on the assay performance was evaluated as well. From Figure 62, it is obvious that the observed effects are in correspondence with those reported previously. A decrease of the incubation time resulted in an increased assay sensitivity, which on the other hand is counter-acted via a decrease in maximal absorbance level.

[Diagram of Figure 61 showing the influence of concentration on I50 and B0 values]

Figure 61. Influence of the concentration of the secondary antibody-enzyme conjugate on the I50 (filled symbols) and B0 (open symbols) values
4.3.2.1.10. **Influence of the chromogen**

Since OPD is frequently applied as a chromogen in peroxidase-based immunoassays, this chromogen was selected initially to conduct the experiments reported. Apart from OPD however, also other chromogens are used, such as ABTS. Saunders (1979) intensively studied the applicability of this chromogen and in fact preferred its use above OPD. Therefore its use was considered as well in the present study. In Figure 63, two competition curves are represented using respectively OPD and ABTS as a chromogen.

It is obvious that maximal absorbance levels using ABTS were considerably lower (mind the difference in two absorbance scales!). On the other hand, lower blanc readings were observed as well. Therefore, the lower maximal absorbance level was not considered as a major problem if ABTS was used as a substrate. It should be noted as well that in other experiments, using another batch of ABTS, higher maximal absorbance levels (up to 0.6) were observed, without any effect on the blanc readings.

As can be observed, the competition curve obtained using ABTS as a chromogen is slightly shifted to the right compared to OPD. This is reflected as well in the estimated $I_{50}$ values: $2.40 \mu M \pm 0.19$ for ABTS and $11.36 \mu M \pm 0.67$ for OPD (95% confidence level). Possibly, the observed improvement in
assay sensitivity is due to the lower blanc absorbance levels if ABTS is used as a chromogen. Also in other experiments in which higher maximal absorbances were obtained, significant improvement in assay sensitivity was obtained if ABTS was used as a chromogen instead of OPD. Therefore, OPD was replaced by ABTS during the course of this study as a chromogen.

![Graph](image)

Figure 63. Influence of the chromogen on the assay performance. (■,□ : OPD, left absorbance scale; ♦,◊ : ABTS, right absorbance scale; filled symbols refer to net absorbance readings, open symbols refer to blanc absorbance readings)

### 4.3.2.2. Specificity of the indirect competitive ELISA

The specificity of the assay was evaluated for a number of compounds. Structural analogues, of which several are allowed to be used within the EU for the production of plastic food contact materials, were included. In addition some other phenolic compounds of which some are known to be xeno-estrogenic (nonylphenol 82, butylhydroxyanisol 92) were studied. Apart from these phenolic compounds however, also some other well known xeno-estrogenic compounds (dibenzyl phthalate 2, butyl benzyl phthalate 3, BADGE 4) present in food contact materials were studied. Results obtained for immunoglobulins isolated from one particular egg (chicken 2, day 42) are summarized in Table 39.
As can be observed from this table, closely structural related compounds show cross reactivity: Cumylphenol 73 and 3,4’-isopropylidene-diphenol 77 showed respectively about 40 and 30 % of cross reactivity. Taking into account that only minor structural differences between these compounds and the target molecule exist (removal of one hydroxyl group in the case of cumylphenol 73 and the different position of one hydroxyl group on the phenolic ring structure for 3,4’-isopropylidene-diphenol 77), it is rather surprising to see that such small molecular differences are detected by the immunoglobulins. Probably the selection of the hapten, used for the production of the immunizing conjugate, could be the reason for the fairly high cross reactivity observed for cumylphenol 73. It is surprising in this regard that for 4-hydroxydiphenylmethane 72 (replacement of one hydroxyl group with one methyl group with respect to bisphenol A) a strong reduction in cross reaction was detected. Possibly the higher apolar character of this compound could be an explanation for this phenomenon. It should be noted in this respect that the cross reactivity observed could be an advantage as well. Probably, molecules with a strong structural similarity as bisphenol A 1, could have a similar toxicological profile. Therefore, the detection of these analogues could be of interest from a food safety point of view as well.

As can be observed, removal of one of the central methyl groups (4, 4’-ethylidenebisphenol 71), reduced cross reactivity drastically up to almost 20 %, while total removal of these central methyl groups (bis-(4-hydroxyphenyl)-methane 70) resulted in even lower molecular recognition. Replacement of the methyl groups with other functional groups gave similar results (2,2-bis-(4-hydroxyphenyl)-perfluoropropane 74; 4,4’-(1-phenylethylidene)bisphenol 75; 4,4’-cyclohexylidenebisphenol 85; 4,4’-dihydroxybiphenyl 88; 4, 4’-dihydroxybenzophenon 89; bis-(4-hydroxyphenyl)-sulphone 90). This indicates that the central methyl groups play an important role in the immunochemical recognition reaction. This is an important observation since the hapten used for antigen synthesis was selected in such a way that these central methyl groups were both present.

Bisphenol A analogues in which the two phenolic groups are separated with a supplementary aromatic ring (4,4’-(1,4-phenylene-diisopropylidene)-bisphenol 86; 4,4’-(1,3-phenylene-diisopropylidene)bisphenol 87), did show some limited cross reactivity. This could not be due to the recognition of one single phenolic group, since cross reactivity versus phenol 78 could not be observed. If on the other hand other functionalities are introduced in the phenolic structure, some molecular recognition seemed to occur, taking into account the results observed for 4-butylphenol 81. Presumably, the presence of an apolar group in combination with the phenolic moiety enhances immunological reactivity. If this apolar group became more voluminous, like in the case of
nonylphenol 82, or smaller, like in the case of the cresols 79 and 80, cross reactivity was reduced again. Therefore, probably butylhydroxyanisol 92 showed some unexpected appreciable cross reactivity. It should be noted in this respect as well that both nonylphenol 82 and butylhydroxyanisol 92 are reported to be xeno-estrogenic compounds, like bisphenol A. Replacement of the apolar moiety with a supplementary hydroxylgroup (dihydroxy benzenes 83 and 84) resulted in low cross reactivity as well. Replacement of the phenolic functionality as such by other groups (benzylalcohol 91, benzoic acid 93) gave similar results.

Other xeno-estrogenic compounds, that can be present in plastic food contact materials, such as the studied phthalates could not be recognized by the immunoglobulins. This clearly indicates that no link between the xeno-estrogenic character and the immunological recognition is present. This is in correspondence with the data observed for another xeno-estrogenic compound, BADGE, which shows some structural similarities with bisphenol A.

Cross reactivity for a selected number of compounds (70, 71, 78, 88, 89, 92) was also studied for eggs from the same chicken isolated on other days. No significant differences could be observed with the data reported in Table 39 (not shown). This does not seem surprising since the immunoglobulins originated from the same chicken. Therefore the same compounds were evaluated with respect to the immunoglobulins isolated from an egg of chicken 5 (day 45). Again, comparable levels of cross reactivity were obtained: bis-(4-hydroxyphenyl)-methane 70 (2 %); 4, 4’-ethylidenebisphenol 71 (19 %); butylhydroxyanisol 92 (14 %).

As a conclusion, the presented assay can be considered as very specific, although for some structural analogues important cross reactivity was observed. Moreover, it seems that comparable levels of cross reactivity were observed irrespective of the data of egg production or of the chicken producing the egg.
Table 39. Cross reactivities of the immunoglobulins isolated from the egg of day 45, from chicken 2

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Name</th>
<th>Cross reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₂,R₃ = CH₃, R₁,R₄ = OH</td>
<td>bisphenol A 1</td>
<td>100</td>
</tr>
<tr>
<td>R₂,R₃ = H, R₁,R₄ = OH</td>
<td>bis-(4-hydroxyphenyl)-methane 70</td>
<td>5</td>
</tr>
<tr>
<td>R₂= CH₃, R₃ = H, R₁,R₄ = OH</td>
<td>4, 4′-ethylidenebisphenol 71</td>
<td>18</td>
</tr>
<tr>
<td>R₁,R₂,R₃ = H, R₄ = OH</td>
<td>4-hydroxydiphenylmethane 72</td>
<td>3</td>
</tr>
<tr>
<td>R₁=H, R₂,R₃ = CH₃, R₄ = OH</td>
<td>4-cumylphenol 73</td>
<td>43</td>
</tr>
<tr>
<td>R₂,R₂ = CF₃, R₁,R₄ = OH</td>
<td>2,2-bis-(4-hydroxyphenyl)-perfluoropropane 74</td>
<td>3</td>
</tr>
<tr>
<td>R₂= CH₃, R₃ = C₆H₅, R₁,R₄ = OH</td>
<td>4,4′-(1-phenylethylidene)bisphenol 75</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>R₁, R₃,R₅,R₇ = CH₃, R₂,R₆ = OH, R₄ = H</td>
<td>4,4-isopropylidene bis(2,6-dimethylphenol) 76</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>R₁, R₂,R₃,R₅,R₇ = H, R₄,R₆ = OH,</td>
<td>3,4′-isopropylidene-diphenol 77</td>
<td>32</td>
</tr>
<tr>
<td>R₁, R₂ = C₄H₉, R₃ = C₆H₅</td>
<td>di-n-butyl phthalate 2</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>R₁= C₄H₉, R₂ = C₆H₅</td>
<td>butyl benzyl phthalate 3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Chemical structure</td>
<td>Name</td>
<td>Cross reactivity (%)</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>R₁, R₃ = H</td>
<td>phenol 78</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>R₁, R₂ = H, R₃ = CH₃</td>
<td>p-cresol 79</td>
<td>0.5</td>
</tr>
<tr>
<td>R₂, R₃ = H, R₁ = CH₃</td>
<td>m-cresol 80</td>
<td>0.5</td>
</tr>
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<td>R₁, R₃ = H, R₂ = sec butyl</td>
<td>4-butylphenol 81</td>
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</tr>
<tr>
<td>R₁, R₃ = H, R₂ = C₉H₁₉</td>
<td>4-nonylphenol 82</td>
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</tr>
<tr>
<td>R₁, R₃ = H, R₂ = OH</td>
<td>1,4-dihydroxybenzene 83</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>R₂, R₃ = H, R₁ = OH</td>
<td>1,3-dihydroxybenzene 84</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>4,4’-cyclohexylidenebisphenol 85</td>
<td>3</td>
</tr>
</tbody>
</table>
Use of Bisphenol A antibodies in enzyme-linked immunosorbent assays

Table 39. continued

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Name</th>
<th>Cross reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Chemical structure" /></td>
<td>4,4’-(1,4-phenylene-diisopropylidene)-bisphenol</td>
<td>86</td>
</tr>
<tr>
<td><img src="image2" alt="Chemical structure" /></td>
<td>4,4’-(1,3-phenylenediisopropylidene)bisphenol 87</td>
<td>0.5</td>
</tr>
<tr>
<td><img src="image3" alt="Chemical structure" /></td>
<td>4,4’-dihydroxybiphenyl 88</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td><img src="image4" alt="Chemical structure" /></td>
<td>4, 4’-dihydroxybenzophenon 89</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Chemical interactions between packaging materials and foodstuffs
<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Name</th>
<th>Cross reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="bis-(4-hydroxyphenyl)-sulphone" /></td>
<td>bis-(4-hydroxyphenyl)-sulphone 90</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td><img src="image2" alt="benzylalcohol" /></td>
<td>benzylalcohol 91</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td><img src="image3" alt="butylhydroxyanisol" /></td>
<td>butylhydroxyanisol 92</td>
<td>10</td>
</tr>
<tr>
<td><img src="image4" alt="benzoic acid" /></td>
<td>benzoic acid 93</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td><img src="image5" alt="bisphenol A diglycidyl ether (BADGE)" /></td>
<td>bisphenol A diglycidyl ether (BADGE) 4</td>
<td>2</td>
</tr>
</tbody>
</table>

*Chemical interactions between packaging materials and foodstuffs*
4.3.2.3. Application of the indirect competitive ELISA for dairy emulsions

Because polycarbonate bottles can be re-used, their application for the packaging of pasteurised milk increases. The use of polycarbonate baby-bottles becomes also more popular than the use of the traditional glass bottles, because of their superior physical properties. However, due to the washing of the bottles, a partial degradation of the polymer can not be excluded. As indicated in the first chapter, an accelerated migration of bisphenol A from the bottle could occur in these circumstances. Therefore it would be interesting to have a tool which is able to check the migration immediately in the food of interest, which in this particular case is milk. Current analytical methods which are available for bisphenol A analysis would require extensive extraction and clean-up steps to tackle this analytical problem as shown in chapter 1. Since milk is an aqueous liquid, it would be possible to apply it directly in the presented assay. This would be a major advantage compared to the traditional analytical techniques.

Initial experiments on whole reconstituted milk revealed a strong reduction in assay quality (increased $I_{50}$ and reduced $B_0$). Because comparable results were obtained for skimmed reconstituted milk, the reduced assay performance could not solely be due to an absorption of bisphenol A by the fat globules rendering it unavailable for immunochemical reactions (results not shown).

Since milk contains an appreciable amount of salts, the ionic strength of the competition buffer was adjusted because of the earlier observations with regard to the influence of ionic strength on the assay performance. From Figure 64, it is obvious that at the ionic strengths of the competition buffer, which are comparable to those normally used if aqueous bisphenol A solutions are analysed, reduced $B_0$ and increased $I_{50}$ levels were obtained, confirming the results mentioned above. By lowering the ionic strength of the competition buffer, competition curves are gradually shifted towards the competition curve observed for aqueous bisphenol A solutions. Consequently, the assay performance could be influenced in such a way that the inhibition curves obtained in milk were almost similar to those obtained in water.

In a supplementary experiment, the difference between milk samples with a varying fat content was evaluated (skimmed, semi-skimmed, whole milk). From Figure 65 it is clear that no differences between the semi-skimmed and whole milk could be observed, while the differences with the skimmed milk were very small. In fact, no significant differences in $I_{50}$ levels were observed for all three samples (26.04 ± 1.84 µM; 23.92 ± 1.32 µM; 26.60 ± 1.38 µM; for skimmed, semi-skimmed and whole milk respectively). Consequently it can be concluded that the reduced assay sensitivity compared to the assay in water can not be due to the absorption of the bisphenol A by the fat globules. Since bisphenol A is an apolar compound it can be expected it is indeed partially absorbed.
by the fat. Therefore the immunglobulins seems able to interact at the water-oil interface inducing immunoochemical reaction the bisphenol A molecules.

Figure 64. Competition curves obtained in skimmed milk at selected ionic strengths of the competition buffer (aqueous bisphenol A solution: ◊ = 700 mM; bisphenol A in milk: • = 140 mM; □ = 220 mM; △ = 300 mM; ▲ = 400 mM; ■ = 600 mM)

The observed differences between the competition curves obtained in milk or in water can be attributed to the complex sample matrix. Proteins or specific interactions with minerals like iodine, as illustrated in a previous paragraph (4.3.2.1.5), could be responsible for the observed differences.

It can be concluded that the assay can be applied for the direct analysis of milk samples, without any need of sample pre-treatment. Because of the observed matrix effects however, it should be realised that a calibration with a sample of similar composition is necessary. Moreover it is clear that due to these matrix effects, assays sensitivity decreases.
4.3.2.4. Application of the indirect competitive ELISA for fatty foods

From the first chapter it could be concluded that migration of bisphenol A is especially important in fatty food matrices. Therefore the applicability of the assay for the quantification of bisphenol A in lipids was investigated. Of course, the lipid extract or the oil can not be used as such in the assay. A draft CEN procedure was prepared for the quantification of bisphenol A in oil. This method is based on an extraction of bisphenol A from the oil using an aqueous methanol solution (50 % v:v) and subsequent HPLC-UV analysis (Franz and Rijk, 1996). If the aqueous methanolic extract could be applied in the assay as such, the instrumental analytical procedure in the CEN method could be avoided. Therefore the assay characteristics were evaluated as a function of the methanol concentration in the competition buffer (Figure 66).

The presence of methanol during the competition step resulted in a significant concentration dependent decrease in the B₀ and I₅₀ levels. At the highest methanol concentration tested, high assay variability was observed as well. This is in correspondence with the results obtained by Abad and Montaya (1997) who investigated the influence of several organic solvents on the characteristics of a carbaryl assay. Probably the denaturing effect of the alcohol on proteins is responsible for these effects.
Despite of the observed effects, the presence of about 10% of methanol resulted in an I_50 value which is still lower compared to those obtained in optimal conditions in milk. Therefore such methanol concentrations are considered to be acceptable and moreover, the dilution necessary to reach this level remains restricted. Taking into account the sensitivity loss due to the dilution of the aqueous methanolic extract and the sensitivity loss due to the presence of methanol, even higher concentrations could be tolerated. Consequently, the presented assay could be applicable for the analysis of lipidic matrices, again taking into account that the overall sensitivity of the assay is rather restricted.

4.3.3. Conclusions

An indirect competitive enzyme-linked immunosorbent assay could be developed using the antibodies isolated from the eggs of BSA-bisphenol A immunized chickens. The sensitivity of the assay was however lower then could be expected from other immunoassays in general and from assays recently developed for bisphenol A analysis (Kodeira et al., 2000; Nishii et al., 2000; Ohkuma et al., 2002). A step-wise approach was followed to increase the assay sensitivity by varying several assay parameters. Only a slight improvement of the assay sensitivity compared to the first experiments could be obtained. From these experiments, it seemed that care should be taken to
avoid matrix dependent effects on assay performance (e.g. salt effects, surface active agents).
Despite the fact that assay sensitivity was lower than expected, the specificity of the assay to various bisphenol A like molecules, which could migrate from food contact materials was evaluated. In addition, also some other phenolic compounds and xeno-estrogenic compounds were investigated. Generally, the assay could be considered to be very specific.
Finally, the applicability of the assay for the analysis of relevant food samples was investigated. Only a slight decrease in assay sensitivity was observed if the assay was applied for the direct analysis of milk. For fatty foods, a simple extraction with an aqueous methanol solution followed by a restricted dilution of the extract to reduce the methanol concentration, similar results could be obtained. Both examples illustrate the potency of the developed assay.
5. Interactions with active and intelligent packaging materials

5.1. Introduction

In order to meet ever more extensive consumer demands and market trends with regard to food and food products, new technologies have been introduced over the years in the food industry. Probably the most important recent development in the area of food contact materials are the so-called active and intelligent packaging systems. Active food packaging can be defined as a material which changes the condition of the packed food to extend its shelf-life and/or improve its safety and its sensory properties. Intelligent packaging materials monitor the condition of the packed food to give information about its quality during its distribution (De Kruijf et al., 2002).

Various kinds of active packaging systems exist and have been recently reviewed by Vermeiren et al. (1999) : oxygen, ethylene or carbon dioxide scavengers; moisture regulators; anti-microbial packaging systems; antioxidants releasers etc. In essence, two important classes can be distinguished: active scavenging systems or absorbers, removing undesired compounds, and active releasing systems or emitters, intentionally releasing compounds such as preservatives to the food. Commercial available indicators monitoring the food quality include time-temperature, leakage or freshness indicators (Ahvenainen et al., 1999; De Kruijf et al., 2002).

Although both technologies are applied frequently in the USA and Japan, introduction within the European market remains restricted. The main reason for their restricted application is the lack of a suitable legislative framework. In addition, no extensive information about consumer and trade acceptability and their environmental impact is available. Therefore in 1999, an EU funded study was started within the framework of the EU FAIR R&D programme, called ‘ACTIPAK’ (CT 98-4170). The project dealt with the evaluation of the safety, effectiveness, economic and environmental impact and consumer acceptance of active and intelligent packaging materials. The project, in which nine research organisations and three industrial partners participated, was coordinated by TNO, Nutrition and Food Research, The Netherlands.

Essentially, the five following objectives could be distinguished within the project:

- review and collect active and intelligent packaging systems
- classify the collected packaging materials based on their composition and their overall migration behaviour
- evaluate the effectiveness, shelf-life extending capacity and microbiological safety of the
collected materials
- evaluate the toxicological, environmental and economic aspects of the collected systems
- draft some recommendations for future legislative amendments with regard to active and intelligent packaging materials.

Basically, the contribution relevant to the presented study, was concentrated on the second and fifth task of the project. It included the following aspects:
- evaluation of the composition of the collected active and intelligent packaging materials
- overall migration studies using official food simulants
- overall migration and specific migration studies using alternative food simulants
- classification of the collected active and intelligent packaging systems
- reflection on the use of active packaging systems taking into account other relevant legislation, with special emphasis on the food additive legislation.

5.2. Materials and Methods

5.2.1. Samples
The active and intelligent packaging systems were obtained from their producers. An overview of the different systems is given in Table 40.

5.2.2. Migration testing with official simulants
Overall migration in volatile simulants (water, 3\% (v:v) acetic acid, 10-15 \% (v:v) ethanol, 95 \% (v:v) ethanol and iso-octane) was measured by contacting the system with the simulants as indicated in Table 40. Stainless steel migration cells were used if possible, although some films were filled as pouches or totally submerged. Sachets were submerged as well in a glass container filled with simulant, which was covered with a glass plate during incubation to avoid excessive evaporation. For sample OS-3, glass bottles filled with simulant were closed by the crown cap and turned upside down during incubation.

For the overall migration in olive oil or sunflower oil, samples were placed in stainless steel migration cells, except sample OS-3, which was filled with olive oil as such. After incubation during 10 days at 40°C, the relevant CEN methods were followed as close as possible.

Overall migration tests for three indicators were carried out by total immersion of the sachet (OI1), by using a one-side test on a especially prepared indicator with a large area (OI2) and by total immersion of two indicators stuck together (CDI).
Other relevant contact conditions (time-temperature combinations) are specified in Tables 42 and 43.

All the data represented are the average of at least three measurements. For the statistical evaluation a 95% confidence interval was applied.

5.2.3. Migration testing with alternative simulants

Samples
Samples were selected on basis of the results of the migration studies using the official aqueous and acidic food simulants. Two iron-based oxygen absorbing systems filled with a powder were considered (OS 1-2). For the moisture regulators, a film containing a moisture absorbing agent (MR-4), a sachet filled with granules (MR-1) and a pad filled with a fibrous material (MR-3) were investigated.

Preparation of the simulant
Agar gel (1-2%, w:w) was prepared by mixing the appropriate amount of agar (Bacteriological agar, n°1, Oxoid, England) with 500 mL of distilled water. An appropriate amount of water was heated up to boiling temperature and was subsequently added to the agar dispersion previously prepared. This mixture was further heated until the solution was clear (near to boiling temperature). Care should be taken to avoid excessive foaming during boiling of the agar solution and to avoid burning of the agar if the highest concentration is used. After a clear solution was obtained, an appropriate amount of acetic acid was added if necessary (3% v:v). If sodium chloride was added to the gel, brine (15% w:v) was used instead of distilled water to prepare the agar.

Once the liquid agar solution is prepared, it is cooled down to about 50 °C. It should be noted that at these temperatures agar is rather viscous and tends almost to solidify. If solidification of the gel would occur before it is applied, gentle reheating may liquefy the gel again.

Different ways to apply the agar gel as a simulant for migration testing are possible.

Migration studies in cell (Moisture absorbing film, MR-4)
The assembled migration cell (TNO, The Netherlands) is weighed before the agar is applied (typically about 1800 g). Afterwards, the cell is filled with agar (typically about 100 g), without application of the packaging material. After solidification of the agar, the cell is weighed again, in order to quantify the amount of agar. Subsequently the cell is opened to apply the packaging material. After incubation, the active packaging material is removed and the cell together with the agar are weighed again, to quantify the remaining agar. Alternatively, the cell can be filled with liquid agar in the presence of the packaging material as well. This approach is not recommended if migration tests at refrigeration temperatures are conducted. Again the amount of agar before and
after the migration test should be quantified. For the migration experiment in meat, a fresh meat sample (steak, Belgian beef, lean meat, about 100 g) is cut into a suitable piece (about 7×7 cm and 2 cm thickness) or is minced. Subsequently, the meat is placed on the film which is applied in a migration cell. After application of the meat, the cell is closed firmly.

Agar was liquefied by adding an equal volume of water to the gel and mixing it. The obtained liquid was further purified and extracted to allow quantification of the compound considered. For the migration to meat, the recovered sample was homogenised in a mixer and a sub-sample was taken for further clean-up and analysis.

Migration experiments were all performed at least in triplicate and every migrate was analysed in duplicate. For the statistical evaluation a 95 % confidence interval was applied.

*Overall migration experiments for other systems*

Liquid agar was pored over the desired active packaging systems. Alternatively, the agar can be pored in a PE box and after solidification, agar slices (about 10×15 cm and 1 cm thickness) are cut from it. Afterwards, the system can be sandwiched between two slices. The latter approach is recommended if migration tests at refrigeration temperatures are performed or if the material absorbs the liquid simulant, like for example via pores.

Reported results are the average of at least triplicate measurements and a 95 % confidence interval was again considered.

*Dry matter content*

Dry matter content of the agar was determined in triplicate by conventional oven drying. An appropriate amount of agar was mixed with dried sea sand in a aluminium dish at 100-105°C until constant weight was reached.

*Overall migration calculation*

Overall migration was calculated as indicated in Figure 68.

*Rhodamine B migration from MR-3.*

The saturation level of MR-3 was determined by submerging the material in distilled water during 30 min followed by a removal of the excess of distilled water by gravity in a funnel and weighing the amount of absorbed water (approximately 15 g/pad).

Pads were spiked with 6 mg of rhodamine B (+99%, Acros, Belgium) using an aqueous solution (2 mg.mL⁻¹). This was accomplished by injection of the rhodamine B solution through the perforated side of the pad using a syringe and a stainless steel needle. In order to reach higher saturation levels, the rhodamine B solution was diluted with water in such a way that the amount of chromogen added to each pad was the same and the desired saturation degree was reached.
Pads were contacted with sliced agar gels (1% w:w) at the indicated temperature. Afterwards, agar gels were mixed and 1 g of agar was dispersed in 50 ml of water and further diluted till a final volume of 100 ml. Rhodamine B was determined by flow injection analysis in a Gilson 122 Fluorometer (USA) using an excitation wavelength of 540 nm and an emission wavelength of 650 nm. Spiked agar gels were used for calibration purposes and readings were corrected for blancs. Extensive flushing of the detection system and the pump with hot water (60°C) is recommended after the analysis.

Migration tests were carried out in triplicate and each migrate was analysed in duplicate. A 95 % confidence interval was considered.

Water activity measurements

Water activity at room temperature was measured using a Novasina Thermoconstantér (Switzerland). The sample was introduced in the apparatus and the reading of the water activity was performed upon reaching equilibrium conditions.

5.3. Results and discussion

5.3.1. Compositional analysis

Several active and intelligent packaging systems were collected, based on a number of criteria including their availability, their functionality and their working mechanism. In addition, priority was given to systems produced within the EU or by the sponsors of the project.

In Table 40 the collected systems are summarized, together with their function, layout and the way the migration tests were performed (see paragraph 5.3.2.).

A first remarkable observation is the complexity of the layout of some of the collected systems. Various systems consisted of multilayered materials such as metallic crown caps with an internal plastic liner or sachets which basically consisted of perforated paper sheets coated with plastic such as polyethylene. In addition, these sachets are filled with various powdery or fibrous substances. These latter sachet-type systems are most frequently added additionally to the packed food, which compared to systems in which the active system is incorporated in the film is a major disadvantage, because in such a way, they do not enhance user-friendliness.

In addition however, this observation is important from a legislative point of view as well, since European plastic food contact material legislation refers only to those systems which are solely composed of plastics, as indicated in the first chapter of this work. Therefore, if legislation is strictly applied, only a part of the collected systems are subjected to this most detailed EU legislation with
regard to food contact materials. Consequently it is obvious that a serious lack in the current legislation on food contact materials exists with regard to these new type of packaging materials. Since the directives on plastic contact materials are the most detailed, they were considered as an adequate tool to evaluate the composition of the various packaging materials studied.

Table 40. Overview of the collected active and intelligent packaging systems

<table>
<thead>
<tr>
<th>Code</th>
<th>Use</th>
<th>Layout</th>
<th>Migration layout</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS1-2</td>
<td>oxygen scavenger</td>
<td>sachet or plastic cap filled with powder</td>
<td>total immersion</td>
</tr>
<tr>
<td>OS3</td>
<td>oxygen scavenger</td>
<td>crown cap with liner</td>
<td>one sided contact</td>
</tr>
<tr>
<td>OS4</td>
<td>oxygen scavenger</td>
<td>film</td>
<td>migration cell</td>
</tr>
<tr>
<td>OS5</td>
<td>oxygen scavenger</td>
<td>film</td>
<td>pouch, one sided</td>
</tr>
<tr>
<td>ES1-2</td>
<td>ethylene scavenger</td>
<td>film or sachet filled with granules</td>
<td>total immersion</td>
</tr>
<tr>
<td>MR1-2</td>
<td>moisture regulator</td>
<td>sachet filled with granules</td>
<td>total immersion</td>
</tr>
<tr>
<td>MR3</td>
<td>moisture regulator</td>
<td>pad filled with fibrous material</td>
<td>total immersion</td>
</tr>
<tr>
<td>MR4</td>
<td>moisture regulator</td>
<td>film containing moisture absorber</td>
<td>total immersion</td>
</tr>
<tr>
<td>AMP1</td>
<td>anti-microbial system</td>
<td>sachet containing anti-microbial agent</td>
<td>total immersion</td>
</tr>
<tr>
<td>AMP2-5</td>
<td>anti-microbial system</td>
<td>film containing anti-microbial agent</td>
<td>migration cell</td>
</tr>
<tr>
<td>AMP6</td>
<td>carbon dioxide releaser</td>
<td>film</td>
<td>total immersion</td>
</tr>
<tr>
<td>OA1</td>
<td>aroma releasing film</td>
<td>film</td>
<td>migration cell</td>
</tr>
<tr>
<td>OA2</td>
<td>aldehyde absorbing film</td>
<td>film</td>
<td>migration cell/pouch</td>
</tr>
<tr>
<td>TTI1-3</td>
<td>time temperature indicator</td>
<td>label</td>
<td>total immersion</td>
</tr>
<tr>
<td>OI1-2</td>
<td>oxygen indicator</td>
<td>sachet</td>
<td>total immersion</td>
</tr>
<tr>
<td>OI1-2</td>
<td>oxygen indicator</td>
<td>label</td>
<td>migration cell*</td>
</tr>
<tr>
<td>CDI</td>
<td>carbon dioxide indicator</td>
<td>label</td>
<td>total immersion</td>
</tr>
</tbody>
</table>

* specially prepared label

As indicated before, European legislation with regard to food contact materials in general and to
plastics in particular is based on compositional restrictions because so-called positive lists are specified (paragraph 1.2.5.3.1). Therefore compositional analysis of the collected active and intelligent packaging systems is of prime importance, although some of them are composed of various materials such as plastics, paper, metal, etc. Based on such analysis, the materials can be evaluated with regard to the compositional restrictions specified in the legislation. In such an evaluation it seemed obvious to consider only those ingredients, which are responsible for the active and intelligent characteristics of the package. These are most probably difficult to replace because of their functionality in contrast to the other non-functional components. Of course, the non-functional ingredients should at the end comply as well with the legislative specifications.

For those intelligent systems which are applied on the outer side of the food package (e.g. time temperature indicators), the migration of the indicators active substances is strongly influenced by the food package on which the material is attached to. As a matter a fact, this food package may act as a functional barrier. Consequently, migration out of such systems is considered of less importance.

Apart from the positive lists specified in the European food contact material legislation, also other legislative restrictions should be considered for the evaluation: food additive, pesticide, hygiene, labelling, etc. (Fabech et al. 2000). Taking especially the food packaging and food additive legislations in mind, the classification as outlined in Table 41 for the active packaging systems is presented with regard to their composition. Considering this table and the food additive legislation however, it should be realised that both restrictions towards the use of the food additive and its concentration are specified. So consequently similar restrictions should be considered for active food packaging materials releasing food additives. Furthermore it should be noted that the use of additives in foodstuffs should be reported on the package. It seems logic that a similar declaration is foreseen for active releasing packaging materials as well. In addition to these aspects also some others should be considered if the system should comply with food additive legislation. These will be discussed in more detail elsewhere (paragraph 5.3.5).

For some of the active systems collected, no identification of the functional ingredient was possible, presumably because it was incorporated in the polymer structure. Consequently its extraction and identification was rendered impossible. In these cases, the compounds in the systems were supposed to be in agreement with the legislation because the contrary could not be concluded from the producers’ information.
Table 41. Classification of active food packaging systems with regard to their composition

<table>
<thead>
<tr>
<th>System</th>
<th>Agreement with list of food contact materials or food additives* or equivalent</th>
<th>No agreement with list of food contact materials and food additives</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>iron, sulphite, sodium chloride, silica polymeric scavenger</td>
<td>permanganate</td>
</tr>
<tr>
<td>ES</td>
<td>zeolite</td>
<td></td>
</tr>
<tr>
<td>MR</td>
<td>silicates, cellulose, sugar derivatives combined with a semi permeable film</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>ethanol, acids, zinc, silver, antimicrobial protein carbon dioxide releaser</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>colorants, volatiles</td>
<td></td>
</tr>
</tbody>
</table>

*food additives can only be used in a restricted amount of foodstuffs

One of the systems seemed to contain permanganate. Serious questions about its use in the presence of foodstuffs can be raised. Although human and animal studies indicate that inorganic manganese compounds have very low acute toxicity by any route of exposure (Pisarczyck, 1995), lethal consequences of oral ingestion of potassium permanganate are described (Young et al., 1996). Previously, Reides (1990) reported an LD_{50} for humans amounting 143 mg.kg\(^{-1}\). The risk however of residual permanganate is most probably negligible because of the strong oxidizing character of the compound. All the migrated permanganate might have reacted with the organic compounds present in the foodstuff. This could be a more serious consequence of the permanganate migration since essential nutrients such as vitamins, amino-acids, (poly)unsaturated fatty acids etc., may be oxidized.

For some of the positively classified active systems, supplementary explanation is necessary. For the iron containing oxygen scavenging systems it should be noted that iron oxide is in the positive list of plastics, together with alloys of iron and other metals such as tin, bronze and cupper. Moreover, tin coated steel plate has been a major food packaging material for decades. For those materials included in the positive lists, a specific migration limit for iron is moreover specified, except for iron oxide (48 mg iron.kg\(^{-1}\)). For all these materials, including the active systems containing iron powder as such, iron will be a major migrating substance in particular foods. Therefore the presence of iron powder in the packaging materials tested, is not supposed to be in conflict with the legislation.
Despite this fact, concerns about increased concentrations of the pro-oxidant ferrous ions in food could be put forward. Sodium chloride and silicate are classified as active components as well, since chloride is reported to catalyse the oxygen scavenging properties of powdered iron and silica improved the oxygen removal rates by the iron, due to the creation of a local high relative humidity (Klein and Knor, 1990). Both compounds are on the positive lists for plastics.

Sulphite is a well-known food additive for its antioxidant properties apart from its anti-microbial character and its colour enhancement properties. It is not included in the positive list for plastics. Its use as a food additive is restricted because of its toxicity (Davidson and Juneja, 1990; Gould and Russell, 1991).

One of the moisture absorbers contains sugar derivatives. These consist of a mixture of compounds of which some minor ingredients are not included on the plastic packaging positive list. The same analogues are approved however as food additives, although similar minor components are present as well. Considering the food additives legislation and its interpretation, a similar approach for the food packaging legislation could be followed. For the sake of completeness it should be noted that the polymer used in this active packaging material is considered essential as well for its proper function. From the analytical data, it could be concluded that the polymer is composed out of listed monomers.

Although silver is not in the positive list for plastics, its use as a food additive is allowed in for example candies for colouring purposes. The anti-microbial protein is a food additive which can be used in several kinds of foods. In addition to the protein, traces of a compound, making the covalent binding between the film and the protein possible, were detected. The same substance however can be traced back as a technical aid present in enzyme treated food products. Therefore, the composition of the material was supposed to be in agreement with the legislation considered. Finally, the colorants and volatile flavour components detected are also listed as food additives and therefore can be used in some particular foods.

As for the intelligent systems, the oxygen indicators contain a redox dye, a reducing and an alkaline compound. Except for the dye, all ingredients are in the positive lists for plastics or are food additives. It should be noted moreover that one of the systems is not intended to come in direct contact with the food itself, since it is placed in a plastic sachet which is introduced in the food package together with the food. The other oxygen indicator is not in direct contact with the food as well, since it is separated from the food by an oxygen permeable plastic film. The carbon dioxide indicator is an acid-base system, which uses a coloured indicator, which is not on the positive list,
nor is a food additive.

5.3.2. Overall migration studies in official food simulants

As indicated in the first chapter, European legislation with regard to plastic food contact materials, prescribes test procedures to be followed to estimate the migration from a food contact material to food simulants (paragraph 1.2.5.3.3). These test procedures specify the type of food simulant to be used, the contact time and contact temperature. Standard procedures for the estimation of the overall migration have been laid down by CEN. Generally these procedures were followed as close as possible throughout the experimental part, although various materials could not be considered as plastics as already mentioned in paragraph 5.3.1. It should be realised however in this respect that for example in the Dutch legislation similar test conditions as those for plastics on the EU level, apply as well for other contact materials as well, like for example paper. Therefore, the applied test conditions can be considered to be relevant. Moreover, in order to compare the various results with each other, similar tests conditions should be used.

Generally overall migration was tested at a time-temperature combination simulating long-term contact at room temperature (Tables 42-43). It should be realised that some of the tested systems are supposed to be applied at refrigeration temperatures. Again to allow proper comparison between the different systems and to avoid confusion about the data obtained, similar test conditions were selected for all systems. By doing so a worst-case scenario was simulated. For one of the antimicrobial systems, releasing ethanol, the loss of volatiles was not taken into account for the calculation of the overall migration level.

If possible, overall migration data are expressed as amounts per unit of surface (mg.dm⁻²). For a large part of the investigated systems however, this approach was not possible because the contact surface was difficult to measure or was not completely relevant (e.g. sachets or indicators). For these systems the overall migration is expressed as amounts per object (mg/object). For the interpretation of the obtained data with regard to current EU legislation the first approach is straightforward. For the second approach an overall migration limit of 60 mg.kg⁻¹ of packed food can be applied. Even then however, a clear interpretation of the overall migration level remains difficult because for most of the sachets no information is available about the minimal amount of food to be contacted with. Therefore its specification can be considered as essential to evaluate migration levels in an objective manner. In order to allow some evaluation in the framework of the presented research, as a first approach it can be supposed that sachets are used in packages containing a minimal amount of 250 g of food, although in practice lower amounts of food are packed with an active packaging as well.
Thus, the overall migration limit for sachets amounts 15 mg/object. It should be stressed however that in reality a case by case evaluation of these systems will be necessary taking into account the expected amount of food to be contacted with the active packaging object.

For the iron containing oxygen absorbers the high overall migration levels in acetic acid are exceeding the limits. Systems were heavily swollen and brown discoloration of both the system ingredients and the residues could be observed. Due to the excessive swelling of one of the systems, floating of the material in the simulant could not be avoided, explaining the low repeatability of the results reported. It is obvious that these high migration levels were mainly due to ferric compounds formed upon electrolytical dissolution of the iron powder (swelling due to hydrogen gas production) and subsequent exposure to air. Also for the other simulants containing water, high migration levels were observed, although a white instead of a brown residue was obtained after contact. Possibly, sodium chloride was the main constituent of the migration residue. For the other oxygen absorbers not containing iron, overall migration levels were in most cases acceptable. One of the ethylene scavenging materials exceeded the overall migration limit in only one particular type of simulant.

One of the moisture regulating systems (MR-4) showed excessive migration in water containing simulants. The migration residue foamed during the evaporation of the simulants and the residue was sticky, viscous and hygroscopic. After contact the moisture regulating system seemed to have taken up large amounts of simulant as well. After contact with 95 % ethanol, a stiffening of the system was observed. Overall migration limits in a limited amount of simulants were exceeded as well by some of the other moisture regulating systems. For the anti-microbial systems, overall migration levels were generally in agreement with current restrictions. For the ethanol releasing systems however care should be taken in the interpretation of the results reported since the migration of ethanol is not considered. If this migration was kept into account, overall migration levels would be too high. The carbon dioxide releasing material showed high overall migration levels as well, especially in 95% ethanol. In the latter case, this was due to dissolution of the film. Systems AMP2 and AMP5 exceeded overall migration levels in respectively the fatty food and acidic food simulant.
Table 42. Overall migration for selected active packaging materials

<table>
<thead>
<tr>
<th>Code</th>
<th>Unit</th>
<th>water 10d, 40°C</th>
<th>3% acetic acid, 10d, 40°C</th>
<th>15% ethanol, 10d, 40°C</th>
<th>olive oil, 10d, 40°C</th>
<th>iso-octane, 2d, 20°C</th>
<th>95% ethanol, 10d, 40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS1</td>
<td>mg/object</td>
<td>617 ± 32</td>
<td>1707 ± 310</td>
<td>796 ± 39</td>
<td>-</td>
<td>2 ± 1</td>
<td>211 ± 25</td>
</tr>
<tr>
<td>OS2</td>
<td>mg/object</td>
<td>88/38/95</td>
<td>467/447/310</td>
<td>90/79/71</td>
<td>-</td>
<td>1 ± 1</td>
<td>41/30/57</td>
</tr>
<tr>
<td>OS3</td>
<td>mg.dm²</td>
<td>1 ± 0</td>
<td>2 ± 0</td>
<td>2 ± 1*</td>
<td>28 ± 0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OS4</td>
<td>mg.dm²</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
<td>8±3</td>
<td>&lt;3°</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OS5</td>
<td>mg.dm²</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1*</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ES1</td>
<td>mg.dm²</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1*</td>
<td>6 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ES2</td>
<td>mg/object</td>
<td>2 ± 1</td>
<td>4 ± 1</td>
<td>2±1</td>
<td>-</td>
<td>18 ± 1</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>MR1</td>
<td>mg/object</td>
<td>&lt;1</td>
<td>967 ± 133</td>
<td>&lt;1</td>
<td>-</td>
<td>&lt;1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>MR2</td>
<td>mg/object</td>
<td>&lt;1</td>
<td>11 ± 3</td>
<td>&lt;1</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MR3</td>
<td>mg/object</td>
<td>9 ± 1</td>
<td>46 ± 8</td>
<td>7±1</td>
<td>-</td>
<td>18 ± 3</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>MR4</td>
<td>mg/object</td>
<td>5333 ± 189</td>
<td>5945 ± 91</td>
<td>6063 ± 307</td>
<td>-</td>
<td>3 ± 1</td>
<td>168 ± 15</td>
</tr>
<tr>
<td>AMP1</td>
<td>mg/object</td>
<td>&lt;1</td>
<td>4 ± 1</td>
<td>2 ± 0</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AMP2</td>
<td>mg.dm²</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1*</td>
<td>42 ± 4</td>
<td>28 ± 0</td>
<td>-</td>
</tr>
<tr>
<td>AMP3</td>
<td>mg.dm²</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
<td>&lt;1</td>
<td>&lt;1°</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AMP4</td>
<td>mg.dm²</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>6 ± 1</td>
<td>2 ± 0°</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AMP5</td>
<td>mg.dm²</td>
<td>5 ± 1</td>
<td>24 ± 3</td>
<td>8 ± 1°</td>
<td>7 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 42 continued.

<table>
<thead>
<tr>
<th>Code</th>
<th>Unit</th>
<th>water 10d, 40°C</th>
<th>3% acetic acid, 10d, 40°C</th>
<th>15% ethanol, 10d, 40°C</th>
<th>olive oil, 10d, 40°C</th>
<th>iso-octane, 2d, 20°C</th>
<th>95% ethanol, 10d, 40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP6</td>
<td>mg.dm⁻²</td>
<td>11 ± 1</td>
<td>20 ± 4</td>
<td>37 ± 10*</td>
<td>73 ± 2</td>
<td>&lt;1</td>
<td>(909 ± 34)</td>
</tr>
<tr>
<td>OA1</td>
<td>mg.dm⁻²</td>
<td>56 ± 3</td>
<td>61 ± 3</td>
<td>62 ± 1*</td>
<td>19 ± 4°</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OA2</td>
<td>mg.dm⁻²</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1*</td>
<td>2 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

# - = not determined
* 10 % ethanol was used instead as a simulant
° sunflower oil was used instead as a simulant

Table 43. Migration behaviour of the gas indicators.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Unit</th>
<th>Water 10d, 40°C</th>
<th>Acetic acid 3% 10d, 40°C</th>
<th>Ethanol 15% 10d, 40°C</th>
<th>Ethanol 95% 10d, 40°C</th>
<th>Olive oil 10d, 40°C</th>
<th>Iso-octane 2d, 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>OI1</td>
<td>mg/object</td>
<td>1 ± 1</td>
<td>&lt;1</td>
<td>3 ± 2</td>
<td>4 ± 1</td>
<td>-#</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>OI2</td>
<td>mg.dm⁻²</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
<td>1 - 3</td>
<td>-</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>CDI</td>
<td>mg/object</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1*</td>
<td>6 ± 1</td>
<td>-</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

# - = not determined
* 10 % ethanol was used instead as a simulant
Interactions with active and intelligent packaging materials

For the flavour and aroma releasing system (OA-1), loss of its colour was observed if contacted with water containing simulants. Overall migration limits were nevertheless also exceeded in fatty food simulants.

Again special care should be taken if the overall migration levels of releasing systems are evaluated. Apart from the restrictions specified in the food packaging legislation, food additive legislation should be respected as well. This implies that specific additives can be used in specific foods, as already emphasized previously. In addition however, maximal additive concentrations should be respected as well. If due to the specific migration of a food additive, the overall migration limits are exceeded, the overall migration could be corrected for the migration of the active compound released, if the food additive legislation is still respected. The corrected overall migration level should of course then be in compliance with the current limit (Figure 67). This principle however may not be used to circumvent the actual overall migration limit. Therefore it could be stipulated that the overall migration limit can be corrected for the migration of food additives if indeed these compounds exhibit their activity in the selected foodstuff. Of course, this proposal should not be applied if the active component is not a food additive nor a food ingredient. Neither it should be applicable for active components that are not intended to be released from the active system (e.g. sodium chloride in oxygen absorber). Again, additional reflections with regard to possible conflicts with food additive legislation will be discussed in more detail later (paragraph 5.3.5).

**Figure 67. Schematic representation of a possible alternative to calculate overall migration from active releasing food packaging systems**

Summarizing, Table 44 shows a classification of the studied active packaging systems according to their overall migration behaviour. It can be observed that only a minority of the tested systems fulfilled the current overall migration limit in all official food simulants. Other systems did only
Interactions with active and intelligent packaging materials

exceed the limit in a few simulants. It should also be noted that both releasing and scavenging active packaging systems exceeded the overall migration limit. Considering their functionality, this was quite surprising for the scavenging systems.

Table 44. Classification of the active systems according to their overall migration behaviour in the official EU food simulants (A = water, B = 3% acetic acid, C = 10-15% ethanol, D = olive oil or equivalents) (based on data shown in Tables 42-43)

<table>
<thead>
<tr>
<th>Overall migration &lt; 10 mg.dm² or equivalent</th>
<th>Overall migration &gt; 10 mg.dm² or equivalent in indicated simulants</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS4-5</td>
<td>OS1-2 (A,B,C,D)</td>
</tr>
<tr>
<td>ES-1</td>
<td>MR4 (A,B,C,D)</td>
</tr>
<tr>
<td>MR-2</td>
<td>AMP1&amp;6 (A,B,C,D)</td>
</tr>
<tr>
<td>AMP3-4</td>
<td>OA1 (A,B,C,D)</td>
</tr>
<tr>
<td>OA2</td>
<td>OS3 (D); ES2 (D); MR3 (B,D); MR1 (B); AMP2 (D) AMP5 (B)</td>
</tr>
</tbody>
</table>

For the intelligent packaging systems, the overall migration behaviour observed agreed with legal limitations (Table 43).

In this part of the study, the official test methods of the European legislator were used to evaluate the overall migration from the various packaging materials. For some of these systems, indeed these methods are appropriate, because they do not differ in their layout from the normal plastic packaging materials, like for example films. On the other hand, it is clear that by immersing a moisture-absorbing sachet in water, a conflict arises with the use of this sachet in real life conditions. Consequently the relevance of the migration data obtained is doubtful. So the question arises whether the simulants proposed by the legislator are realistic for some of these new types of packaging materials. This question is especially relevant because some of the systems are used in contact with solid foodstuffs, containing appreciable amounts of moisture (e.g. oxygen scavenger in packed prepared meat). Migration in solid food matrices could be much smaller compared to liquid matrices, because of the restricted diffusion. Moreover, a soaking of the active packaging sachets by the liquid simulant is avoided. For fatty food simulants at high temperatures, modified polyphenylene oxide has been used already as a solid food contact material. Because especially in aqueous food simulants high overall migration values were observed, a solid aqueous simulant
interactions with active and intelligent packaging materials seem to be more appropriate for testing these kinds of active packaging materials. This will be discussed in the next paragraph.

5.3.3. **Migration studies with alternative food simulants**

5.3.3.1. **Introduction**

As indicated above (paragraph 5.3.2), for some of the tested active packaging materials, the unrealistic methodology to simulate migration, was considered as a possible reason for the high migration levels observed. Especially for the aqueous and acidic food simulants extremely high overall migration levels were obtained for some oxygen absorbers and moisture regulators. Most of these packaging systems are intended to be in contact with fresh or minimally processed foods such as (prepared) meat or fish, vegetables, etc. According to EU regulations, water and/or acetic acid should be used, among other simulants, to perform migration tests for these types of foods. If such migration experiments were performed, the considered active packaging systems would get soaked, thus possibly accelerating the migration process. If similar migration tests would be performed using real food matrices, such as meat, soaking could possibly be prevented. Although these foods are moisture-rich, the water is rendered macroscopically immobile since it is physically entrapped. Moreover, mobility of the migrant in the food would be restricted because of its solid nature. Therefore migration levels are expected to be lower than those observed in the liquid simulant. Of course, preference would be given to a simulant instead of a real food to conduct migration experiments. This simulant should be similar to the food considered: moisture-rich, but not liquid. Moreover, the simulant should be easily standardised, preferably cheap and easy to use and manipulate.

Agar gels seemed to be an appropriate choice as an alternative simulant for water. Similarly, acidified agar gels could be used to study migration to acidic moisture-rich solid foods. Therefore, these alternative food simulants are considered to study the overall and specific migration from selected active food contact materials.

5.3.3.2. **Results and discussion**

5.3.3.2.1. **Remarks with regard to the development and principle of the method**

Agar gels contain usually 1% (w:w) of agar. Therefore the water content is very high resulting in very high water activities (typically 0.98 or higher). Due to the addition of acetic acid to the aqueous phase, no solidification of the gel occurred at a 1% (w:w) agar concentration, requiring the use of
high agar concentrations (2% w:w). Apart from agar, also the use of gelatine could be considered. At temperatures of 40°C no solid material is obtained, restricting the practical applicability of such an alternative. Since the migration experiments in the official food simulants were performed at 40°C, preference was given to the use of agar gels in order to be able to compare the migration levels obtained.

Boiling temperatures are necessary for dissolving the agar in water. It is obvious that care should be taken when the material to be tested is contacted with the alternative simulant in its liquid state, because of its elevated temperature. In fact two options are possible.

In the first case, the hot simulant is cooled down near its melting temperature (about 50°C) and contacted at this temperature with the test specimen. After application of the simulant, it should be cooled down as soon as possible to the desired incubation temperature.

The second option consists of cooling down the agar to the desired incubation temperature or until the gel solidifies. Subsequently, agar slices can easily be cut from the solid agar block and the test specimen can be sandwiched in between two agar slices. Because of the rather flexible nature of the solid agar gel, close contact between the test specimen and the simulant is still achieved. The latter approach is also recommended if due to its structure, the test specimen could absorb the simulant (e.g. MR-3). If this occurs, it can easily be seen because of the presence of solid gel particles inside the test specimen. This second option could also be applied using a standard migration cell. Once the agar is solidified in the cell, it can be opened to apply the packaging film, without any risk of loss of the simulant.

Overall migration measurements are based on the difference in the total dry matter content of the agar gel before and after contact as shown in Figure 68. Since dry matter content is determined by oven drying, only components with a comparable volatility as water can be added to the gel to change its characteristics, like for example its pH. Otherwise, a loss of dry matter (= non volatile substances) could occur due to the negative migration from the simulant to the test specimen. Therefore the use of acetic acid did not present any problem in this regard, since it is also evaporated during the drying process. As a further consequence only specific migration measurements can be performed if non volatile substances, like for example sodium chloride, are added to the agar gel. Of course, care should be taken to avoid analytical interferences between the substances added and the analytes of interest.

Since the total dry matter content of the gel should be known after contact, it is important to recover
all the simulant and quantify it. Using liquid simulants, this is fairly easily achieved. For solid simulants, problems may occur like for example observed in the initial experiments using moistened silica gel. These experiments failed because it was impossible to recover completely the powdery silica gel, which stuck partially to the test specimen. As a consequence, results obtained from migration experiments, were not repeatable. Agar gels on the other hand can be applied as one piece, which are quite firm and easy to handle.

In order to determine the dry matter content it is important that a homogenous sample is obtained. Because of the solid structure it can be possible that the highest dry matter content is found near the contact surface with the test specimen. Similarly, due to evaporation of water at the agars surface, an increased dry matter content can be observed at these places as well. Therefore, after total recovery of the agar gel, it was weighed and mixed in a blender to homogenize it. From this mixture, sub-samples were taken to determine the relative dry matter content. Since the total amount of recovered simulant is known, the total dry matter content can easily be calculated. Similar data can be obtained from the gel before contact and therefore overall migration can be calculated as shown in Figure 68.

Similar as to the official methods, only non volatile migrants can be quantified. Moreover, the presented methodology for overall migration measurement is a gravimetric technique as well. In contrast to the official methods, no differences in weight as such are recorded but merely changes in the total dry matter content. Taking into account the analytical limitations of the dry matter content analysis via oven drying and the relatively high amount of dry matter initially present (typically 1000 mg for a 1% w:w agar gel), it can be expected that the sensitivity of the overall migration measurements using the proposed alternative simulant will be of a different level compared to the overall migration measurements using the analogue official simulants. Therefore the method is only applied on those active systems which, in migration studies using the official simulants, proved to exhibit high overall migration levels.

For the specific migration measurements, it seemed sufficient to liquefy the agar by the addition of water and subsequent mixing. Alternatively this mixture could be heated until the agar melts down. Afterwards classic clean-up and analytical techniques were able to quantify the specific migrants.
5.3.3.2.2. **Overall migration from iron based oxygen absorbers**

It was observed that the overall migration from iron-based oxygen absorbers was very high in the official aqueous and acidic food simulants (Tables 42 and 45). However, using the alternative aqueous simulant, a nearly six-fold reduction in overall migration from OS-1 was observed. Similar results were obtained for OS-2, although the reduction was somewhat more restricted (two-fold). Interestingly, for this sample, repeatability was much better if agar was used as a simulant instead of water. As indicated previously (paragraph 5.3.2), overall migration measurements in the liquid simulants were not repeatable because the material tended to float during the incubation. Since the agar is a solid material it is able to prevent the active packaging material from floating.

Although the active packaging materials were not soaked, their content was completely moistened. This moistening could be expected because of the high difference in water activity between the active packaging material and the agar gel. It is important to realise that although the water is physically trapped in the agar gel, it is still very mobile because of the high water activity. In contrast to the official aqueous simulant, water is released in a somewhat controlled manner. Combining this phenomenon with the restricted diffusion in the solid simulant, probably explains the significant reduction in overall migration observed. Because of these lower overall migration levels, the amount of food to be contacted with such active materials can be reduced drastically, if only the overall migration limit is taken into account (60 mg.kg⁻¹ food). For OS-1 however, the minimal amount of food which can be contacted remains unrealistically high (about 2 kg!).
Therefore the practical applicability of these systems in contact with for example meat, remains questionable.

Table 45. Overall migration from oxygen scavengers in aqueous and acidic food simulants after incubation during 10 days at 40°C (reported data are the averages of at least 3 measurements)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Simulant</th>
<th>Overall migration (mg/object)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS1</td>
<td>water*</td>
<td>617 ± 32</td>
</tr>
<tr>
<td>OS1</td>
<td>1 % agar</td>
<td>113 ± 22</td>
</tr>
<tr>
<td>OS1</td>
<td>3 % acetic acid*</td>
<td>1707 ± 310</td>
</tr>
<tr>
<td>OS1</td>
<td>2% agar, 3% acetic acid</td>
<td>1484 ± 190</td>
</tr>
<tr>
<td>OS2</td>
<td>water*</td>
<td>63 ± 27</td>
</tr>
<tr>
<td>OS2</td>
<td>1 % agar</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>OS2</td>
<td>3 % acetic acid*</td>
<td>392 ± 136</td>
</tr>
<tr>
<td>OS2</td>
<td>2% agar, 3% acetic acid</td>
<td>529 ± 25</td>
</tr>
</tbody>
</table>

*adopted from Table 40

Using the alternative acidic solid food simulant, again a reduction in the average overall migration level for OS-1 was observed compared to the level obtained in the official analogue. The reduction however was not significant. For OS-2, even higher overall migration levels were obtained. Probably in the latter case, this was again due to the fact that by using the agar gel, floating of the absorber can be prevented and therefore more intense contact between the simulant and the test object is possible. It should be noted that for both absorbers, the agar showed cracking after incubation in the acidic agar gels. This is probably due to the production of hydrogen gas, due to the electrolytic dissolution of the iron in the acidic environment.

So in contrast to the use of the solid aqueous food simulant, the use of acidified agar gels did not result in a significant reduction of the overall migration levels. No explanation for these results could be found. It should be noted however that the amount of iron present in the samples is considerably higher compared to the amounts of sodium chloride. In addition, the migration process of iron is partially governed by the electrochemical dissolution of the metal. It is unclear whether the diffusion of protons in the agar would proceed faster compared to the diffusion of the other ions of interest (Fe²⁺, Na⁺ and Cl⁻) and therefore could minimize the effect of the solid simulant. Nevertheless, it is obvious that the use of these active packaging materials in moisture rich acidic foods is not suitable, even if the water is physically entrapped.
5.3.3.2.3. **Overall migration from moisture regulators**

Using the official aqueous and acidic food simulants, it was observed that for some of the moisture regulators tested, high overall migration levels were observed, as indicated in Table 46. After contacting MR-4 with the alternative solid aqueous simulant (10 days, 40°C), strong reduction in the amount of agar was observed together with an equivalent uptake of water by the active packaging material (about 40 g on a total of 100 g). Surprisingly, no significant differences could be observed between the results obtained using the official aqueous simulant, even if the incubation time was drastically reduced. Presumably, migration proceeded very fast for this particular system, since maximal migration levels were already obtained after 24 h of incubation at 40°C. Shorter incubation times were not considered because they would not be relevant with regard to the system studied. Therefore experiments were performed at lower temperatures using both the official and alternative aqueous food simulant. A two fold reduction in the overall migration level was observed using agar as a simulant. These results are in agreement with those observed for the iron-based oxygen scavengers, illustrating again the potential of the use of agar gels as more realistic simulants for overall migration measurement to aqueous solid foods. It should be realised however that even by using the alternative simulant, the overall migration level exceeded the limit. Consequently the considered sample is not appropriate to be contacted with aqueous foodstuffs, even if the food is not liquid.

In order to change the water activity of the agar gel, sodium chloride was added. As can be observed however, results obtained were unrealistic. This could be expected because of the negative migration of the salt to the active packaging material as explained before (paragraph 5.3.3.2.1).

For the other moisture regulators, especially the overall migration levels in the official acidic food simulant were high. Using the acidified agar gel, overall migration was reduced almost two-fold for MR-1. This is in contrast to the results previously obtained for the iron-based oxygen absorbers. Despite the strong reduction in overall migration, it should be noted that MR-1 is intended to be used as a desiccant in dry foods. Consequently, the practical importance of the obtained results remains rather limited.

For MR-3, variable and inconsistent results were obtained using the alternative acidic simulant. Probably this is due to the analytical limitations of the method presented. Only if the overall migration is sufficiently high, reliable results can be obtained. As for MR-3 overall migration levels in the official acidic simulant was relatively low compared to the other systems considered, it seems...
that the methodology is not suitable to assess these low migration levels. This example clearly illustrates the analytical limitations of the alternative simulants in overall migration measurements.

Table 46. Overall migration from moisture regulators in the official and alternative aqueous and acidic food simulants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Simulant</th>
<th>Incubation time/temperature</th>
<th>Overall migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR-4</td>
<td>water-cell 10 days - 40°C</td>
<td>674 ± 37 mg.dm⁻²</td>
<td></td>
</tr>
<tr>
<td>MR-4</td>
<td>1% agar-cell 10 days - 40°C</td>
<td>679 ± 40 mg.dm⁻²</td>
<td></td>
</tr>
<tr>
<td>MR-4</td>
<td>1% agar-cell 1 day - 40°C</td>
<td>675 ± 9 mg.dm⁻²</td>
<td></td>
</tr>
<tr>
<td>MR-4</td>
<td>water 2 days - 5°C</td>
<td>503 ± 40 mg.dm⁻²</td>
<td></td>
</tr>
<tr>
<td>MR-4</td>
<td>1% agar 2 days - 5°C</td>
<td>224 ± 26 mg.dm⁻²</td>
<td></td>
</tr>
<tr>
<td>MR-4</td>
<td>1% agar, 15% NaCl-cell 10 days - 40°C</td>
<td>-2270 ± 23 mg.dm⁻²</td>
<td></td>
</tr>
<tr>
<td>MR-1</td>
<td>3% acetic acid* 10 days - 40°C</td>
<td>967 ± 133 mg/object</td>
<td></td>
</tr>
<tr>
<td>MR-1</td>
<td>2% agar, 3% acetic acid 10 days - 40°C</td>
<td>443 ± 142 mg/object</td>
<td></td>
</tr>
<tr>
<td>MR-3</td>
<td>3% acetic acid* 10 days - 40°C</td>
<td>45 ± 8 mg/object</td>
<td></td>
</tr>
<tr>
<td>MR-3</td>
<td>2% agar, 3% acetic acid 10 days - 40°C</td>
<td>-226/-31/225 mg/object</td>
<td></td>
</tr>
</tbody>
</table>

*adopted from Table 42

5.3.3.2.4. Specific migration from MR-4

All migration experiments were performed at refrigerated temperatures because in the overall migration studies it was revealed that rapid equilibrium is reached at 40°C. Moreover, meat was used as well as a ‘simulant’ to compare the behaviour of agar with real food matrices. Results are summarized in Table 47.

Two migrants could be identified in the migrate and the reported results refer to the main component (about 90 % w:w). Comparing the specific migration data observed in water with the overall migration data presented in Table 46, it can be concluded that the quantified migrant is only a part of the total migrate from the active packaging material. Probably other compounds migrating from the film itself were involved, which were not detected with the applied analytical method. Similar as in Table 46, it seems that the specific migration is influenced by the temperature in a comparable manner as the overall migration.
More interesting however, a nearly two-fold reduction in the specific migration is observed using agar as an alternative aqueous solid food simulant. Performing a comparable migration experiment in meat, it seemed that specific migration levels were similar to those obtained in agar. This result clearly illustrates the suitability of an agar gel to evaluate migration to solid moisture-rich foods. The introduction of the alternative simulant is not intended to circumvent the existing migration limits, by changing the methodology to assess migration. The results presented in Table 47 clearly illustrate that more realistic migration levels are obtained in agar, which are though still higher compared to those observed in meat.

As a further elaboration of the solid aqueous simulant, brine was added to the agar gel to reduce its water activity (about 0.9). An additional reduction in the specific migration was obtained compared to the levels observed for normal agar. This indicates that, if necessary, the properties of solid aqueous food simulant can be further adjusted in such a way that better correspondence with the water activity of the food of interest can be obtained. Water activity is clearly a major driving force in determining the specific migration of the active compound considered. Similar conclusions could be drawn by comparing the specific migration levels obtained in water and in brine respectively.

Since the specific migration levels in brine were significantly higher compared to those obtained in its solid equivalent, it can be concluded that water activity is not the only factor of importance. Probably the physical entrapment of the water in a solid gel structure and the reduced mobility of the migrants in the solid simulant seem to be of importance as well.

5.3.3.2.5. Specific migration from MR-3

Some concern exists about the possible accelerated migration from moisture absorbing pads if these
reach their saturation level. Therefore, migration from pads at different saturation levels to agar gels was studied. Since no specific migrants could be identified in these active packaging materials, they were spiked with rhodamine B, which is a fluorescent chromogen.

Results indicate that by increasing the water content of the pad, lower amounts of rhodamine B migrated to the agar (Table 48). Possibly, due to the lower concentrations of rhodamine B in the aqueous phase of the pad at higher saturation levels, equilibrium with the agar is reached at lower chromogen concentrations in the simulant. Consequently, migration was not enhanced due to higher saturation levels of the pad.

Table 48. Migration of rhodamine B from moisture absorbing pad ad different saturation levels (10 days at 5°C)

<table>
<thead>
<tr>
<th>Saturation level (%)</th>
<th>rhodamine B (mg/pad)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.85 ± 00.6</td>
</tr>
<tr>
<td>60</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>80</td>
<td>0.31 ± 0.03</td>
</tr>
</tbody>
</table>

5.3.3.3. Conclusions

Agar gels are able to simulate overall migration to solid moisture-rich food from iron-based oxygen absorbers and moisture regulators in a much more realistic manner compared to the official aqueous food simulant. The usefulness of agar to assess overall migration measurements is however somewhat restricted.

For specific migration experiments, agar seems very promising as an alternative aqueous food simulant. Additional experiments on other relevant food contact materials seems necessary to confirm the results of this research.

Despite of the introduction of this more realistic approach to simulate migration from some active food packaging systems to solid moisture-rich foods, the classification of the active packaging materials as shown in Table 44, does not change. Therefore it should be concluded that some of the active packaging systems studied, simply exhibited too high migration levels to be applied in contact with foodstuffs.

5.3.4. Classification of active and intelligent packaging systems

On the basis of their compositional data and their observed migration behaviour the studied active
and intelligent packaging systems can be classified. Therefore, the scheme as outlined in Figure 69 was used. Five different categories can be distinguished (category A-E). For systems listed in category A, only minor legal problems can be expected if they would be applied in practice. For all other systems, applicability is questionable due to various reasons or combinations thereof (e.g. non listed components, too high migration levels etc.).

In this classification exercise, the fact whether a packaging system is solely composed of plastics or not is not taken into account. The presented classification uses EU legislation on food contact materials for plastics as a reference. As could be concluded from the first chapter, this legislation can be considered as the most detailed regulation on food contact materials which is applicable in the whole EU. Therefore it seemed reasonable to use these criteria as a starting point for the classification. Other legislative restrictions (specific migration limits, food additive, labelling, etc.) should be considered as well before a particular material can be approved. These are not considered in the presented classification.

Category A systems comply with current overall migration and compositional restrictions for plastic food contact materials. Only four of the nineteen tested active materials (MR2, AMP3, ES1, OA2) can be classified in this category. Systems classified as A, can be composed out of other materials as plastics as well. Admission to bring these on the EU market should be requested in each individual member state. It is obvious that such a procedure is very tedious. This fact combined with the limited number of the evaluated systems classified in category A, clearly indicates that a (uniform) legislation in the EU for these types of food packaging materials is needed to guarantee their break-through on the EU market.

For the intelligent systems only those applied at the outside of the package (TTI 1-3) are classified in this category. Although these materials contain not listed substances, their presence is considered of minor importance because of the presence of a functional barrier reducing the likely migration of these substances to minimal and probably safe levels. It is nevertheless clear that carcinogenic and mutagenic substances cannot be used even if an adequate functional barrier is present.

If the packaging complies with overall migration restrictions, but if the active compounds are not listed, although toxicological data are available or they are listed as food additives, it is classified in category B. For the oxygen scavenging films OS4-5 and the oxygen indicators OI1-2 relevant toxicological data with regard to the active ingredients were found so they can be classified as such. Similarly the anti-microbial packaging AMP4 could be classified in this category. Some of the studied systems contained however suspected mutagenic compounds. This clearly illustrates that
care should be taken in the interpretation of such a classification. A detailed evaluation of the toxicological data is necessary to evaluate the safety of the systems classified as such. If toxicity data of the active component allow their uptake in the positive lists, the materials involved could be classified in category A.

![Classification scheme for intelligent and active packaging materials](image)

Figure 69. Classification scheme for intelligent and active packaging materials

If the packaging material does not meet the migration limit, although it complies with the positive lists, it is classified as category C. In most cases, the migration limit is not respected because too high amounts of the active component migrate from the active packaging material (e.g. ethanol). On the other hand, also other compounds may migrate into excessive amounts, even if they are not
intended to do so (e.g. sodium chloride for some oxygen scavengers). Both toxicological relevant (e.g. iron TDI 0.8 mg/kg bodyweight) and not relevant compounds (e.g. sodium chloride) might be involved. Following systems were classified in this category: OS1-2, MR1, MR3 and AMP1-2. Curiously only a limited amount of active releasing systems could be classified as such (AMP1-2). As already indicated before, such systems could be classified in category A, if a corrected overall migration level as introduced in Figure 67, would be applied. For the scavenging systems, such a correction can not be tolerated.

If the packaging system fails on both aspects of the packaging legislation (overall migration and positive list), but if the compounds present are food additives or if relevant toxicological information is available, they are classified in category D. Following systems could be classified as such: OS-3, MR4, OA1, ES2 and AMP5. If the toxicity data of the active compounds enable their incorporation on the positive list, the systems could be classified in category C.

If no toxicological data are available or the component is not a food additive and the systems passes the migration tests, the systems are classified in category E. None of the collected active systems fell into this category. Since for the carbon dioxide indicator, several components were present for which no toxicity data were available, it was classified as such. If toxicity data would become available, the system could be classified as B. System AMP6 could not be classified at all, since it exceeded the migration limits and no adequate toxicity data on the active components were available.

In Table 49 an overview of the classification is presented. It is supposed that the materials tested can be contacted with all kinds of foodstuffs. Of course, some active packaging materials will only be used with some specific foodstuffs. Therefore, adjustment of the proposed classification, taking into account the intended use of the active packaging material, can be made. Alternatively, the use in a particular kind of foodstuff, in which the overall migration limit was exceeded, could be excluded. By doing so the following active systems, initially classified as C, could be classified as A if their use in the indicated food classes is avoided: MR1 (acidic foods), AMP2 (fatty foods) and MR3 (acidic and fatty foods).
Table 49. Classification of the collected active and intelligent systems according to the scheme presented in Figure 69

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>No classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OS4-5</td>
<td>OS1-2</td>
<td>OS3</td>
<td>ES1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MR2</td>
<td>MR1&amp;3</td>
<td>MR4</td>
<td>AMP3</td>
<td>AMP4</td>
</tr>
<tr>
<td></td>
<td>AMP3</td>
<td>AMP1-2</td>
<td>AMP5</td>
<td>OA2</td>
<td>OA1</td>
</tr>
<tr>
<td></td>
<td>TTI1-3</td>
<td>OI1-2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Despite the reduction observed in the overall migration levels from OS1-2 using the more realistic moisture-rich solid food simulant, the levels obtained were still too high. Consequently it seems reasonable to classify these systems in category A if their use is restricted to dry food only.

If the ethylene and the oxygen scavenger ES2 and OS-3 are not contacted with fatty foods, they can be classified in category B. Similarly, an adjusted classification for AMP5 can be proposed if contact with acidic foods is avoided.

Finally, it should be noted that for a lot of foods necessitating the use of the fatty food simulant in migration tests, a reduction factor applies. Consequently the restrictions for fatty food contact can be further refined. Taking all these aspects into account, an adjusted classification table can be presented (Table 50).

Table 50. Adjusted classification of the collected active systems according to the scheme presented in Figure 69 and keeping into account restrictions in use

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS1-2 (only in dry foods)</td>
<td>OS3 (not in fatty foods)*</td>
</tr>
<tr>
<td></td>
<td>ES2 (not in fatty foods)*</td>
</tr>
<tr>
<td>MR1 (not in acidic foods) ; MR3 (only dry foods)</td>
<td>AMP2 (not in fatty foods)*</td>
</tr>
<tr>
<td></td>
<td>AMP 5 (not in acidic foods)</td>
</tr>
</tbody>
</table>

*unless a reduction factor of at least 2 (ES2), 3 (OS3) or 4 (AMP2) applies
5.3.5. Possible conflicts with regard to food additive legislation

5.3.5.1. Introduction

As already stressed before, apart from the food contact material regulations, also other food legislation should be considered to implement active and intelligent packaging systems. Fabech et al. (2000) presented recently a comprehensive overview of the legislation which could be relevant in this respect.

Food additive legislation may be one of the most important to consider and therefore some of the main conflicts which can be identified will be discussed here in some more detail, together with some propositions how these problems could be solved.

Basically, since intelligent packaging materials are designed to monitor the (quality of the) packed food, a conflict with food additive legislation is not likely to occur. A more complex situation arises if active packaging materials are considered, because these can be designed in such a way that substances deliberately migrate to the food.

From the collected active packaging materials, it became clear that both releasing and absorbing systems can be distinguished. It is obvious that especially the releasing systems are a matter of concern in the present discussion. Experimental migration studies however revealed that also absorbing active systems may transfer particular components of interest to the food, like for example sodium chloride (food ingredient) or sugar alcohols (food additive). If these compounds can fulfil a technical function in the food, they should be regarded as food additives and consequently, the active packaging material in concern should be considered as a releasing system as well. Otherwise, the migrants should be regarded as contaminants and food additive legislation should not be considered.

These experimental observations clearly indicate that not only the releasing active packaging systems but also the absorbing materials are of importance if the legislative aspects of food additives, are discussed with respect to active packaging materials.

Supplementation of additives to the food via the packaging material is foreseen in the legislation since it is specified that their addition may serve a technological purpose during the packaging, transport or storage of the food. This is exactly what the purpose of active packaging is: fulfilling a technological function in the food while the food is already packed. Although food additive legislation basically agrees with the principle of active releasing packaging materials, some major
problems with this regulation may occur.

5.3.5.2. Problem identification and solution

Problems with regard to migration

As indicated above, the concentration of the active compound should be high enough to ensure that the active packaging system is really effective. Due to migration however, the concentration of the active compound in the food can become that high that the migration restrictions are not respected. As suggested already before (Figure 67), a possible solution could be to subtract the migration of the active compound from the overall migration level. This corrected level should be in compliance with current restrictions. If the specific migration level is exceeded, the use of the material should be rejected since these specific migration levels are based on toxicological considerations. Of course maximal concentration as specified in the food additive legislation should be respected as well. This might necessitate a specification about the minimal amount of food to be contacted with the material.

The migration of the active compound to (semi-) solid foods probably creates a non uniform distribution of the component in food, due to restricted diffusion. Therefore, those parts of the food in close contact with the contact material will contain the highest concentrations of the additive. It might be possible that from a safety point of view, that particularly in those parts, too high concentrations are reached.

A similar problem is observed for the non uniform distribution of pesticides on crops. In this particular area, the concept of the acute reference dose has been recently applied, which could also be helpful in this particular case. It is obvious that too high local concentrations of the active compound should be avoided.

Problems with positive lists specified for food additives and labelling

Food additive legislation foresees lists in which the use of an additive is tolerated for a particular foodstuff. As already stated before, it is clear that these regulations should be respected, even if the supplementation of the additive proceeds via the packaging material. Therefore, proper instructions for use and application should be provided together with the active packaging material. It is obvious as well that the presence of the food additive in the food should be labelled, even if it originates from the packaging material.
Problems with regard to the acceptable daily intake

Toxicological relevant compounds used as food additives, are characterised with an acceptable daily intake value. This value is used as a tool to set maximal concentrations present in the food. A regular evaluation of the food consumption and the concentration of the particular food additive enables to calculate the estimated daily intake of the compound. If this estimated intake is higher then the acceptable intake, legislation will be changed by lowering the maximal concentrations in the food. It is possible that due to the application of the new packaging technology discussed, the intake of some additives will increase and might cause too high daily intakes. Consequently, the intake of additives via the packaging material should also be monitored, similar as the monitoring of the additives supplemented in the traditional way to the food. If necessary, maximal levels in some foods should be changed in order to guarantee food safety.

Problems about the presence of impurities

For food additives, some purity criteria are specified. Migrants from a releasing packaging material, which are not due however to the presence of the food additive, should not be considered as impurities of the additive itself.

A more important problem could arise due to the accelerated degradation of the food additive because of the processing of the active packaging material (e.g. antioxidants in an extruded plastic film). Food additive legislation considers the additive even if it is present in an altered form. So consequently possible reaction products should be identified to allow toxicological evaluation if necessary. Both the original compound and its side-products should be taken into account to evaluate the compliance with the qualitative restrictions specified in both food contact and food additive legislation.

Problems with regard to active and ordinary migrants

It has been suggested that deliberate addition of substances to the food via the contact material should be declared in the ingredient list. Of course not every migrant should be mentioned. In addition, a plastic additive can be a food additive at the same time, but its migration may not serve a technical function in the food. So consequently a difference between an active and an ordinary migrant, not fulfilling a technical function in the food, should be made. Only those migrants which are active should be indicated on the ingredient list of the packed food. This approach however could create some problems since traditional food contact materials such as tin cans or wood can be considered to be active and consequently the active ingredients (tin, wood components) should be declared. On the other hand, if the claimed activities of a packaging material are not in
correspondence with the activity of the migrating substances, it could be an indication that other migrating species are involved.

As indicated above, the active compound released by an active packaging material may not fulfil its technical function to the food. This could be due to several reasons which are not relevant in the present discussion, but experimental evidence of such cases have been demonstrated in other parts of the ‘ACTIPAK’ project. The contact material should then be considered as an ordinary food packaging material, for which the food contact material legislation applies.

For all these problems, it should be emphasised as well that food legislation does not tolerate the presence of compounds which normally should not be present in the food as such. So basically if the compound is not fulfilling a function in the food, its presence should be regarded in detail, to evaluate the cause of its presence and to decide whether its presence can be tolerated or not.

5.3.5.3. Conclusions

Most probably this list of possible conflicts with food additive and other related legislation and active food packaging materials is not complete. In addition, some of the solutions suggested can be replaced by others. Nevertheless this short overview indicates that major problems may arise with regard to food additive legislation if active packaging materials are to be implemented.

To make the situation even more complicated, it should be realised that apart from conflicts with food additive legislation, also other legal problems can be expected. In addition, concern about the microbiological safety of some of the systems applied, arises as well. Therefore it seems reasonable to assume that a general solution to these legal and other problems will only be available within a number of years. In order to enable food industry to apply this new technology sooner, it could be suggested that a European committee of experts, as could for example be present in the European Food Authority, evaluates petitions by a case by case methodology. Considering the reflections about the possible conflicts with food additive legislation, the scheme as outlined in Figure 70 could be applied for the evaluation of active food packaging materials. Particularly for the active releasing materials, compliance with food legislation will very much depend on the type of food to be contacted with the material. Therefore the case by case strategy for the evaluation of these packaging systems, will always be necessary up to a certain extend, even if dedicated legislation would be come available in the time to come.
5.3.6. Conclusions

With regard to the composition, a first important conclusion is that a lot of active and intelligent packaging systems are very complex. Apart from plastics, other materials such as paper, metals, minerals etc. are being used. Various of these complex layouts, are not supposed to enhance the user-friendliness of these materials because most of them should be added separately to the packed food. Some active materials on the other hand are simple plastic films. Therefore the present legislation for plastics applies only for a minority of the tested materials.

If this legislation is considered however to classify the systems, it seemed that both conflicts with
regard to their composition and their migration behaviour occurred. The use of an alternative aqueous solid food simulant, which proved to simulate migration in a much more realistic manner than the official aqueous food simulant, for some of the evaluated systems, did not allow to change these conclusions.

Apart from the food contact material legislation, also other food legislation should be considered in the evaluation of these materials. Food additive legislation is an important example, especially with regard to the releasing active systems.

Generally it could be concluded that a dedicated regulation seems to be necessary to permit breakthrough of these materials on the EU market and to guarantee their safe introduction and use in Europe. Because of the multitude of legislative problems which can be expected if active or intelligent packaging systems are to be implemented, a case by case evaluation by for example the European Food Authority seems appropriate if indeed application of these new technologies on the EU-level should be realised within the near future.
6. General conclusions

The first part of the experimental work of this study about chemical interactions between foods and packaging materials, considered the chemical characterisation of polyglycerol esters. These compounds are a group of complex plastic additives. The analytical scheme presented consisted essentially of two steps: the identification of the polyglycerol moiety of the esters followed by a further analysis of the esters themselves. Polyglycerols could be determined quantitatively up to the tetruglycerols, while qualitative analysis up to heptaglycerol was possible using a gas chromatographic separation, after derivatisation of the polyols into trimethyl silyl ethers. A possible identification of various non cyclic and cyclic isomers of di-and triglycerol could be presented as well.

For the analysis of the polyglycerol esters, a combined liquid and gas chromatographic analytical method was presented. The liquid chromatographic pre-fractionation of the sample seemed necessary because several components co-eluted during the gas chromatographic analysis. Using column chromatography, standards with sufficient purity of the following compounds were isolated: di-esters of di- and triglycerol and mono-esters of di-and triglycerol. Consequently, qualitative analysis of these compounds would be possible using the methodology presented. Mono-esters of tetruglycerol could be analysed qualitatively as well. In addition, a possible identification of various isomers of the mono-esters of diglycerol was presented.

The second part dealt with an evaluation of immunological methods as an alternative analytical methodology to study the specific migration. Therefore a model compound, bisphenol A, was selected and an enzyme-linked immunosorbent assay was developed. Bisphenol A was converted into a suitable hapten which could be covalently bound to proteins. In such a manner, an immunizing antigen could be produced which was injected in chicken hens. The polyclonal antibodies could conveniently be isolated from the yolk of the eggs, the hens produced. Using a bisphenol A coating antigen, it could be revealed that the IgY antibodies showed reactivity towards bisphenol A.

Using the same coating antigen and a secondary anti IgY antibody from rabbits, which was coupled to a peroxydase, the isolated antibodies were applied in an indirect competitive enzyme-linked immunosorbent assay. The assay proved to be quite sensitive towards possible matrix effects such as the ionic strength and the presence of surface active components like proteins. It was moreover revealed that the sensitivity of the assay was lower compared to the classical instrumental methods
or to recently developed immunoassays for bisphenol A using poly- and monoclonal mammalian antibodies. Variation of several experimental parameters did not result in a spectacular increase in assay sensitivity.

Nevertheless, the assay proved to be very specific towards bisphenol A. In addition the loss of sensitivity in the direct analysis of milk could be restricted as well.

In the last part of this study, the interactions between food and active and intelligent packaging systems were investigated. This part consisted of a EU FAIR R&D research project, called ‘ACTIPAK’ (CT 98-4170).

It was revealed that the studied packaging systems were complex in their composition. Only a few materials were solely composed of plastics. Despite this observation, EU food contact material legislation with regard to plastics was considered to evaluate the collected systems. Problems with both their composition (positive list) and their overall migration behaviour were observed. Only 20% of the tested systems did not present any problems. These observations clearly indicate the need for a dedicated EU legislation to allow brake-through of active and intelligent food packaging systems on the EU market.

The use of an alternative aqueous solid food simulant was evaluated for some oxygen absorbers and moisture regulators. The agar gels seemed to simulate the migration to moisture-rich solid foodstuffs such as meat, in a much more realistic way, compared to the official aqueous food simulant.

In addition to the problems of their composition and their migration behaviour, active and intelligent packaging materials may be in conflict with other food legislative aspects. Food additive legislation can be considered as an important example in this respect, especially for active packaging materials. Because of the multitude of legislative problems which can be expected if active or intelligent packaging systems are to be implemented, a case by case evaluation by the European Food Authority seems appropriate in order to guarantee food safety and quality.
6. General conclusions

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8. Annex 1: abbreviations

ABTS: 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ADI: acceptable daily intake [mg.kg\(^{-1}\) bodyweight.day\(^{-1}\)]
AMP: anti-microbial packaging materials
BADGE: bisphenol A diglycidyl ether
BCR: Bureau of certified reference materials
BSA: bovine serum albumin
CDI: carbon dioxide indicator
CEN: European centre for normalisation (Centre Européen de Normalisation)
DCC: N,N’-dicyclohexylcarbodiimide
DEHP: di-(ethyl-2-hexyl)phthalate
DIP: di-iso-octyl phthalate
DMF: dimethylformamide
EDI: estimated daily intake [mg.kg\(^{-1}\) bodyweight.day\(^{-1}\)]
EDTA: ethylenediamine tetra-acetic acid
ELISA: enzyme-linked immunosorbent assay
ES: ethylene scavenger
EU: European Union
FTIR: Fourier transform infra red spectroscopy
GC: gas chromatography or gas chromatograph
GC-MS: gas chromatography coupled to mass spectrometry
GLC: gas liquid chromatography – capillary gas chromatography
HMDS: hexamethyldisilazane
HPLC: high pressure liquid chromatography
HPLC-UV: high pressure liquid chromatography coupled to ultra violet detection
HS: head space
ID: internal diameter
Ig A: immunoglobulins A
Annex 1: abbreviations

Ig G: immunoglobulins G
Ig M: immunoglobulins M
IgY: egg yolk immunoglobulins, similar to IgG
IRRM: Institute for reference materials and measurements
  (former BCR)
JECFA: Joint Expert Committee on Food Additives
LC-MS: liquid chromatography coupled to mass spectrometry
LC: liquid chromatography
MAFF: Ministry of Agriculture, Fisheries and Foods of the United Kingdom
MPPO: modified polyphenoloxide or Tenax®
MR: moisture regulator
MS: mass spectrometry
MTDI: maximum tolerable daily intake
NMR: nuclear magnetic resonance
OA: other active packaging material
OI: oxygen indicator
OS: oxygen scavenger
OVA: ovalbumin
PA: polyamide
PAN: polyacrylonitrile
PBS: phosphate buffered saline
PC: polycarbonate
PE: polyethylene
PET: polyethyleneterephthalate
PP: polypropylene
PS: polystyrene
PTFE: polytetrafluoroethylene
PTWI: provisional tolerable weekly intake \([\text{mg.kg}^{-1}\text{bodyweight.week}^{-1}]\)
PVAC: polyvinylacetate
PVAL: polyvinylalcohol
PVC: polyvinylchloride
Annex 1: abbreviations

PVDC: polyvinylidenechloride
RT: room temperature
SCF: Scientific Committee for Food of the European Union
SDS-PAGE: sodium dodecyl sulphate polyacryl
gel electrophoresis
Simulant A: water
Simulant B: 3 % (v:v) acetic acid
Simulant C: 10 or 15 % (v:v) ethanol
Simulant D: olive oil or equivalent
SML: specific migration limit [mg.kg⁻¹ food]
t-ADI: temporary ADI [mg.kg bodyweight⁻¹.day⁻¹]
TBAF: N,N,N-tributyl-1-butylammonium fluoride
tBCDS: tert-butyl(chloro)dimethylsilane
TDI: tolerable daily intake [mg.kg bodyweight⁻¹.day⁻¹]
TFA: trifluoro acetic acid
THF: tetrahydrofurane
TLC: thin layer chromatography
tMCS: trimethylsilylchloride
TNBS: 2,4,6-trinitrobenzenesulfonic acid
t-TDI: temporary TDI [mg.kg bodyweight⁻¹.day⁻¹]
TTI: time temperature indicator
USA: United States of America
US: United States (of America)
VCM: vinyl chloride monomer
UV: ultra violet

Chemical interactions between packaging materials and foodstuffs

A : contact surface between the polymer and the food \([\text{cm}^2]\)

\(A_P\) : effect of the polymer on the diffusion

B : absorbance

\(B_0\) : absorbance in absence of analyte

b : constant related to the effect of the molecular weight of the migrant on diffusion \([\text{mole.g}^{-1}]\)

C : concentration of a compound \([\text{g.cm}^{-3}]\)

c : constant related to the effect of the temperature on the diffusion \([\text{K}^{-1}]\)

\(C_F\) : concentration of a component in the food \([\text{mg.kg}^{-1}]\) or equivalent

\(C_F,\text{daily}\): concentration of the indirect food additive in the daily diet \([\text{mg.kg}^{-1}]\) or equivalent

\(C_F,\text{daily,j}\) : concentration of the indirect food additive in the daily diet, due to contact with material j \([\text{mg.kg}^{-1}]\) or equivalent

\(C_F,\text{experimental}\) : experimentally observed concentration of a component in the food \([\text{mg.kg}^{-1}]\) or equivalent

\(C_F,e\) : equilibrium concentration of a component in the food \([\text{g.cm}^{-3}]\)

\(C_F,j\) : concentration of the indirect food additive in food contacted with material j \([\text{g.cm}^{-3}]\)

\(C_F,\text{predicted}\) : predicted concentration of a migrating component in the food \([\text{kg.m}^{-3}]\)

\(C_F,t\) : concentration of a component in the food at time t \([\text{g.cm}^{-3}]\)

\(C_P,0\) : initial concentration of a component in the polymer \([\text{g.cm}^{-3}]\)

\(C_P,t\) : concentration of a component in the polymer at time t \([\text{g.cm}^{-3}]\)

\(C_P,e\) : equilibrium concentration of a component in the polymer \([\text{g.cm}^{-3}]\)

\(C_F\) : consumption factor of a particular food contact material

\(C_F,j\) : consumption factor of food contact material j

C/M : contact material

D : diffusion coefficient \([\text{cm}^2.\text{s}^{-1}]\)

\(D_F\) : diffusion coefficient of a compound in the food \([\text{cm}^2.\text{s}^{-1}]\)
Annex 2: symbols

DP : diffusion coefficient of a compound in the polymer [cm².s⁻¹]
DP,f : diffusion coefficient of a food component in the polymer [cm².s⁻¹]
DP,m : diffusion coefficient of a migrant in the polymer [cm².s⁻¹]
D₀ : diffusion coefficient [cm².v⁻¹]
d : ion concentration [M]
E : environment
Eₐ : activation energy of diffusion [kJ.mole⁻¹]
Em : emission wavelength [nm]
Ex : excitation wavelength [nm]
e : the base number of the natural logarithm (ln(e) = 1)

erf(z): error function of the variable z
erfc(z) : 1-erf(z)
F : food
f : charge
f_j : food type distribution factor of contact material j
I : ionic strength [mM]
I₅₀, compound : concentration of the analyte at which the absorbance equals half of the maximal absorbance in an ELISA
Jₓ, Jᵧ, Jₜ : mass flux of a component, in the respectively the direction of the x, y and z-axis
Jₓ(x); Jₓ(x+∆x) : mass flux of a component, in the direction of the x-axis at place x and x+∆x respectively
Jᵧ(y); Jᵧ(y+∆y) : mass flux of a component, in the direction of the y-axis at place y and y+∆y respectively
Jₜ(z); Jₜ(z+∆z) : mass flux of a component, in the direction of the z-axis at place z and z+∆z respectively
Kₚ/F : partition coefficient of a component between the food and the polymer
k : rate constant of a chemical reaction of order m [(cm³.g⁻¹)m.s⁻¹]
Lₚ : thickness of the polymer [cm]
M : molecular weight [g.mole⁻¹]
m_F,₀ : initial amount of a migrating substance in the food [g]
Annex 2: symbols

\( m_{F,t} \): amount of a migrating substance in the food at time \( t \)  \([g]\)
\( m_{F,e} \): amount of a migrating substance into the food at equilibrium \([g]\)
\( m_{P,0} \): initial amount of a migrating substance in the polymer \([g]\)
\( m_{P,e} \): amount of a migrating substance in the polymer at equilibrium
\( M_r \): molecular weight of the migrant \([g.mole^{-1}]\)
\( m \): order of a chemical reaction
\( n \): mathematical help variable
\( P \): polymer or plastic
\( R \): universal gas constant= 8.314 \([J.mole^{-1}.K^{-1}]\)
\( R_f \): retention factor in TLC experiments
\( q_n \): positive root of the trigonometric equation \( \tan(q_n) = -\alpha q_n \)
\( S \): lower asymptote to the competition curve as defined in formula [43]
\( T \): absolute temperature \([K]\)
\( T_g \): glass transition temperature \([K]\)
\( t \): time \([s]\)
\( V_F \): volume of the food \([cm^3]\)
\( V_P \): volume of the polymer \([cm^3]\)
\( x \): concentration of the analyte (formula [43])
\( z \): dimensionless factor as defined in equation [19]

\( \alpha \): dimensionless factor as defined in equation [14]
\( \beta \): dimensionless factor as defined in equation [22]
\( \Delta x, \Delta y, \Delta z \): small step in respectively the \( x, y, z \), direction \([cm]\)
\( \delta \): chemical shift in NMR \([ppm]\)
\( \xi \): constant related to the dependence of the diffusion coefficient upon \( M_r \)
\( \psi \): constant related to the activation energy of the diffusion \([J.g^{1/3}.mole^{2/3}]\)
\( \mu_F \): chemical potential of a component in the food \([J.mole^{-1}]\)
\( \mu_P \): chemical potential of a component in the polymer \([J.mole^{-1}]\)
\( \pi \): the number pi
ω₁: mass fraction of a solvent in a polymer