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New investigations into macular pigment optical density

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NEW INVESTIGATIONS INTO MACULAR PIGMENT OPTICAL DENSITY

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Macular pigment (MP) is the collective name for three carotenoids, lutein, zeaxanthin and meso-zeaxanthin, which are found at high concentrations in the central macula. The macular carotenoids, like all carotenoids, are entirely of dietary origin. The term 'macular pigment optical density' (MPOD) refers to the peak concentration of MP in the retina, which varies from one individual to the next and is measurable in vivo. On account of its blue-light-filtering and antioxidant properties, MP has become a subject of interest with respect to age-related macular degeneration (AMD), the hypothesis being that MP helps to protect against AMD; the higher the MPOD, the lower the risk for AMD.

Recently, a new MPOD-measuring device, the MPS 9000 (MPS), entered the ophthalmic market. Using this device, the research described here aimed to contribute new information to the MP literature. A second MPOD instrument, the Macular Pigment Reflectometer, was also used at times, but a reliability study (included in the thesis) demonstrated that it was unsuitable for use on its own.

First, a series of exploratory investigations were undertaken to maximize the accuracy and consistency of MPOD measurements taken with the MPS; a protocol was established that substantially improved repeatability. Subsequently, a series of MPOD-based studies were conducted on anisometropia, South Asian race, blue-light-filtering contact lenses, and dietary modification with kale. The principle findings were as follows: interocular MPOD differences were not attributable to interocular refractive error differences; young adults of South Asian origin had significant gender-related MPOD differences (males>females, $p<0.01$), and they also had significantly higher MPOD than Caucasians ($p<0.0005$); wearing blue-light-filtering contact lenses for eight months did not affect MPOD; and dietary modification with kale for 16 weeks did not increase MPOD.

This body of research adds new insights to MP knowledge, which in turn may contribute to MP knowledge in the context of AMD.

Keywords: heterochromatic flicker photometry; kale; lutein; macular degeneration.

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CONTENTS

LIST OF APPENDICES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACD	Anterior chamber depth
AF	Autofluorescence
AL	Axial length
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
BMI	Body mass index
BVS	Best vision sphere
CL	Contact lens
cm	Centimetre
C-only	Central only
CP	Central and peripheral
CR	Coefficient of repeatability
CV	Coefficient of variation
dB	Decibel
DS	Dioptre sphere
FR	Fundus reflectometry
g	Gram
HFP	Heterochromatic flicker photometry
HPLC	High-performance liquid chromatography
Hz	Hertz
IOL	Intraocular lens
K	Keratometry (corneal curvature)
kcal	Kilocalorie
L	Lutein
LE	Left eye
LED	Light-emitting diode
LogMAR	Logarithm of the minimum angle of resolution
mcg	Microgram
mg	Milligram
mm	Millimetre
MP	Macular pigment
MPOD	Macular pigment optical density
MPR	Macular Pigment Reflectometer
MPS	MPS 9000, M|POD, QuantifEYE
MZ	Meso-zeaxanthin

n	Number of subjects
nm	Nanometre
RE	Right eye
RPE	Retinal pigment epithelium
RRS	Resonance Raman spectroscopy
SD	Standard deviation
UK	United Kingdom
USDA	United States Department of Agriculture
UV	Ultraviolet
UV CL	Ultraviolet-filtering contact lens
VA	Visual acuity
VEP	Visual evoked potential
W	Watt
Z	Zeaxanthin

CHAPTER 1: INTRODUCTION

Macular pigment (MP) is the collective name for three carotenoids, lutein (L), zeaxanthin (Z) and meso-zeaxanthin (MZ), which are found at high concentrations in the central macula, to the exclusion of all other carotenoids (Bone et al. 1993). They are only available to the body by dietary intake of foodstuffs or supplements containing them (Malinow et al. 1980; Neuringer et al. 2004). In this opening chapter, the dietary origins of the macular carotenoids and their pathway to the eye are briefly described, followed by an account of the history of MP discovery, its functions, the methods by which it can be quantified, its associations, and finally the evidence for its main proposed purpose.

1.1 Lutein and zeaxanthin

Lutein and Z are part of the carotenoid family of pigments, or more specifically they are xanthophyll hydroxycarotenoids (Snodderly et al. 1984b; Khachik et al. 1992). Xanthophylls (Greek for 'yellow leaf'), and in indeed all carotenoids, are entirely of dietary origin, confirmed in the case of L and Z by Malinow et al. (1980) and later by Neuringer et al. (2004) with their studies on monkeys. Compared with monkeys raised on a diet containing xanthophylls, Malinow et al. found that monkeys raised on xanthophyll-free diets had no detectable xanthophylls in their blood serum (or retina). Neuringer et al. reported the same, but armed with a greater level of knowledge at that point in time, they were able to report more precisely that no L or Z was detectable in the monkeys' serum (or retina). Furthermore, following supplementation with L and Z, these carotenoids did become evident in the serum (and retina), thus proving that while the primate body cannot synthesize L and Z, it can still absorb L and Z even after years of xanthophyll deprivation (the monkeys were 8 to 16 years old before the supplementation began).

Good sources of L and/or Z include green vegetables, particularly spinach, kale and courgettes; brightly-coloured vegetables, particularly corn and orange peppers; egg yolks (the result of a high carotenoid intake by hens); certain spices, namely, paprika (made from peppers); and perhaps less intuitively, pistachio nuts (Sommerburg et al. 1998; Perry et al. 2009; Kay et al. 2010; USDA 2010). Although many fruits do contain L/Z, the levels are not especially high, with the exception of the goji berry, which contains a considerable amount of Z (Zhou et al. 1999; Leung et al. 2001).

Following ingestion and digestion of appropriate food, L and Z are believed to be absorbed from the small intestine via a mainly protein-mediated mechanism (Reboul et al. 2005), after which they are transported to the liver on chylomicrons (Erdman et al.

1993; Gärtner et al. 1996), a type of lipoprotein, before being released into the plasma on other forms of lipoprotein. Although it is widely acknowledged that L and Z are transported on lipoproteins, it is not entirely clear which ones are involved. For example, Erdman et al. (1993), and Goulinet and Chapman (1997), reported that L and Z were distributed fairly evenly between low-density lipoproteins and high-density lipoproteins, whereas Cardinault et al. (2005), Wang et al. (2007) and Loane et al. (2010b) found that there was a preference for high-density lipoproteins. Either way, lipoproteins are the carrier method by which L and Z are delivered to bodily tissues, including the retina.

The delivery of L and Z to the retina is not well understood, but in recent years the idea of retinal uptake via xanthophyll-binding proteins has grown in momentum (Bernstein et al. 1997; Yemelyanov et al. 2001; Bhosale et al. 2004; Bhosale et al. 2009a), and Z-specific and L-specific binding proteins have now been identified in the human retina (Bhosale et al. 2004; Bhosale et al. 2009a).

1.2 Macular pigment

Macular pigment is the name used to describe the combined presence of L and Z in the retina, and in particular the fovea, where their concentration is greatest (Bone et al. 1985; Bone et al. 1988; Handelman et al. 1988). Although a conspicuous yellow pigmentation at the macula (or more specifically, the fovea) had been noted for well over 150 years (Nussbaum et al. 1981), it was George Wald in the 1940s who first suggested that MP was composed of a xanthophyll such as L (Wald 1945, 1949). Forty years later, his suggestion was proven to be correct, with official identification made by Bone and colleagues in 1985 (Bone et al. 1985). Using high-performance liquid chromatography (HPLC) on human donor retinas, these researchers also noted the presence of Z, and in 1993 they identified the third carotenoid, MZ, which is a stereoisomer of Z (Bone et al. 1993). Unlike L and Z, MZ is not found in foods typical of a human diet (Maoka et al. 1986). As a result, it is undetectable in serum, and was thought by Bone et al. to be the result of L transformation within the retina (Bone et al. 1993). This was confirmed by Johnson et al. (2005) as part of their series of studies concerning the nutritional manipulation of monkeys raised on diets absent of L and Z. To the present day, L, Z and MZ remain the only carotenoids to be located in the retina, despite there being at least 14 major carotenoids (e.g., lycopene, beta-carotene) circulating in the human body (Khachik et al. 1997a; Khachik et al. 1997b), and although many ocular structures do contain L and Z (e.g., the lens and uvea) (Yeum et al. 1995; Bernstein et al. 2001), it is the retina that holds the majority (Bernstein et al. 2001).

Anatomically, the macula covers a circular area of approximately 5.5 mm (19°) diameter in the central retina (Remington 1998), with the fovea accounting for the middle 1.5 mm (5.2°) diameter, followed by the parafovea with a width of 0.5 mm (i.e., 2.6-4.3° eccentricity from the centre of the fovea), and then the perifovea with a width of 1.5 mm (i.e., 4.3-9.5° eccentricity from the centre of the fovea). The spatial distribution of MP peaks in the central 0.5 mm (1.7°) of the fovea, with a rapid decline in concentration from 0.25 mm eccentricity onwards (Bone et al. 1988). It reaches near zero levels by 2 mm (7°) eccentricity, but it never disappears completely, even in the far peripheral retina (Bone et al. 1988). In the central fovea, up to 2.3 mm eccentricity, Z (and to a lesser degree, MZ) is the dominant carotenoid over L (Bone et al. 1988; Handelman et al. 1988; Bone et al. 1997), with a L:Z ratio of 1:2.4 at 0.25 mm eccentricity. Beyond 2.3 mm, L becomes dominant, and the L:Z ratio swaps over by 5.8 mm eccentricity onwards, such that the amount of L is more than double that of Z (Bone et al. 1988).

Within the retinal layers, MP is primarily located in the cone photoreceptor axons of the rod-free central fovea, also known as Henle's fibres (Snodderly et al. 1984a; Snodderly et al. 1984b). Beyond this point, MP continues to be located in photoreceptor axons (here part of the outer plexiform layer), but it is also present in high concentrations in the interneurons of the inner plexiform layer (Snodderly et al. 1984a; Snodderly et al. 1984b; Trieschmann et al. 2008). Somewhat surprisingly, until very recently the retinal layer localization of MP had only been established in monkey retinas, but in 2008 the same arrangement was confirmed in humans (Trieschmann et al. 2008). In addition to the plexiform layers, between 10 and 25 percent of MP has been located in rod photoreceptor outer segments in the perifoveal and peripheral retina (Sommerburg et al. 1999; Rapp et al. 2000). As per MP distribution in general, the concentration is higher in the perifovea than the periphery, and the ratio of L:Z rises with increasing eccentricity (Rapp et al. 2000). Of note, there do not appear to be any published studies that have looked for MP in cone photoreceptor outer segments.

The selective accumulation of L and Z in the retina and their high concentration in the fovea suggests that their presence may not be a mere coincidence, and that MP might be there to serve a bigger purpose. A good place to start in attempting to elucidate any such purpose is to consider the proposed functions of MP, of which there are two.

First and foremost, it has been conclusively established that MP is a blue light filter. Its absorption spectrum peaks around 460 nm, and it reaches a minimum in the

ultraviolet/violet (around 380 nm) and green (around 540 nm) regions of the electromagnetic spectrum (Wyszecki and Stiles 1982; Snodderly et al. 1984b; Handelman et al. 1991; Bone et al. 1992). Research demonstrates that short-wavelength (blue) visible light is more damaging to the retina than longer-wavelength visible light (Ham et al. 1976; Ham et al. 1978), so with its pre-receptoral location, MP is well placed to attenuate the amount of blue light reaching the photoreceptors and beyond, thus limiting the subsequent damage that could ensue (Junghans et al. 2001). In fact, L and Z have been proven as more efficient blue light filters than some other carotenoids (beta-carotene and lycopene) (Junghans et al. 2001), making their sole carotenoid presence in the macula all the more evocative. In particular, L appears to be better than Z in terms of blue light absorption efficiency, which is believed to be the result of its two orthogonal orientations (compared with Z's one orientation) in lipid membranes making it more suited to absorbing blue light from all directions (Sujak et al. 1999; Junghans et al. 2001).

For three main reasons, the retina is subjected to oxidative stress, i.e., cell damage or death that is caused by reactive oxygen species such as free radicals, singlet oxygen and hydrogen peroxide (Beatty et al. 1999; Beatty et al. 2000b). Firstly, it is exposed to a lot of visible light, secondly, it uses large amounts of oxygen, and thirdly, it contains a high number of polyunsaturated fatty acids (Beatty et al. 1999; Beatty et al. 2000b). Light and oxygen consumption, in isolation or in combination, result in the generation of reactive oxygen species, while membrane-bound polyunsaturated fatty acids are highly susceptible to cell damage by free radical-induced lipid peroxidation (Beatty et al. 1999; Beatty et al. 2000b). As discussed above, short-wavelength visible light has been shown to be more damaging to the retina than longer-wavelength visible light, so it could well be that MP helps to protect against oxidative stress passively in its capacity as a blue light filter. However, there is also the second proposed function of MP to consider, which is its role as an antioxidant.

Antioxidants are substances that can mount a defence against oxidative stress, and carotenoids, including xanthophylls like L and Z, are known antioxidants (Sies and Stahl 1995), which is why MP is thought to exercise this property in the retina. Numerous in vitro retinal studies have demonstrated the antioxidant abilities of L and Z (e.g., Sundelin and Nilsson 2001; Wrona et al. 2004; Kim et al. 2006; Chucair et al. 2007; Bhosale et al. 2009b; Li et al. 2009; Nakajima et al. 2009; Li et al. 2010; Sasaki et al. 2010), but the most persuasive evidence for a MP antioxidant function comes from Khachik et al. (1997a), who identified the direct oxidation products of L and Z in human donor retinas (and monkey retinas); of note, the oxidation products were not attributable to postmortem

events. Whereas L has been reported to be a better blue light filter than Z (Sujak et al. 1999; Junghans et al. 2001), it seems that Z may take the lead in terms of antioxidant ability, with some studies demonstrating that Z may be a more effective antioxidant than L (Kim et al. 2006; Trevithick-Sutton et al. 2006).

The outer and inner plexiform layer location of MP is not especially conducive to an antioxidant role, so it may be that the blue-light-filtering characteristics of MP are more important than the antioxidant characteristics. However, it is not to be forgotten that MP is also present in the outer segments of perifoveal rods (Sommerburg et al. 1999; Rapp et al. 2000), an area with considerable oxidative stress due to a very high polyunsaturated fatty acid count (Beatty et al. 2000b). Consequently, a valuable antioxidant function for MP does seem likely (Rapp et al. 2000; Subczynski et al. 2010).

Although MP invariably reaches its greatest concentration in the central fovea, the actual amount of MP here is not fixed; it varies widely between individuals, and as with the earlier-mentioned nutritional manipulation investigations on monkeys (Neuringer et al. 2004; Johnson et al. 2005), it can be increased in many humans by regular consumption of foods or supplements rich in L and/or Z (e.g., Hammond et al. 1997a; Wenzel et al. 2006b; Connolly et al. 2010). The measured level of MP in its peak area of concentration at the fovea has long been known as macular pigment optical density (MPOD) (Bone and Sparrock 1971), and logically, thinking of the two proposed functions of MP, it would be anticipated that the higher the MPOD, the greater the capacity for blue light absorption and combating of oxidative stress. Indeed, it has been estimated that an 'average' MPOD results in 20 to 40 percent absorption of 460 nm light, whereas a very high MPOD results in 90 to 98 percent absorption of 460 nm light (Hammond and Caruso-Avery 2000; Landrum and Bone 2001).

1.3 Measuring macular pigment optical density in vivo

The ex vivo techniques, such as HPLC, that have been used to measure MPOD in donor and animal retinas have proved invaluable for establishing the components of MP and its spatial location within the retina. Nonetheless, they are clearly not suitable for use in living populations, and therein lies their limitation. Fortunately, the spectral and spatial aspects of MP have afforded the development of in vivo techniques that can measure MPOD in the vast majority of individuals. These in vivo techniques are noninvasive, and they are normally categorized under one of two headings: psychophysical (requiring a response from the subject) or objective (requiring minimal input from the subject). Each method has confirmed the large range of MPOD in human population samples, from

virtually no MP to greater than one log unit optical density, with average levels ranging from 0.16 (Wüstemeyer et al. 2003) to 0.69 (van de Kraats et al. 2008), depending on the method used and/or the study population. A detailed review of the in vivo MPOD measurement techniques has been published (Howells et al. 2011) and can be found in appendix 1, so here only the basic measurement principles of each approach are described.

PSYCHOPHYSICAL TECHNIQUES

The psychophysical methods of measuring MPOD include the following:

Threshold spectral sensitivity (e.g., Brown and Wald 1963; Bone and Sparrock 1971; Stabell and Stabell 1980; Pease and Adams 1983; Pease et al. 1987).
Colour matching (e.g., Ruddock 1963; Moreland and Bhatt 1984; Moreland and Alexander 1997; Davies and Morland 2002).
Dichroism-based measurements (e.g., Bone 1980; Bone et al. 1992).
Minimum motion photometry and apparent motion photometry (see below).
Heterochromatic flicker photometry (see below).

The first three of the psychophysical methods have now been largely superseded by heterochromatic flicker photometry (HFP) and, to some extent, minimum motion photometry. This is in part due to their increased level of difficulty and/or the longer time needed to perform them (Hammond et al. 2005).

HETEROCHROMATIC FLICKER PHOTOMETRY

Developed by Ives in the early 1900s (cited by Viner 2003), HFP has so far been the most commonly used of all the techniques for measuring MPOD. As such, it is often used as a standard against which other techniques are validated (e.g., Delori et al. 2001; Neelam et al. 2005; van de Kraats et al. 2006; Bone et al. 2007a; Canovas et al. 2010), although at present there is no true 'gold-standard' in vivo measure of MPOD.

The use of HFP to measure MP levels was first described over 30 years ago by Werner and Wooten (1979), but the technique wasn't elaborated on until 1987, in a key paper by Werner and co-workers (Werner et al. 1987). Since then, HFP has been developed and used by numerous research groups investigating MP. Key papers incorporating detailed descriptions and variations of the technique include Hammond and Fuld (1992), Hammond et al. (1997c), Landrum et al. (1997), Wooten et al. (1999), Beatty et al. (2000a), Mellerio et al. (2002), Bone and Landrum (2004), Snodderly et al. (2004), Tang

et al. (2004), Iannaccone et al. (2007), Stringham et al. (2008), and van der Veen et al. (2009a). All other studies using HFP to measure MPOD tend to use the instruments originally designed or developed by these investigators.

In conjunction with many of the MPOD techniques, HFP makes use of the spectral absorption properties and retinal location of MP. Essentially, HFP determines MPOD by presenting a light stimulus of two alternating wavelengths at the fovea and at an eccentric retinal area (technically the parafovea or perifovea but often referred to interchangeably as either the parafoveal or peripheral measure). The wavelengths are chosen such that one is a short-wavelength blue light that is maximally absorbed by MP, and the other is a longer-wavelength green to yellow light that is not absorbed by MP. If the lights are alternated at an appropriate frequency and their luminances are not perceived to be equal by the subject, the stimulus will appear to flicker; the perceived colour of the stimulus will be an amalgamation of the two source colours (Beatty et al. 2000a; Bone and Landrum 2004; Snodderly et al. 2004; Loane et al. 2007). Typically, the radiance (often also termed intensity) of the blue light is adjusted by the subject until the observed flicker is minimized (e.g., Hammond et al. 1997c; Landrum et al. 1997; Wooten et al. 1999; Mellerio et al. 2002; Snodderly et al. 2004; Tang et al. 2004; Stringham et al. 2008). This occurs when there is an equiluminance match between the blue and green lights (Loane et al. 2007; Kirby et al. 2009). The procedure is carried out with the subject looking directly at the stimulus (i.e., the foveal measure), and then it is repeated with the subject looking away from the stimulus so that the eccentric measure (where MP is assumed to be negligible) can be made (Snodderly and Hammond 1999). Since MP reaches its highest concentration in the fovea, it will absorb some of the blue light before it reaches the photoreceptors, whereas in the area of negligible MP, very little blue light will be absorbed. Consequently, a greater radiance of blue light is required at the fovea than at the parafovea to appreciate minimal flicker. The log ratio of the radiance of blue light needed at the fovea compared with that needed at the parafovea gives a measure of MPOD (see formula 1). It should be noted that the chief (but not only) purpose of the eccentric measure is to cancel out the effect of any blue light absorption by the crystalline lens (which happens as a consequence of age-related lens yellowing) so that the final density value is a representation of MP alone, and not MP plus lens yellowing (Makridaki et al. 2009) (see formula 2).

$MPOD = \log (R^f_{\lambda s} / R^p_{\lambda s}) - \log (R^f_{\lambda \gamma} / R^p_{\lambda \gamma})$

Formula 1 Example calculation for macular pigment optical density (MPOD), from Stringham et al. (2008). $R^f_{\lambda s}$ = radiance of a peak MP absorption wavelength, e.g., 460 nm, measured at a foveal location. $R^p_{\lambda s}$ = radiance of a peak MP absorption wavelength, measured at an eccentric location, e.g., 7°. $R^f_{\lambda \gamma}$ = radiance of a negligible MP absorption wavelength, e.g., 570 nm, measured at a foveal location. $R^p_{\lambda \gamma}$ = radiance of a negligible MP absorption wavelength, measured at an eccentric location.

$B\ fov \times T_{lens} \times T_{MP} = B\ ref \times T_{lens}$ (i)

$T_{MP} = B\ ref / B\ fov$ (ii)

$MPOD = \log 1/T_{MP} = \log (B\ fov / B\ ref)$ (iii)

Formula 2 Derivation of MPOD, from Snodderly and Hammond (1999). The transmission of blue light through the MP (T_{MP}), and the transmission of blue light through the lens (T_{lens}) is taken into account. B fov and B ref are the radiances of blue light needed to minimize flicker at the fovea and parafovea (reference) respectively. Since T_{lens} is assumed to be the same at the fovea and parafovea, it is removed from equation (i). The final equation (iii) is a simplified version of formula 1.

MOTION PHOTOMETRY

The minimum motion paradigm was initially described 100 years ago by Stumpf (1911), although this went largely unnoticed until its translation into English by Todorović in 1996 (Todorović 1996; Moreland 2004). With parallels to HFP, it refers to the perceived reduction in motion of a moving square or sine wave grating as equiluminance of the colours involved is reached. The concept was taken up for use in photometry by both Moreland (1980, 1982) and Anstis and Cavanagh (1983), but in subtly different ways. This then led to the use of minimum motion photometry for in vivo measurement of MP (e.g., Moreland and Bhatt 1984; Moreland et al. 1998).

Many of the principles described for HFP also apply for motion photometry, i.e., a wavelength of light at the peak of MP absorption is compared with a wavelength of light not absorbed by MP, at central and eccentric locations. Moving square wave gratings are used, with the bars being alternately illuminated by the two light wavelengths. The radiance of the longer-wavelength stimulus is adjusted until the motion appears to either slow down or change direction (Moreland 1980, 1982; Anstis and Cavanagh 1983),

depending on the method being employed. The slowing down of the grating is minimum motion photometry, whereas the reversal of grating movement is known as apparent motion photometry (Moreland 2004). As with HFP, different radiances of the test wavelength are required for equiluminance at the foveal and parafoveal positions, on account of the higher levels of MP at the fovea. A log ratio of these radiances provides a measure of MPOD (Moreland et al. 1998; Moreland et al. 2001; Robson et al. 2003; Moreland 2004; Robson et al. 2005; Robson et al. 2006; Robson and Parry 2008).

OBJECTIVE TECHNIQUES

The objective methods for measuring MPOD are:

Fundus reflectometry
Fundus autofluorescence
Resonance Raman spectroscopy
Electrophysiology using visual evoked potentials

FUNDUS REFLECTOMETRY

Quantitative measurement of light reflected from the fundus is known as fundus reflectometry (FR), and the researchers Brindley and Willmer (1952) were the first to adopt this technique. Their aim was to estimate MPOD in vivo by comparing light reflected at the macula with light reflected from a peripheral area of retina. Since then, FR has gone on to become the most widely used of the objective methods for MPOD measurement, although many improvements and variations have happened along the way.

When light enters the eye it passes through many structures, including the cornea, the lens, the retina and the choroid. Some of these structures (and their components) will reflect a small part of the light, whilst others will absorb part of it. Through measurement of reflected light from the retina and choroid, FR is able to assess several ocular features, including MP (Berendschot and van Norren 2004).

Although there are several variations on the reflectometry procedure, there are two methods that predominate. The first is a comparison technique, similar to that used in HFP. Light reflected from the fovea is compared with light reflected from an eccentric retinal area, using two wavelengths (one absorbed by MP and one not) or using a spectrum of wavelengths. Since MP absorbs rather than reflects certain wavelengths, there will be a difference in the observed reflectance at the fovea and periphery, owing to

the assumed lack of MP at the eccentric site. Researchers who have used this method include Brindley and Willmer (1952), van Norren and Tiemeijer (1986), Delori and Pflibsen (1989), Elsner et al. (1998), Berendschot et al. (2000), Delori et al. (2001), Bour et al. (2002), Wüstemeyer et al. (2002), and Cardinault et al. (2003).

The second core technique is known as a spectral analysis (Berendschot and van Norren 2004). As the name suggests, this involves the analysis of a spectrum of reflected light from a spot of light on the retina. To achieve this, a detailed optical model of the pathways of light in the eye is required. A number of optical models of increasing complexity have been proposed over the years, from van Norren and Tiemeijer (1986) through to van de Kraats and van Norren (2008). Probably the most familiar optical model is that derived by van de Kraats et al. (1996), which has been used to work out MPOD in several studies (e.g., Berendschot et al. 2000; Berendschot et al. 2002b; Berendschot and van Norren 2005; van de Kraats et al. 2006; Kanis et al. 2007a, 2007b). In essence, the density of MP is determined using its known spectral characteristics, and by taking into account the amount of light reflected at the internal limiting membrane, the photoreceptor discs and the sclera (van de Kraats et al. 1996; Berendschot et al. 2000; Berendschot and van Norren 2005; van de Kraats et al. 2006; Kanis et al. 2007b). The densities of the lens, melanin and blood are likewise calculated.

FUNDUS AUTOFLUORESCENCE

One of the newer methods for MPOD measurement relies on the intrinsic fluorescence, or autofluorescence (AF), of lipofuscin in the retinal pigment epithelium (RPE). Lipofuscin in the RPE is a waste product of photoreceptor outer segment phagocytosis, and it accumulates with age (Delori 1994; Delori et al. 1995; von Rückmann et al. 1995). When excited with light wavelengths of 400 to 590 nm, lipofuscin fluoresces, emitting light in the wavelength range 520 to 800 nm (Delori 2004). Delori (1994) was the first to develop a technique for fundus AF, with the primary aim being to measure lipofuscin. Further studies by Delori et al. (1995) and von Rückmann et al. (1995) provided evidence for lipofuscin being the main fluorophore in AF. It was their observations of a decrease in AF at the macula that lead to the use of AF as a means for measuring MPOD.

To recap, the absorption spectrum of MP is in the range of 380 to 540 nm, and the absorption spectrum of lipofuscin is in the range of 400 to 590 nm. Since MP is located anterior to lipofuscin, incoming light directed at the fovea will be absorbed by MP before it reaches the lipofuscin, provided the wavelength of the light is within the absorption range of MP. Accordingly, there will be an attenuation of lipofuscin fluorescence at the macula;

the more MP present, the higher the level of attenuation. By comparing the emitted AF at the fovea and parafovea of two excitation wavelengths, one that is well absorbed by MP and one that is not, MPOD can be calculated (Delori et al. 2001).

Two AF procedures exist for measurement of MP. The first is a comparison method as used in HFP and some forms of FR; the emitted fluorescence is collected from a foveal and parafoveal sampling area, and then compared to give a measure of MPOD (e.g., Delori et al. 2001). The second and more common procedure is an imaging method whereby up to 32 images (Robson et al. 2003; Robson et al. 2005) are taken in succession with one or two wavelengths. The images are aligned (manually or using dedicated software) and averaged, then a greyscale index of intensity is used to generate density maps of MP, which includes a measure of peak MPOD. Key studies using the AF imaging technique include those of Wüstemeyer et al. (2003), Berendschot and van Norren (2005), Delori et al. (2006), Liew et al. (2006), Trieschmann et al. (2006), and Wolf-Schnurrbusch et al. (2007).

RESONANCE RAMAN SPECTROSCOPY

With the exception of electrophysiology methods, resonance Raman spectroscopy (RRS) is the most recent MPOD technique and, arguably, the most controversial. First described by Bernstein et al. (1998), RRS takes advantage of L and Z's ability to exhibit a phenomenon called Raman scattering (Koyama et al. 1988). Over the last ten years, the use of RRS to measure MPOD has quickly gained momentum, with many papers published on its use (Ermakov et al. 2001; Bernstein 2002; Bernstein et al. 2002; Gellermann et al. 2002a; Gellermann et al. 2002b; Zhao et al. 2003; Bernstein et al. 2004; Ermakov et al. 2004; Gellermann and Bernstein 2004; Ermakov et al. 2005; Neelam et al. 2005; Hogg et al. 2007; Obana et al. 2008; Sharifzadeh et al. 2008).

When monochromatic light is directed at a molecule, some of the light is scattered. Most of the light is scattered elastically (Rayleigh scattering), but a small proportion is scattered inelastically (Raman scattering). When this inelastic scattering happens, there is a wavelength shift of the incident light, known as a Raman shift; the shift in wavelength is molecule-specific, and therefore the back-scattered light can be collected and analysed to identify the molecule in question (Bernstein et al. 2004). Usually the Raman signal is very weak, and as a result, is not easily identified. However, if the incident wavelength overlaps with the absorption spectrum of the molecule, a large resonance enhancement of the Raman-scattered light occurs, and the molecule can be recognized. Carotenoids, including L and Z, are an excellent example of this. When excited by 488 nm argon laser

light, they exhibit a resonance enhancement of up to five orders of magnitude (Bernstein 2002), with three characteristic Raman spectral peaks (Koyama et al. 1988; Bernstein et al. 1998; Gellermann et al. 2002a). The Raman line with the strongest peak is subsequently quantified into Raman counts, an MPOD measurement unit that is unique to this technique.

The Raman method is completely different from almost all other MPOD techniques, in that it measures absolute levels of MP in a one-mm (3.5°) area, with no peripheral consideration at all. The researchers in this field claim that this is acceptable because the signal is derived directly from the pigment itself, rather than relying on light that must travel to deeper layers of the retina (Gellermann et al. 2002a; Gellermann and Bernstein 2004), and that furthermore, the signal is only strong enough to register at carotenoid concentrations found in the macula, rather than in any other ocular structures (Bernstein et al. 1998).

ELECTROPHYSIOLOGY – VISUAL EVOKED POTENTIALS

The first suggestion that visual evoked potentials (VEPs) could potentially be used to detect MP was made over ten years ago by Moreland et al. (1998). This was investigated further by Robson et al. (2006) some time later. However, it is only recently that this particular technique has looked like it could be a truly viable method for measuring MPOD. Using steady-state VEPs, Robson and Parry (2008) measured MPOD across a range of eccentricities in three subjects. Blue-green gratings on a colour monitor were employed, and these same gratings were also used to measure MPOD with HFP. The VEP and HFP results were compared with each other, as well as with the equivalent MPOD as measured by minimum motion photometry. This required a correction factor on the part of the VEP and HFP results, to allow for the overlapping phosphor emissions of the blue and green stimuli. The correlation between all three techniques was excellent ($r \geq 0.94$, $p < 0.0005$, in all cases), suggesting that steady-state VEPs have potential as a valid, objective method for measuring MP and its distribution.

1.4 Macular pigment and age-related macular degeneration

Having such a diverse range of MPOD techniques is useful for MP research, but it does present difficulties for those wishing to compare MPOD values between techniques. Each method has its own benefits and limitations, and there is no clear ideal choice, as highlighted in a recent paper by Beatty et al. (2008). Nevertheless, the fact that the opportunity to measure MP in living subjects exists at all, despite any inherent limitations, must be a good thing, and owing to the availability of these techniques, a multitude of

studies have been able to investigate MPOD and its possible associations. Table 1.1 (page 33) is a summary of all the main variables to date that have been reported to have a statistically significant association with MPOD in at least one study. What is clear from the list of variables is that many of them are things that have also been linked to some degree with age-related macular degeneration (AMD), and this is no coincidence, for the two properties of MP discussed earlier have led to a growing belief that MP may have a role in protecting against this visually debilitating eye disease (Snodderly 1995; Beatty et al. 1999; Loane et al. 2008; Carpentier et al. 2009).

Time and again, AMD is reported to be the leading cause of irreversible visual impairment in Western societies (e.g., Leibowitz et al. 1980; Cooper 1990; Evans et al. 2004; Bunce and Wormald 2008). It is characterized in various stages by the development of degenerative age-related changes in the macula, such as soft drusen, hyperpigmentation and hypopigmentation of the RPE, geographic atrophy, RPE detachments, haemorrhages, and scarring (Bird et al. 1995). The exact aetiology of AMD is not yet fully understood, but it appears to be a highly multifactorial disease that is likely to have more than one underlying cause (AREDS 2000; Clemons et al. 2005). As a result, many factors (both modifiable and unmodifiable) could increase or decrease the risk of developing the disease, although to date, the only three undisputed risk factors are increasing age, family history and smoking (Smith et al. 2001; Connell et al. 2009).

One of the major theories for the pathogenesis of AMD is that of oxidative stress (Beatty et al. 2000b), and linked to that there is the possibility of a secondary pathogenesis in the form of cumulative exposure to short-wavelength light (Algvere et al. 2006). These factors were mentioned earlier in the context of MP function, and this is why MP has become a subject of interest for AMD, the hypothesis being that MP helps protect against AMD; the higher the MPOD, the lower the risk is for AMD. The evidence to support this hypothesis is diverse and complex, but the following is a summary of the pertinent points.

AMD AND MPOD PARALLELS

Numerous studies have examined MPOD with respect to putative risk factors for AMD, in order to investigate whether there are any shared variables for the likelihood of AMD occurrence and the likelihood of low MPOD. Table 1.1 includes all the risk factors that have been examined. The evidence, like that for many of the proposed AMD risk factors, is inconsistent. However, it should perhaps be pointed out that the studies that dedicate their investigations to one specific variable (e.g., smoking, gender, iris colour), as with the series of experiments by Hammond and colleagues in the 1990s and 2000s, for example

(Hammond et al. 1995; Hammond et al. 1996a; Hammond et al. 1996b; Hammond et al. 1996c; Hammond et al. 1997b; Hammond et al. 1998; Hammond et al. 2002; Hammond and Wooten 2005), nearly always find a significant result, maybe because there are appropriate numbers of subjects in each group being examined, unlike the more all-encompassing studies. The relationship between MPOD and smoking, for instance, is often hindered by a lack of current or ex-smokers in the sample population (see table 1.1).

Taking the three established risk factors for AMD – increasing age, family history and smoking – it is interesting that the largest MPOD study to date that sought to establish risk factors for low MPOD (828 healthy subjects aged 20-60 years) obtained a statistically significant lower MPOD for each of these variables, i.e., lower MPOD with increasing age, family history of AMD, and a history of cigarette smoking (Nolan et al. 2007b).

OBSERVATIONAL STUDIES

In a thorough review by Beatty et al. (2004), the inter-relationships between dietary L and Z, serum L and Z, and MPOD were discussed. Unsurprisingly, these three variables were found to be correlated more often than not; the relationship between dietary L/Z and serum L/Z appears to be the most consistent, while the relationship between dietary L/Z and MPOD seems to be the least consistent (Beatty et al. 2004, and see table 1.1). The latter is probably the result of variations between subjects in terms of how effectively the L/Z they ingest is absorbed, transported and eventually deposited in the retina or other bodily tissues and organs. Nevertheless, studies that examine dietary L/Z as well as serum L/Z and MPOD with respect to AMD are still important in the quest to determine whether MP can protect against or delay the progression of AMD, and for that reason those studies are included here.

Of the cross-sectional or case-control observational studies that have compared healthy-eyed subjects with AMD subjects, four have reported a statistically significant decreased risk for AMD in subjects with a high dietary intake of L and Z (Seddon et al. 1994; Snellen et al. 2002; SanGiovanni et al. 2007; Seddon et al. 2010), while one found no association (Morris et al. 2007). Another study only found an association for subjects in a younger age bracket rather than the whole study population (Mares-Perlman et al. 2001). For the equivalent result in terms of serum L and Z, three studies have reported a statistically significant decreased risk for AMD (Gale et al. 2003; Delcourt et al. 2006; Zhou et al. 2011), and four found no association (Mares-Perlman et al. 1995; Mares-Perlman et al. 2001; Cardinault et al. 2005; Dasch et al. 2005). Interestingly, all three of the significant

results were stronger for serum Z than for serum L, thus suggesting that Z may have a bigger influence on AMD than L. For MPOD (see below for details), eight studies have reported a statistically significant lower mean MPOD in AMD subjects (mostly early AMD) than healthy-eyed subjects (Beatty et al. 2001; Bone et al. 2001; Bernstein et al. 2002; Wüstemeyer et al. 2002; Trieschmann et al. 2003; Obana et al. 2008; Nolan et al. 2010; Schweitzer et al. 2010), and six found no significant difference (Berendschot et al. 2002b; Ciulla and Hammond 2004; Koh et al. 2004; Kanis et al. 2008; LaRowe et al. 2008; Moeller et al. 2009). However, one of the 'insignificant' studies only comprised a total of 13 subjects (Koh et al. 2004), and another did report a statistically significant lower mean MPOD in late AMD subjects (as opposed to early) compared with healthy subjects (Kanis et al. 2008).

Of the longitudinal observational studies that have examined the incidence of AMD over time in a cohort of healthy-eyed subjects, two have reported a statistically significant decreased risk for AMD in subjects with a high dietary intake of L and Z (Tan et al. 2008; Ho et al. 2011), while five found no association (VandenLangenberg et al. 1998; Flood et al. 2002; van Leeuwen et al. 2005; Moeller et al. 2006; Cho et al. 2008). There have been no equivalent studies for serum L and Z, but there has been one for MPOD, which did not find any significant link with AMD incidence, although out of 419 healthy subjects at the start, only 30 developed early AMD (and two late AMD) (Kanis et al. 2007a). Of interest, an MPOD research group based in Waterford, Ireland, has plans to take part in a collaborative ageing study that will assess, among other things, MPOD at regular intervals over many years in a 'nationally representative sample' of 8000 participants minimum (aged 50 years and over), in order to comprehensively examine whether MPOD is related to AMD (Nolan et al. 2010).

In addition to the observational studies already discussed, very recently MPOD has been investigated in a group of 369 individuals with various stages of age-related maculopathy (ARM; in other words, early AMD). The researchers found no statistically significant differences in MPOD between the various stages (Dietzel et al. 2011). They also analysed MP levels in the ARM study eye while taking into account the disease status of the opposite eye. The results were not as perhaps anticipated, in that the mean MPOD of study eyes with ARM and opposite eyes with AMD (MPOD≈0.64, n=24) was significantly greater than the mean MPOD of study eyes with ARM and either no ARM (MPOD≈0.55, n=49, p=0.0207) or ARM (MPOD≈0.53, n=55, p=0.0070) in the opposite eye (Dietzel et al. 2011). The authors speculated that this difference might have been caused by previous L/Z supplement intake in those subjects diagnosed with AMD.

INTERVENTIONAL STUDIES

Some studies have reported on the effects in AMD subjects of increased L and/or Z via dietary modification and supplementation. These have been focused on observing improvements in visual function, rather than the clinical appearance of AMD, although two large-scale studies are now in progress (Neelam et al. 2008; AREDS2 2011).

Of the interventional studies that have examined the effect of L/Z supplementation on various aspects of visual function (e.g., visual acuity, glare response and contrast sensitivity), five have reported a statistically significant improvement (Olmedilla et al. 2001; Falsini et al. 2003; Richer et al. 2004; Cangemi 2007; Parisi et al. 2008) and one no improvement (Bartlett and Eperjesi 2007). It may be that the L dosage in the supplement used by the latter study was not high enough to bring about an improvement (Bartlett and Eperjesi 2007), as its 6 mg of L was lower than the amount used in all the other studies – dosage ranged from 8 to 15 mg of L, and only two supplements included Z (Cangemi 2007; Parisi et al. 2008). Richer et al. (2004) reported a concurrent increase in MPOD with visual function improvements, but this was the only study that measured MPOD. That said, there have been a host of observational and interventional investigations into visual function and MPOD in healthy-eyed participants (see table 1.1), and these have recently been reviewed by Loughman et al. (2010b) and Stringham et al. (2010). One final intervention study used a dietary modification approach; eleven AMD patients were instructed to considerably increase their consumption of spinach. The results, again, were positive (Richer 1999).

At the time of writing, there have not been any, nor do there appear to be any plans for, interventional studies on healthy subjects assessing whether L/Z supplementation is capable of reducing the incidence of AMD.

Out of all the observational and interventional studies, whether the findings were significant or not, it is noteworthy that with the exception, perhaps, of the study by Dietzel et al. (2011), none have reported a relationship between high dietary L/Z, high serum L/Z or high MPOD, and an increased risk for AMD, nor a relationship between L/Z supplementation and reduced visual function.

It is unfortunate that so few human studies have assessed MPOD in longitudinal and interventional AMD studies. In animal studies it has been shown that the retinas of quail and monkeys (which both selectively accumulate L and Z at the macula) fed a

xanthophyll-deficient diet are more susceptible to light-induced damage than animals fed a standard or xanthophyll-supplemented diet (Thomson et al. 2002; Barker et al. 2011), and interestingly, Thomson et al. reported that following Z supplementation, the amount of retinal damage was inversely related to the amount of MP but was unrelated to serum Z, which is perhaps suggestive of a more important role for MPOD in AMD than for dietary or serum L/Z (Thomson et al. 2002), although evidently there is some level of correlation between all three.

MPOD IN SUBJECTS WITH AMD VERSUS HEALTHY-EYED SUBJECTS

Convincing experimental evidence for an MPOD-AMD link comes from work by Bone et al. (2001) and Beatty et al. (2001). Bone and colleagues used HPLC to determine the amount of retinal L and Z in 56 donors with AMD and 56 donors without AMD (age, gender and race-matched controls). They found that the AMD eyes had significantly less L and Z across the whole retina (divided into inner, middle and outer regions, concentric with each other and centred on the fovea), therefore indicating that low MPOD is causally associated with AMD, rather than being the result of degenerative processes in the macula (Bone et al. 2001). Beatty and colleagues used living subjects and HFP to assess MPOD in nine individuals with and without AMD. The AMD subjects each had advanced AMD in one eye but no clinical signs of it in their other eye. The healthy eyes were designated 'high-risk' and their MPODs were compared in a case-control approach with nine healthy-eyed subjects, matched for age, gender, smoking status, iris colour and lens density. The mean MPOD of the high-risk eyes (0.147±0.144) was significantly lower than the mean of the control eyes (0.311±0.206, p=0.015). In fact, eight out of the nine high-risk eyes had significantly lower MPOD than their matched control eye (Beatty et al. 2001). Although not every in vivo study has found AMD subjects to have lower MPOD than healthy-eyed subjects (e.g., Berendschot et al. 2002b; LaRowe et al. 2008), a 'high-risk' versus control study like this one has only been repeated once; the result was the same as that for Beatty et al., using a different in vivo measurement technique (Obana et al. 2008).

CLINICAL OBSERVATIONS

Clinical evidence for MP being protective against AMD comes from observations that the central fovea, i.e., where MP peaks, tends to be the last area affected by geographic atrophy (Maguire and Vine 1986; Sarks et al. 1988; Schatz and McDonald 1989). Weiter and co-workers localized the spared areas in 40 cases of annular maculopathy to the exact presence of MP (Weiter et al. 1988). Furthermore, monkeys reared on a long-term

L and Z-free diet (discussed earlier) went on to develop drusen-like pathology (Malinow et al. 1980; Feeney-Burns et al. 1989).

Overall, the evidence regarding the role of MP in AMD is not conclusive, but given the multifactorial nature of AMD, this is not altogether unexpected. What's more, it is highly likely that an individual's MPOD is equally multifactorial, therefore adding to the inconsistency. Nevertheless, the evidence is highly suggestive of an association, and as one of the few modifiable potential factors in AMD, its continued investigation is extremely important.

1.5 Summary

Much progress has been made in our understanding of MP over the last 25 years, and with recent improvements in MPOD measurement technology, this is set to continue. Moreover, there are now three MPOD instruments available to optometrists and ophthalmologists for use in everyday clinical practice (the M|POD - Tinsley Ophthalmic, Redhill, Surrey, UK; the MacuScope - Macuvision Europe, Solihull, West Midlands, UK; and the Zeiss Visucam 200 - Carl Zeiss, Jena, Germany), which in time will hopefully allow MPOD testing to reach a wider populace. Using one of these devices, the body of research described in the upcoming chapters seeks to add to the growing conglomerate of MP knowledge.

Table 1.1 Summary findings of studies that have examined MPOD with respect to various dietary, lifestyle, ocular and physical factors. (For reference details, see appendix 2.)

Variable	Number of studies investigating the variable's association with MPOD and finding:		Comment
	Significant link (p < 0.05)	Insignificant link (p > 0.05)	
Adipose tissue lutein	Positive in 2 studies (M only)[18,46] Inverse in 1 study (F only)[46]	1 study (F only)[18]	18 = FR 46 = HFP
Age	Positive in 7 studies[9,26,27,43,56,95,101] Inverse in 19 studies[4,6,10,11,12,32,38,39,43,54,66,67,68,71,73,75,85,105,107]	38 studies[3,5,10,12,13,14,16,18,19,20,21,22,23,25,38,41,42,44,45,46,51,56,58,59,63,64,72,77,79,80,81,84,91,92,93,100,105,106]	Positive = HFPx3, AFx3, FRx2 Inverse = HFPx11, RRS*x7, AFx1, FRx1 Insignificant = HFPx20, FRx7, AFx6, HPLCx4, Colour matchingx1, Histologyx1 51 = significant association found for older vs younger subjects in 'central dip' MPOD profile. 81 = insignificant for both subject groups.
AMD	Inverse in 8 studies[6,11,16,73,75,84,90,104]	7 studies[9,23,47,49,52,55,65]	Inverse = RRSx2, AFx1, FRx2, HFPx2, HPLCx1 Insignificant = HFPx5, FRx3 73 = only 12/64 subjects with early AMD. Lack of significance may have been due to lack of power and/or L supplement use in 23, 52 & 65.
Body fat	Inverse in 2 studies[40,68]	2 studies[46,68]	All HFP. 40 = association driven by body fat ≥ 27%. 68 = association in M only.
Body mass index	Inverse in 7 studies[18,19,40,51,63,68,72]	8 studies[18,19,46,67,68,71,73,99]	All HFP except 18 (= FR). 18 & 68 = association in M only. 19 = insignificant when BMI analysed as a continuous variable but significant with BMI split into two groups. 40 = association driven by BMIs ≥ 29.
Diabetes (presence of)	Inverse in 3 studies[25,58,63]	1 study[65]	25 = colour matching 58 = AF 63 = HFP 65 = HFP
Dietary lutein and/or zeaxanthin *NB This doesn't include any supplementation studies.*	Positive in 10 studies[15,19,22,24,63,68,70,71,99,101]	7 studies[1,6,29,33,34,51,65]	All HFP. 68 = significant for Z only. 1 = insignificant for both subject groups. 29 = insignificant for both subject groups.

Variable	Number of studies investigating the variable's association with MPOD and finding:		Comment
	Significant link (p < 0.05)	Insignificant link (p > 0.05)	
Family history AMD	Inverse in 1 study[71]	4 studies[51,61,63,102]	All HFP except 102 (= AF). 61 = significantly higher serum L in family history subjects; authors suggest impaired retinal uptake in these subjects.
Fat intake	Positive in 1 study (M only)[34] Inverse in 1 study (F only)[34]	7 studies[22,24,33,63,68,71,101]	All HFP.
Foveal architecture	10 studies[1,2,29,30,50,57,72,80,81,96]	6 studies[48,50,72,75,88,96]	1 = HFP, positive for foveal thickness in both subject groups. 2,29,80 = HFP, foveal thickness (2 = positive for both subject groups; 29 = measurements on CHM subjects only). 30 = FR, foveal cone photopigment distribution corresponded with MP distribution. 50 = HFP, positive for foveal width & FPPS, not foveal thickness. 57 = AF & HFP, foveal thickness. 72 = HFP, positive significance for foveal width, not foveal thickness (except in non-white subjects). 81 = AF, positive for foveal thickness in both subject groups. 96 = HFP, positive for foveal thickness, not foveal width. 48 = FR, foveal thickness. 75 & 88 = RRS, foveal thickness.
Fruit and vegetable intake	Positive in 2 studies[19,64]	4 studies[18,22,63,65]	All HFP except 2 (= FR). 64 included eggs. 65 may have been influenced by concurrent low fat intake in high fruit & vegetable group.

Variable	Number of studies investigating the variable's association with MPOD and finding:		Comment
	Significant link (p < 0.05)	Insignificant link (p > 0.05)	
Gender	M>F in 9 studies[5,8,18,34,39,54,64,71,103] F>M in 1 study[73]	29 studies[4,9,10,11,19,22,24,26,43,44,45,46,47,51,66,67,72,75,80,81,82,89,91,94,95,101,102,105,107]	Significant = HFPx8, FRx2 Insignificant = HFPx16, FRx7, AFx7, RRSx5 73 = HFP, not significant in final multiple linear regression model. No significance in 19 & 24 despite higher L/Z intake in F than M. 81 = insignificant for both subject groups. 94 = only 23 subjects (5M, 18F) – lack of power?
Genetics	5 studies[17,33,56,60,99]	None	17 = FR, gene study. 33 = AF & HFP, 76 MZ twin pairs & 74 DZ twin pairs; MPOD more highly correlated in MZ than DZ, with MZ heritability estimates of 0.85 (AF) & 0.67 (HFP). 56 = HFP, statistically similar MPOD in 5/10 identical twin pairs. 60 = HFP, gene study. 99 = HFP, spousal study; results were suggestive of genetics playing a bigger role than other factors in MPOD.
High cholesterol	Inverse in 2 studies[71,80]	4 studies[18,58,61,63]	All HFP except 58 (= FR).
Iris colour	Dark>Light in 7 studies[22,35,39,51,64,80,103]	10 studies[10,11,18,26,28,43,44,63,71,72]	Significant = all HFP. Insignificant = HFPx7, FRx3, AFx2, RRSx2. 18 & 43 = few dark compared with light – lack of power? Lack of significance in 44 confounded by a sample of black subjects with low MPOD.
Lens changes	Inverse in 2 studies[8,37]	4 studies[23,25,43,67]	8 = FR, lens optical density. 37 = HFP, lens density in subjects aged ≥48 years. 23 = HFP, cataract mean MPOD not different from normals, but lack of significance may have been due to lack of power and/or L supplement use. 25 = colour matching, lens density. 43 = HFP & RRS, lens status, but very few subjects graded higher than zero – lack of power? 67 = HFP, lens thickness, inverse trend with MPOD but not significant.

Variable	Number of studies investigating the variable's association with MPOD and finding:		Comment
	Significant link (p < 0.05)	Insignificant link (p > 0.05)	
Race	Whites>Non-whites in 1 study[44] Non-whites>Whites in 2 studies[72,102]	2 studies[11,35]	44 & 72 = HFP 102=AF 11=RRS 35=HFP Very few non-white subjects in 11 & 35 – lack of power?
Serum lutein and/or zeaxanthin *NB This doesn't include any supplementation studies.*	Positive in 20 studies[1,2,7,15,19,22,24,29,34,51,61,63,67,68,70,71,80,83,91,99] Inverse in 1 study[75]	8 studies[1,18,29,33,52,66,69,98]	Positive = HFPx17, AFx1, FRx1, HPLCx1 Inverse = RRS Insignificant = HFPx7, FRx1 1 = positive in non-RP subjects, insignificant in RP subjects. 2 = positive in both subject groups. 29 = positive in non-CHM subjects, insignificant in CHM subjects. 18 = almost positive significance in M (p<0.06). Compared with the majority of significant studies, 4 of the 8 insignificant studies had few subjects (29: n=13 [CHM subjects], 33: n=20 [10 MZ twins], 52: n=13, 69: n=4) – lack of power?
Smoking	Inverse in 9 studies[26,36,39,47,64,70,71,73,103]	19 studies[10,11,18,19,22,24,26,28,43,44,51,63,65,67,72,81,82,91,102]	Inverse = HFPx7, AFx1, FRx1 Insignificant = HFPx11, AFx6, FRx3, RRSx2 70 = a link between serum L/Z & MPOD became insignificant for heavy smokers (≥20/day). 11,19,43,44,65,72,91 = no or very few current smokers in these studies – lack of power? 51 = significant association found for smokers vs non-smokers in 'central dip' MPOD profile. 81 = insignificant for both subject groups.
Sunlight exposure	Inverse in 1 study[64]	2 studies[22,63]	All HFP. 64 = < 3 hours sun exposure per day compared with > 3 hours or regular sunbathing/sunbeds.
Supplement use of lutein (self-reported) *NB This doesn't include any supplementation studies.*	Positive in 3 studies[11,13,44]	1 study[75]	11 = RRS 13 = HPLC 44 = HFP 75 = RRS

Variable	Number of studies investigating the variable's association with MPOD and finding:		Comment
	Significant link (p < 0.05)	Insignificant link (p > 0.05)	
Visual function *NB Includes visual function changes as a result of L/Z supplementation studies.*	Positive in 11 studies[29,38,41,62,74,76,77,78,87,88,97]	5 studies[31,53,62,74,101]	All HFP. 29 = central target absolute threshold (measurement on CHM subjects only). 38 = age-related visual sensitivity. 41 = critical flicker fusion threshold. 62 = various visual performance measures; MPOD associations with contrast sensitivity & VA. 74 = various visual performance measures; some level of improvement in glare disability & light/dark adaptation after supplementation. 76 = contrast thresholds. 77 = temporal visual function. 78 = Amsler grid, contrast sensitivity & VA in a group of subjects with AMD. 87 & 88 = glare disability & photostress recovery. 97 = photophobia light thresholds. 31 = gap acuity & hyperacuity. 53 = mesopic contrast acuity thresholds, light scatter & wavefront aberrations. 101 = age-related visual sensitivity.

* The marked decline in MPOD with age demonstrated in studies using RRS is the subject of much debate (see appendix 1).

AF = autofluorescence. BMI = body mass index. CHM = choroideraemia. CSR = central serous retinopathy. DZ = dizygotic. F = females. FPPS = foveal pit profile slope. FR = fundus reflectometry. HFP = heterochromatic flicker photometry. HPLC = high-performance liquid chromatography. L = lutein. M = males. MPOD = macular pigment optical density. MZ = monozygotic. n = number of subjects. RP = retinitis pigmentosa. RRS = resonance Raman spectroscopy. VA = visual acuity. Z = zeaxanthin.

Reference 1 = RP and healthy-eyed subject group. Reference 2 = Stargardt disease/cone-rod dystrophy and healthy-eyed subject group.

Reference 29 = CHM and healthy-eyed subject group. Reference 80 = RP subject group. Reference 81 = CSR and healthy-eyed subjects.

Other variables that have been investigated with respect to MPOD include alcohol consumption, blood pressure, education, exercise, melanin optical density, and refractive error. No significant associations have been observed.

CHAPTER 2: INVESTIGATING THE USE OF A NOVEL MACULAR PIGMENT MEASUREMENT DEVICE

In 2007, a new instrument for measuring MPOD was released into the ophthalmic domain. Though known by several different names (M|POD, MPS 9000, QuantifEYE), here it will be referred to as the MPS. At the beginning of the research period there was no published data on its repeatability, so it was important to establish how to use the MPS to its full potential, in order that data could be interpreted appropriately and future studies could be suitably designed. It was also hoped that this information would aid eye care practitioners using the device in clinical practice. This chapter describes the exploratory studies undertaken.

2.1 Background and rationale

The MPS (Tinsley Ophthalmic, Redhill, Surrey, UK) uses the well-established technique of HFP, but it employs a novel approach to this. Instead of responding to minimal or no flicker (as per traditional HFP), subjects respond to the *appearance* of flicker as the blue-green alternation rate is decreased at 6 Hz from a starting level of 60 Hz (van der Veen et al. 2009a). This is above the critical flicker fusion frequency for the test conditions and therefore subjects do not perceive any flicker initially. Rather than the radiance of one wavelength being adjusted by the subject, a sequence of blue-green ratios is used, and these are inverse-yoked to ensure that overall luminance remains constant. The instrument also offers the possibility of estimating MPOD from a central measure alone, the peripheral measure being estimated from the age of the subject and their expected level of lens yellowing (Pokorny et al. 1987; van de Kraats and van Norren 2007; Makridaki et al. 2009).

As mentioned above, there were no published studies on the repeatability of the MPS at the time of data collection, nor was there any information regarding the validity of using the age-based peripheral estimate to derive MPOD. Therefore, the aims of the studies described here were to gather such data. Unsurprisingly, there have been several papers published in the meantime (Makridaki et al. 2009; van der Veen et al. 2009a; Bartlett et al. 2010c; de Kinkelder et al. 2011).

2.2 Methods

SUBJECTS

The main repeatability study involved 25 subjects (4 males, 21 females) who were recruited by email and by word-of-mouth, and consisted of staff and students from Aston

University. They were all in good general health, with ages ranging from 20 to 50 years (mean±SD: 29.4±6.9 years). Refractive error (best vision sphere, BVS) was between +1.00 DS and -10.50 DS (mean±SD: -2.25±3.25 DS – all eyes averaged). Visual acuity (VA) was measured under standard testing conditions using a logMAR letter chart; all eyes had VAs of 0.06 logMAR (Snellen 6/7.5[+2]) or better. Exclusion criteria were: age younger than 18 years; VA worse than 0.2 logMAR (Snellen 6/9.5); and the presence of any ocular disease. Nine of the subjects had prior experience with the MPS.

Five subjects (1 male, 4 females), aged 24 to 61, took part in an additional 'intense intra-session repeatability' study. Four were familiar with the MPS, and three had participated in the main study. Refractive error (BVS) was between plano and -7.50 DS, and all participants had VAs of -0.1 logMAR (Snellen 6/4.8) or better in the eye being tested (the right eye).

A further five subjects (2 males, 3 females), aged 21 to 47, took part in an additional 'intense inter-session repeatability' study. Three were familiar with the MPS, two had participated in the main study, and three had participated in the 'intense intra-session repeatability' study. Refractive error (BVS) was between +3.00 DS and -7.50 DS, and all participants had VAs of -0.04 logMAR (Snellen 6/6[+2]) or better in the eye being tested (the right eye).

Aston University's Ethics Committee approved the studies. All subjects signed an informed consent form, and all procedures adhered to the tenets of the Declaration of Helsinki.

MPS 9000

The MPS is a small, portable device (figure 2.1). It was set up on a standard adjustable table, and participants were directed to rest their forehead on the upper part of the instrument's eyepiece, such that the eye being tested was comfortably centred on the appropriate target. The central target of the MPS is a one-degree circular stimulus composed of blue (465 nm) and green (530 nm) light-emitting diodes (LEDs) on a thirty-degree, white LED-generated background (van der Veen et al. 2009a) – see figure 2.2. For the foveal ('central') test, each subject looked directly at the stimulus while the alternation rate between the blue and green was automatically ramped down from 60 Hz. At the point when they first became aware of the stimulus flickering, the subject pressed a response button and this plotted a point on a graph that was visible to the investigator via a linked computer screen. The process then started again. The first five responses were

used to determine the flicker sensitivity of the subject. Based on this, the main part of the test began, with the subject responding to their first perception of flicker throughout a series of green-blue ratios, until a V-shaped curve was plotted on the screen (figure 2.3) – the minimum point on the curve corresponds to equiluminance of the blue and green lights (van der Veen et al. 2009a). For the central test the minimum point on the curve represents the amount of blue light absorption at the fovea. The process was then repeated for the perifoveal ('peripheral') test: the subject's gaze was directed to a larger target (1.75°), red in colour and eight degrees eccentric from the centre, but the task was still to respond to their first awareness of flicker in the central stimulus. Accordingly, the minimum point on the peripheral curve represents the amount of blue light absorption at an eccentric retinal area assumed to have negligible MP.

Figure 2.1 The MPS 9000 (also known as M|POD or QuantifEYE), shown with a linked computer that reveals the resulting MPOD output.

Central test: subject fixates and responds to flicker of blue target.

Peripheral test right eye: subject fixates left red target and responds to flicker of blue target.

Peripheral test left eye: subject fixates right red target and responds to flicker of blue target.
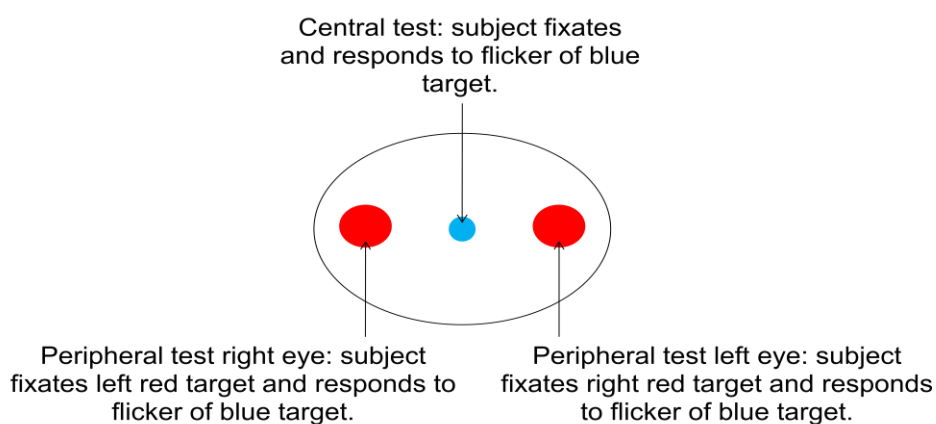
Figure 2.2 The stimuli setup of the MPS (not to scale).
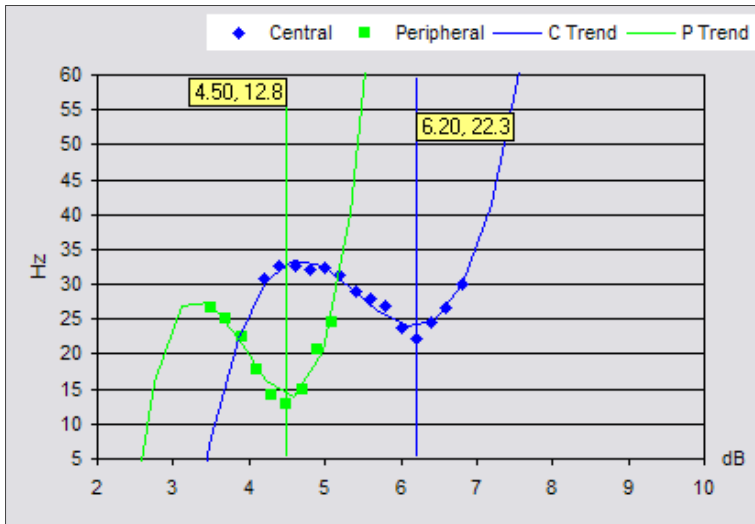
Figure 2.3 Example central (right) and peripheral (left) curves, as generated by a subject using the MPS.

The minimum point on the peripheral curve should always be at a lower dB luminance than the central curve. This is because the x-axis represents the attenuation of the green part of the green-blue ratio (Makridaki et al. 2009; van der Veen et al. 2009a), so as the numbers increase along the axis, the amount of blue in the stimulus is increasing, while the amount of green is decreasing. Since more blue light is absorbed at the fovea than the perifovea (due to the presence of MP), a greater amount of blue light is required to achieve equiluminance. The difference between the central and peripheral minima determines the MPOD – the larger the difference, the higher the MPOD.

All subjects were tested by the same investigator (OH). An explanation of how to perform the MPS test was given verbally, using a prepared instruction sheet (see appendix 3). Subjects wore their habitual distance spectacles/contact lenses, if appropriate, and the eye not being measured was occluded to avoid distraction. The test room was occupied only by the operator and subject, providing a quiet, calm environment.

For the main 25-subject study, each participant had their right eye (RE) and left eye (LE) assessed alternately, with three repeats per eye in a single session, i.e. R1, L1, R2, L2, R3, L3. Before the central and peripheral tests of R1 and L1, a short practice test was conducted (the practice option is part of the MPS software). The RE was always tested first. Because the MPS generates a visible curve as subjects respond to flicker, it provides a way for the operator to assess whether the curve adheres to the expected V-shape, and hence gives an indication as to the accuracy of the MPOD value produced. In this study, if the operator thought that the curve was of very poor quality, the result was

discarded and repeated. The total procedure time, including regular, short breaks to help avoid fatigue, was approximately 45 minutes. The decision to investigate within-session MPOD data (rather than between-session) was made for two reasons. Firstly, at the time, all the future research projects were anticipated to be cross-sectional in nature, and as such the aim was to determine how best to acquire an accurate, one-off measure. Secondly, other members of the research group were already conducting a clinically-driven, inter-session repeatability study on the MPS, with one central and one peripheral measure being taken in one eye per subject (Bartlett et al. 2010c).

For the ancillary intra-session study, each participant's MPOD was measured ten times consecutively, in the RE only. The total procedure time, including regular, short breaks to help avoid fatigue, was between 45 and 60 minutes.

For the ancillary inter-session study, each participant's MPOD was measured five times consecutively, in the RE only. The same method was repeated 7 to 14 days later. Again, the total procedure time was between 45 and 60 minutes.

CENTRE-ONLY MPOD

As mentioned earlier (section 2.1), the MPS also offers an MPOD calculation using the central flicker test alone. To recap from chapter 1, the main purpose of the peripheral test is to cancel out the effect of any blue light absorption by the crystalline lens, so that the final density value is a representation of MP alone, and not MP plus lens yellowing. With the MPS, a fixed peripheral minimum is offered in place of a subject actually undertaking the peripheral flicker test. It is estimated on the basis of the subject's age and their subsequent expected level of lens yellowing, generated from the data of Pokorny et al. (1987) and van de Kraats and van Norren (2007). The amount of lens yellowing is known to increase with age, and therefore the central and peripheral curves are expected to shift rightwards, as a result of the extra blue light absorption by the lens (Makridaki et al. 2009). (To illustrate this, figure 2.4 shows the curves of two subjects, one aged 25 and the other aged 82, both with MPOD values of 0.41.) Because the perifoveal area at eight degrees eccentricity is assumed to be free from MP, and is therefore just a measure of lens yellowing, the peripheral minimum point is potentially predictable from the aforementioned data on the aging lens. This is how the MPS can provide an MPOD value from the subject undertaking the central test alone.

In this chapter, MPOD derived using the measured central and measured peripheral minima will be referred to as CP (central and peripheral), whereas MPOD derived using

the measured central minimum and the age-based peripheral estimate minimum will be referred to as C-only (central testing only).

Figure 2.4 Central (filled symbols) and peripheral (unfilled symbols) curves of two subjects, MM and TG.

The horizontal distance between the central and peripheral minima are the same for both subjects and equate to an MPOD of 0.41, but the curves of the older subject are shifted rightwards as a result of the higher amount of age-related lens yellowing in this subject. (It is also evident that the temporal frequencies at which the minima occur are lower for the older subject.) The arrows indicate the minima for subject TG. NB The temporal frequency unit 'dB' is incorrect and should read 'Hz'. (Reprinted from Makridaki et al. 2009.)

SAMPLE SIZE

A sample size of 25 is in line with many studies examining the repeatability of MP measuring devices (e.g., Hammond et al. 1995; Ciulla et al. 2001b; de Kinkelder et al. 2011). For the ancillary studies, the objective was to gain a slightly more in-depth understanding of how many measures ought to be conducted in a single session for consistent results, and since it was intended that the data would be analysed on an individual basis, it was felt that five subjects per study were sufficient to achieve this.

STATISTICS

Microsoft Excel and IBM SPSS were used for data analysis. The means of the three repeated MPOD measures were compared using a one-way repeated measures ANOVA or its non-parametric equivalent, as appropriate in terms of normality. Simple calculations were used to work out mean individual standard deviations and coefficients of variation,

and repeatability was determined using Bland-Altman analysis and plots (Bland and Altman 1986). CP and C-only comparisons were made using paired-samples t-tests or the non-parametric equivalent (as appropriate), along with Pearson's r or Spearman's rho correlations and Bland-Altman analysis. Interocular MPOD comparisons were approached in the same way.

NB In every study chapter (chapters 2 to 8), a p-value of less than 0.05 was considered statistically significant, and for all t-tests and correlations (and their non-parametric equivalents), the two-tailed significance level was used.

2.3 Results

MEAN MPOD AND WITHIN-SESSION REPEATABILITY

Table 2.1 is a summary of the mean MPODs for the 25 participants under the various test conditions. Also shown are the results of the analyses conducted to assess whether any statistically significant differences existed between the means of the repeated MPOD measurements; there appeared to be no such differences, indicating an absence of any overall learning or fatigue effect throughout the measurements.

Test condition	Mean (±SD) MPOD			Repeated measures analysis	
	Measure 1	Measure 2	Measure 3	Statistic	Significance (p)
CP RE	0.36 ± 0.17	0.31 ± 0.13	0.32 ± 0.14	$\chi^2 = 3.098$	0.213
CP LE	0.33 ± 0.14	0.35 ± 0.15	0.32 ± 0.13	F = 2.943*	0.073
C-only RE	0.39 ± 0.15	0.37 ± 0.13	0.36 ± 0.14	$\chi^2 = 1.914$	0.384
C-only LE	0.37 ± 0.14	0.36 ± 0.13	0.36 ± 0.14	F = 0.390	0.679

Table 2.1 Mean MPODs for 25 subjects (as determined using the MPS), plus information on whether the three means within each test condition were significantly different from one another.

CP = central and peripheral testing derived MPOD. C-only = central testing only derived MPOD (peripheral estimate provided by the MPS, based on subject age). RE = right eye. LE = left eye.

'F' refers to a one-way repeated measures ANOVA, which was used where each data set was normally distributed. Otherwise, Friedman's ANOVA ('χ^2') was used.

*For CP LE, the assumption of sphericity was not met according to Mauchly's test, so with the sample size in mind (n=25), multivariate test statistics were used for interpretation.

The mean individual standard deviations of the MPOD values are given in table 2.2 for each test condition. In other words – taking 'CP RE' as an example – the standard deviation of each individual's three CP RE MPOD values was worked out, followed by calculation of the mean of all 25 standard deviations. Also shown are the mean individual coefficients of variation, calculated from the aforementioned individual standard deviations, and divided by the mean of an individual's three MPOD values. Of note, the LE statistics were lower than those of the RE, and similarly, the C-only statistics were lower than the CP statistics. Paired-samples t-tests revealed that these differences were statistically significant ($p < 0.01$) for all but CP RE versus CP LE, yet even these differences were approaching significance ($p = 0.055$ for standard deviation and $p = 0.067$ for coefficient of variation) – see table A4.1 in appendix 4 for details.

Test condition	Mean SD (±SD) between measures 1, 2 and 3	Mean CV (±SD) between measures 1, 2 and 3 (%)
CP RE	0.063 ± 0.047	20.0 ± 12.6
CP LE	0.047 ± 0.030	14.9 ± 9.3
C-only RE	0.038 ± 0.027	11.0 ± 6.9
C-only LE	0.024 ± 0.021	6.3 ± 5.4

Table 2.2 The means of the individual standard deviations and the individual coefficients of variation (CV) of three repeated MPOD measurements in the 25 subjects, demonstrated for each of the four test conditions.

Accurate analysis of test-retest data can be achieved using the coefficient of repeatability (CR) (Bland and Altman 1986), which gives the 95 percent confidence limits for the amount of difference between two sets of results. It is calculated as 1.96 multiplied by the standard deviation of the differences between two sets of data, and indicates the amount of change that can occur between readings and still be classed as measurement noise. The within-session CRs for MPOD values measured with the MPS are shown in table 2.3. Taking the first and second CP RE tests as an example, the data suggests that when the same operator is taking consecutive MPS readings within the same session, MPOD differences of less than or equal to 0.25 are not clinically significant (figure 2.5). Analogous to the mean individual standard deviations and coefficients of variation, the LE MPOD values had consistently better repeatability than the RE, and the C-only MPOD values had consistently better repeatability than the CP MPOD values.

Comparison	Mean difference	SD of mean difference	Coefficient of repeatability
CP R1 – R2	0.049	0.128	0.25
CP R2 – R3	-0.008	0.085	0.17
CP L1 – L2	-0.018	0.092	0.18
CP L2 – L3	0.032	0.072	0.14
C-only R1 – R2	0.022	0.068	0.13
C-only R2 – R3	0.002	0.058	0.11
C-only L1 – L2	0.008	0.043	0.08
C-only L2 – L3	-0.002	0.046	0.09

Table 2.3 Determination of coefficient of repeatability (CR) values for the MPS – see text for details.

R1, L2, etc. = right eye measure 1, left eye measure 2, etc.
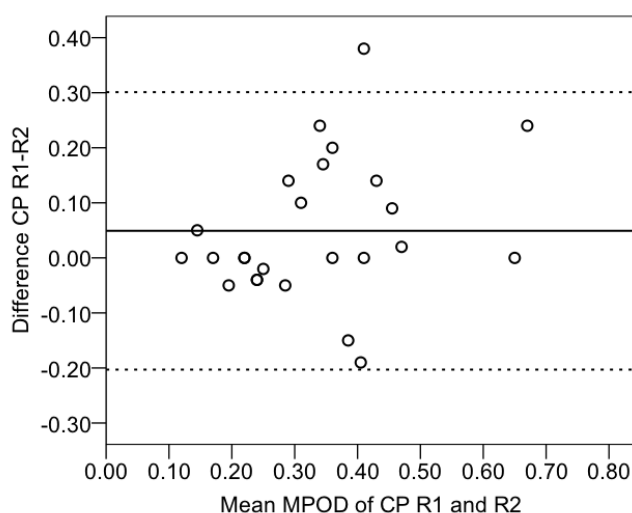
Figure 2.5 Bland-Altman plot representing the difference in CP MPOD readings between measures R1 and R2, compared with the mean of both measures.

The solid line represents the mean difference, and the dashed lines represent the 95% confidence limits. NB There are two overlapping points.

CP MPOD VERSUS C-ONLY MPOD

Looking at table 2.1, it appears that the mean CP MPOD was always lower than the C-only MPOD. Paired-samples t-tests and Wilcoxon signed rank tests (as appropriate) comparing the CP and C-only mean MPODs did confirm this in most cases (see table 2.4). Regardless of these differences, however, the two methods were, unsurprisingly (given that the central reading was common to both measures), highly correlated in all cases (table 2.4), thus suggesting a possible systematic difference. This was investigated further by calculating the 95 percent limits of agreement for CP and C-only MPOD (table

2.5), as per Bland-Altman analysis (Bland and Altman 1986), i.e., 95 percent of CP/C-only differences are expected to lie between these limits. While the mean differences show that CP MPOD is consistently lower than C-only MPOD, the limits of agreement indicate that one cannot dismiss the fact that some individuals' MPODs were actually higher with the CP strategy (see figure 2.6 for details). That said, these limits of agreement should be regarded with caution, particularly for R1, as they are likely to be influenced by the weaker repeatability of the CP strategy. To help overcome this potential problem, the same analyses were conducted using the averages of the three CP and the three C-only MPOD values. The results are presented in table 2.6 and figure 2.7, and confirm a statistically significant difference between the means, along with a highly significant correlation, for both the RE and LE MPOD. Moreover, the Bland-Altman analyses emphasize the bias toward lower CP values, but indicate a good level of agreement (as determined by the 95 percent limits of agreement), hence indicating that a correction factor could possibly be made to determine actual MPOD (CP) when using the C-only test strategy. Nevertheless, these findings are only truly relevant if it is intended for participants in future studies to always complete three measurements.

Comparison	Difference (significance, p)	Correlation (significance, p)
CP and C-only R1	z = -1.856 (p = 0.063)	r = 0.904 (p<0.0005)
CP and C-only R2	t = -3.927 (p = 0.001)	rho = 0.806 (p<0.0005)
CP and C-only R3	z = -3.067 (p = 0.002)	rho = 0.866 (p<0.0005)
CP and C-only L1	z = -2.840 (p = 0.005)	r = 0.895 (p<0.0005)
CP and C-only L2	z = -1.07 (p = 0.285)	r = 0.919 (p<0.0005)
CP and C-only L3	z = -3.464 (p = 0.001)	r = 0.929 (p<0.0005)

Table 2.4 Comparisons of the mean CP MPOD with the mean C-only MPOD, along with the correlations between CP and C-only MPOD.

't' refers to a paired-samples t-test, which was used where the differences between each data set were normally distributed. Otherwise, the Wilcoxon signed rank test ('z') was used. 'r' refers to Pearson's r, which was used where both data sets were normally distributed. Otherwise, Spearman's rho was used.

Comparison	Mean difference	SD of mean difference	SD x 1.96*	95% limits of agreement^
CP – C-only R1	-0.027	0.072	0.141	-0.17 to 0.11
CP – C-only R2	-0.055	0.070	0.137	-0.19 to 0.08
CP – C-only R3	-0.045	0.059	0.116	-0.16 to 0.07
CP – C-only L1	-0.037	0.064	0.126	-0.16 to 0.09
CP – C-only L2	-0.012	0.058	0.114	-0.13 to 0.10
CP – C-only L3	-0.045	0.051	0.099	-0.14 to 0.05

Table 2.5 Determination of the 95% 'limits of agreement' for CP versus C-only MPOD.

*This represents the 95% confidence limit, and means that 95% of the CP/C-only differences are expected to lie within the value calculated here. NB This is the same calculation as used to determine the CR, but with the CR, the mean difference should be close to zero, and so there is no need for any further arithmetic.

^The limits of agreement are calculated as: mean difference ± (SD x 1.96), and reflect the mean difference bias, e.g., for CP – C-only L3, the CP MPOD is expected to be no more than 0.14 below the C-only MPOD, and no more than 0.05 above it (in 95% of cases).
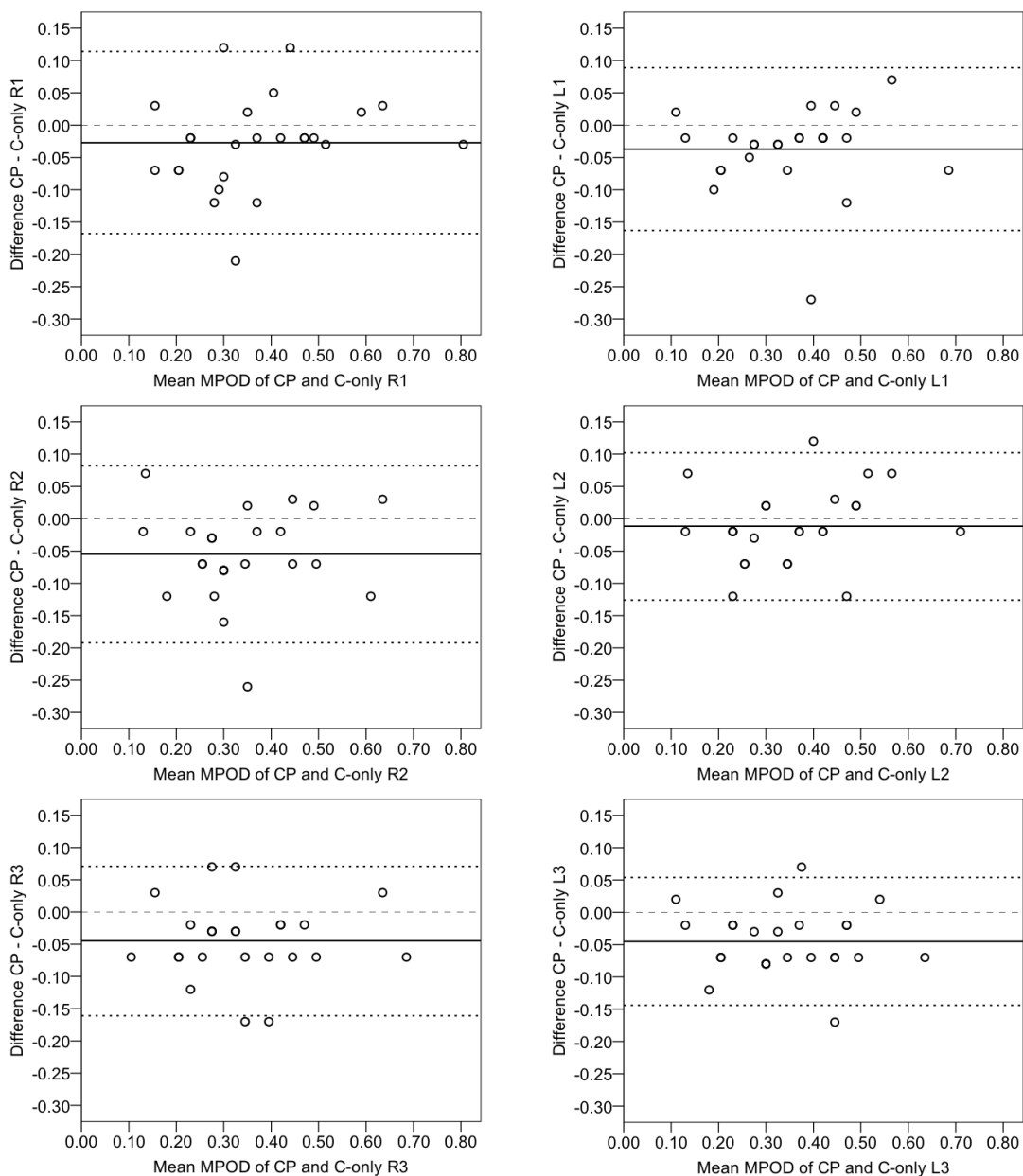
Figure 2.6 Bland-Altman plots representing the difference between CP and C-only MPOD readings, compared with the mean of both measures.

The solid line represents the mean difference, the short dashed lines represent the 95% limits of agreement, and the long dashed line is set to zero in order to aid visualization of how many CP readings were lower than C-only readings. NB There are several overlapping points in each plot.

	CP – C-only RE	CP – C-only LE
Difference (significance, p)	t = -4.224 (p<0.0005)	t = -3.632 (p = 0.001)
Correlation (significance, p)	r = 0.925 (p<0.0005)	r = 0.947 (p<0.0005)
Mean difference	-0.045	-0.032
SD of mean difference	0.053	0.044
SD x 1.96	0.103	0.085
95% limits of agreement	-0.15 to 0.06	-0.12 to 0.05

Table 2.6 Statistical analyses of CP versus C-only MPOD, using the averages of the three CP and the three C-only MPOD values.
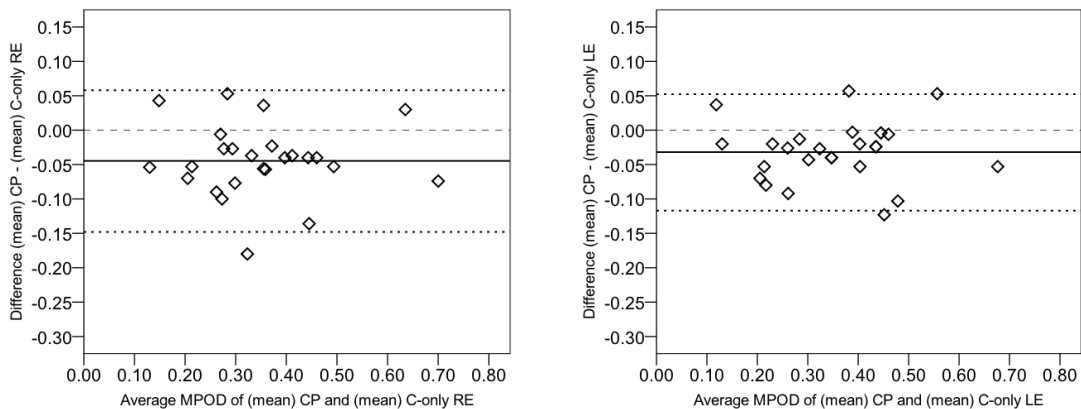
Figure 2.7 Bland-Altman plots representing the difference against mean for the combined CP and C-only MPOD readings.

INTEROCULAR COMPARISONS

Paired-samples t-tests and Wilcoxon signed rank tests (as appropriate) comparing right and left mean MPODs showed no statistically significant interocular differences (see table A4.2 in appendix 4 for details). Interocular correlations were also high, with Pearson's r/Spearman's rho values ranging from 0.66 to 0.85 for the CP data, and from 0.87 to 0.92 for the C-only data (p<0.0005 in all cases – see table A4.2 in appendix 4). The slightly weaker correlations for the CP interocular MPOD are probably a manifestation of the poorer repeatability of the CP data in general. Indeed, Bland-Altman constructions revealed a similar pattern; C-only interocular agreement was better than CP, with both methods showing increasing interocular agreement for the later measurements (see table 2.7). To help remove some of the possible repeatability bias, the same analyses were conducted using the averages of the three RE and the three LE values. The results are presented in table 2.8 and figure 2.8, and confirm the lack of statistical difference between the right and left means, combined with a very high correlation, and a good level of interocular agreement as determined by the 95 percent confidence limits, for the CP

and C-only MPOD. The confidence limits also appear to be within the expected variation for repeated measurements (table 2.3), so taken together with the other statistics, it would appear that an MPS MPOD measurement in one eye alone should accurately reflect MPOD in the opposite eye too (but see discussion).

Comparison	Mean difference	SD of mean difference	SD x 1.96
CP R1 – L1	0.026	0.123	0.241
CP R2 – L2	-0.041	0.101	0.198
CP R3 – L3	-0.001	0.076	0.149
C-only R1 – L1	0.016	0.076	0.150
C-only R2 – L2	0.002	0.053	0.104
C-only R3 – L3	-0.002	0.050	0.098

Table 2.7 Determination of the 95% confidence limits (i.e., SD x 1.96) for RE versus LE MPOD, i.e., 95% of R/L differences are expected to lie within the calculated value.

	CP RE – LE	C-only RE – LE
Difference (significance, p)	t = -0.637 (p = 0.530)	t = 0.651 (p = 0.521)
Correlation (significance, p)	r = 0.911 (p<0.0005)	r = 0.947 (p<0.0005)
Mean difference	-0.008	0.006
SD of mean difference	0.057	0.044
SD x 1.96	0.111	0.087

Table 2.8 Statistical analyses of RE versus LE MPOD, using the averages of the three RE and the three LE MPOD values.
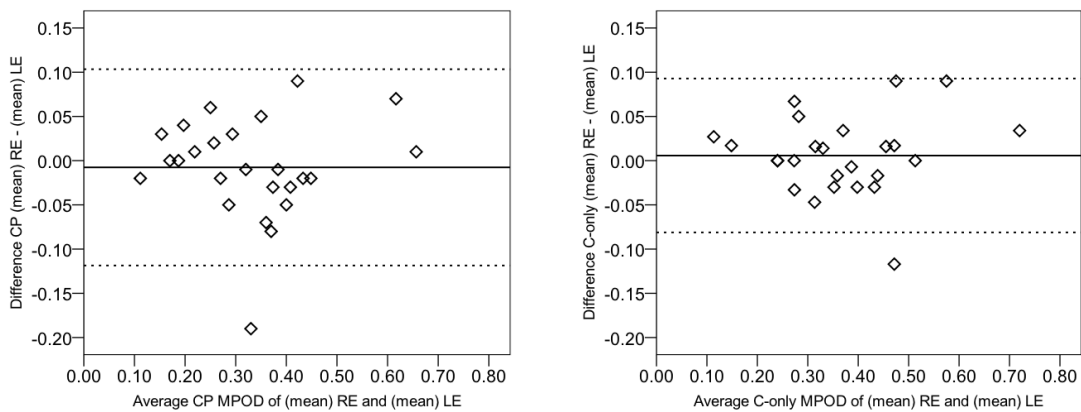
Figure 2.8 Bland-Altman plots representing the difference against mean for the combined RE and LE MPOD readings.

ANCILLARY INTRA-SESSION STUDY

The aim of this smaller study was to investigate the variation in results of ten intra-session MPOD measurements in a row, in an attempt to provide some further insight into how many repeat measures need to be carried out on the MPS before consistent data is obtained. For each of the five participants, running means, standard deviations and coefficients of variation were calculated over the course of the ten measures (table 2.9).

Observation of the resulting data showed no clear set pattern for when the above-mentioned consistent data was achieved. Only one participant's MPOD standard deviation and coefficient of variation decreased throughout the ten measures (subject 1). For the rest the figures seemed reasonably stable by two to four measures. For one subject there was a considerable amount of CP MPOD inconsistency all the way through, but nevertheless, the standard deviations and coefficients of variation still reached a plateau early on (subject 4). As per the earlier studies, CP MPOD was subject to more disparity than C-only MPOD. For the most part, this was probably just a consequence of having two variables in play (i.e., C and P) rather than one (see discussion), but for subject four it would seem that it was a specific difficulty with the peripheral task, since their C-only data is as good as the other subjects.

Despite the standard deviation and coefficient of variation calculations, it was probably the simplest marker that proved to be the most informative, namely the running MPOD mean. For all subjects, i.e., including one and four, the running mean was within 0.05 units of the final MPOD mean (at measure ten) by measure three of the CP data, and by measure two of the C-only data.

		MEASURE									
		1	2	3	4	5	6	7	8	9	10
CENTRE AND PERIPHERAL											
Subject 1	MPOD	0.55	0.26	0.36	0.46	0.46	0.36	0.41	0.31	0.41	0.41
	Running mean MPOD	N/A	0.41	0.39	0.41	0.42	0.41	0.41	0.40	0.40	0.40
	Running SD	N/A	0.21	0.15	0.13	0.11	0.10	0.09	0.09	0.09	0.08
	Running CV (%)	N/A	50.6	37.8	30.7	26.6	25.0	22.8	23.5	21.9	20.6
Subject 2	MPOD	0.26	0.26	0.22	0.26	0.26	0.26	0.26	0.31	0.31	0.26
	Running mean MPOD	N/A	0.26	0.25	0.25	0.25	0.25	0.25	0.26	0.27	0.27
	Running SD	N/A	0	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.03
	Running CV (%)	N/A	0	9.4	8.0	7.1	6.4	5.9	9.2	10.4	9.9
Subject 3	MPOD	0.50	0.46	0.41	0.46	0.41	0.41	0.36	0.46	0.36	0.41
	Running mean MPOD	N/A	0.48	0.46	0.46	0.45	0.44	0.43	0.43	0.43	0.42
	Running SD	N/A	0.03	0.05	0.04	0.04	0.04	0.05	0.04	0.05	0.05
	Running CV (%)	N/A	5.9	9.9	8.1	8.6	8.5	10.7	10.2	11.3	10.7
Subject 4	MPOD	0.26	0.12	0.22	0.36	0.26	0.07	0.31	0.07	0.22	0.02
	Running mean MPOD	N/A	0.19	0.20	0.24	0.24	0.22	0.23	0.21	0.21	0.19
	Running SD	N/A	0.10	0.07	0.10	0.09	0.11	0.10	0.11	0.10	0.11
	Running CV (%)	N/A	52.1	36.1	41.4	35.4	48.8	44.8	52.8	49.1	59.8
Subject 5	MPOD	0.36	0.26	0.31	0.31	0.26	0.22	0.26	0.26	0.31	0.26
	Running mean MPOD	N/A	0.31	0.31	0.31	0.30	0.29	0.28	0.28	0.28	0.28
	Running SD	N/A	0.07	0.05	0.04	0.04	0.05	0.05	0.04	0.04	0.04
	Running CV (%)	N/A	22.8	16.1	13.2	13.9	17.3	16.4	15.6	14.9	14.4
CENTRE-ONLY											
Subject 1	MPOD	0.53	0.38	0.38	0.43	0.48	0.38	0.43	0.38	0.43	0.43
	Running mean MPOD	N/A	0.46	0.43	0.43	0.44	0.43	0.43	0.42	0.42	0.43
	Running SD	N/A	0.11	0.09	0.07	0.07	0.06	0.06	0.06	0.05	0.05
	Running CV (%)	N/A	23.3	20.1	16.4	14.8	14.7	13.4	13.3	12.4	11.7
Subject 2	MPOD	0.24	0.24	0.19	0.24	0.24	0.24	0.24	0.24	0.24	0.24
	Running mean MPOD	N/A	0.24	0.22	0.23	0.23	0.23	0.23	0.23	0.23	0.24
	Running SD	N/A	0	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.02
	Running CV (%)	N/A	0	12.9	11.0	9.7	8.8	8.1	7.6	7.1	6.7
Subject 3	MPOD	0.53	0.53	0.48	0.48	0.43	0.48	0.43	0.48	0.43	0.48
	Running mean MPOD	N/A	0.53	0.51	0.51	0.49	0.49	0.48	0.48	0.47	0.48
	Running SD	N/A	0	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04
	Running CV (%)	N/A	0	5.6	5.7	8.5	7.7	8.5	7.9	8.2	7.8
Subject 4	MPOD	0.24	0.24	0.24	0.29	0.29	0.29	0.29	0.24	0.29	0.24
	Running mean MPOD	N/A	0.24	0.24	0.25	0.26	0.27	0.27	0.27	0.27	0.27
	Running SD	N/A	0	0	0.03	0.03	0.03	0.03	0.03	0.03	0.03
	Running CV (%)	N/A	0	0	9.9	10.5	10.3	10.0	10.1	9.8	9.9
Subject 5	MPOD	0.24	0.29	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
	Running mean MPOD	N/A	0.27	0.26	0.25	0.25	0.25	0.25	0.25	0.25	0.25
	Running SD	N/A	0.04	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02
	Running CV (%)	N/A	13.3	11.2	9.9	8.9	8.2	7.6	7.2	6.8	6.5

Table 2.9 Intra-session MPOD values for five subjects performing ten measurements in a row, along with a running mean, standard deviation and coefficient of variation.

Example: Running mean at measure 3 = mean MPOD of measures 1-3. Running SD at measure 3 = SD of measures 1-3. Running CV = Running SD at measure 3/Running mean at measure 3 (x100 for percentage).

ANCILLARY INTER-SESSION STUDY

It was suggested that a small inter-session repeatability study would be an additional helpful way of determining how many repeat readings ought to be taken for reliable MPOD data. Inter-session CRs were calculated based on: (1) just the first MPOD reading

at each visit, (2) the average of the first two readings at each visit, (3) the average of the first three readings at each visit, and so on up to the average of all five readings (table 2.10). For CP and C-only MPOD, it appears that by three measures, the CR is low and stable.

		Mean difference visit 1 – visit 2	SD of mean difference	Coefficient of repeatability
CP	Measure 1 only	-0.040	0.085	0.17
	Average of measures 1-2	-0.035	0.048	0.09
	Average of measures 1-3	-0.020	0.030	0.06
	Average of measures 1-4	-0.032	0.034	0.07
	Average of measures 1-5	-0.029	0.033	0.07
C-only	Measure 1 only	-0.010	0.065	0.13
	Average of measures 1-2	-0.010	0.029	0.06
	Average of measures 1-3	-0.013	0.014	0.03
	Average of measures 1-4	-0.022	0.015	0.03
	Average of measures 1-5	-0.022	0.016	0.03

Table 2.10 Determination of inter-session CR values (calculated as 1.96 multiplied by the standard deviation of the mean difference) for the MPS (n=5) – see text for details.

2.4 Discussion

The exploratory investigations described in this chapter were designed to find out how to use the MPS to its full potential, in order that meaningful, reliable results on an individual's MPOD would be achievable for future studies.

With the CP MPS testing strategy, the mean MPOD for 25 subjects ranged from 0.31±0.13 (R2) to 0.36±0.17 (R1). These values are largely in line with previously reported MPS means: 0.34±0.17 (n=5616 – Makridaki et al. 2009), 0.40±0.15 (n=26 – van der Veen et al. 2009a), 0.40±0.17 (n=40 – van der Veen et al. 2009c), 0.35±0.14 (n=40 – Bartlett et al. 2010c), 0.39±0.17 (n=23 – de Kinkelder et al. 2011). With the C-only strategy, the mean MPOD ranged from 0.36±0.14 (R3 and L3) to 0.39±0.15 (R1). This is in line with the only other reported C-only mean of 0.35 (SD unknown) (Makridaki et al. 2009).

In a study by van der Veen et al. (2009a), 26 subjects repeated MPS measurements (CP) five times in a single session, and the mean individual standard deviation was 0.067±0.033. The results of the present investigation are not directly comparable because there were only three repeats per eye, and the same eye was not tested

consecutively. Nevertheless, the figures are very similar, with the CP RE mean individual standard deviation being 0.063±0.047. Nolan et al. (2004), using another HFP device, took six MPOD readings in a single session. Their 100 participants produced a mean coefficient of variation of 16.14%, which is slightly lower than the CP RE variation in this study (20.0%), but again the results are not directly comparable.

In the other test conditions (CP LE, C-only RE, C-only LE), the mean individual standard deviations and coefficients of variation decreased (see table 2.2), which seems to indicate, firstly, that subjects were better (or at least more consistent) at completing the MPS task after the first measure, and secondly, that the C-only strategy produces more repeatable MPOD results than the CP strategy. The latter of these findings may be the result of the C-only MPOD having just one variable in play (i.e., the central minimum) rather than the two variables of CP MPOD (i.e., the central and peripheral minimum), or it could be that participants found the central test less demanding – and hence more repeatable – than the peripheral test, or it might be a combination of both these things. To assess these possibilities, the original central and peripheral minimum values were considered separately. The resulting mean individual standard deviations and coefficients of variation are given in table 2.11. It appears that the peripheral values are more variable than the central values. On comparing the means statistically, though, the differences were significant only for the LE coefficients of variation (t(24) = -2.563, p=0.017). This would seem to suggest that it is probably the combined effect of the C-only MPOD having just the one variable involved, and the central test giving slightly more consistent results, that leads to its overall better repeatability.

Test condition	Mean (±SD), dB	Mean SD (±SD) between measures 1, 2 and 3	Mean CV (±SD) between measures 1, 2 and 3 (%)
Minimum C RE	6.13 ± 0.54	0.16 ± 0.11	2.6 ± 1.7
Minimum P RE	4.77 ± 0.28	0.18 ± 0.12	3.7 ± 2.5
Minimum C LE	6.11 ± 0.52	0.10 ± 0.08	1.6 ± 1.4
Minimum P LE	4.72 ± 0.24	0.15 ± 0.14	3.1 ± 2.7

Table 2.11 The means of the individual standard deviations and the individual coefficients of variation of three repeated central (C) and peripheral (P) minimum measures, demonstrated for the RE and LE. The means of the minimum values are also provided (second column) to help put the subsequent standard deviations and coefficients of variation into context.

The within-session CRs determined in this study are not directly comparable with any previously published HFP data. Three studies have reported a within-session CR for non-MPS HFP instruments, but these have been calculated on the basis of all repeat tests rather than concentrating on only two tests at a time. The reported formulae for these CRs vary slightly, or are not provided at all in one case (Nolan et al. 2004). Koh et al. (2004) took five consecutive MPOD readings, and the CR was 0.035±0.016 (RE) and 0.041±0.014 (LE), calculated as $1.96.\sqrt{2}.SD^2$. Nolan et al. (2004) took six readings, and the CR was 0.025±0.011 (calculation not provided). Berendschot and van Norren (2005) took five readings, and the CR was 0.19, calculated as $2.\sqrt{2}.SD$. In an attempt to compare the present study's results, a formula advised by Bland and Altman (1999) for determining repeatability on replicate measures has been used: $1.96.\sqrt{2}.\sqrt{variance}$. In other words, this means working out the variance of each subject's three MPOD values, then calculating the mean within-subject variance and the square root of this, followed by multiplying by 2.77 (i.e., $1.96\sqrt{2}$). Note that this formula is subtly different from that used by Berendschot and van Norren (2005), in so much as the mean within-subject standard deviation does not automatically equal the square root of the mean within-subject variance. The resulting CRs in the present study of 25 subjects were 0.22, 0.15, 0.13 and 0.09 for CP RE, CP LE, C-only RE and C-only LE, respectively. (For the purposes of comparison, in table 2.12 these CR values are shown alongside the original CRs.) The figures are most similar to the CR of 0.19 obtained by Berendschot and van Norren, and given that the formulas are very similar, it seems that the MPS is as good as other HFP devices when it comes to within-session repeatability.

Comparison	Coefficient of repeatability
CP R1 – R2	0.25
CP R2 – R3	0.17
CP R1 to R3	**0.22**
CP L1 – L2	0.18
CP L2 – L3	0.14
CP L1 to L3	**0.15**
C-only R1 – R2	0.13
C-only R2 – R3	0.11
C-only R1 to R3	**0.13**
C-only L1 – L2	0.08
C-only L2 – L3	0.09
C-only L1 to L3	**0.09**

Table 2.12 Coefficient of repeatability values for the MPS (n=25).

For the purpose of a one-off measurement, the CR values as found here (tables 2.3 and 2.12) are probably acceptable. Given that the CR reduced with the later measurements, though, it would seem wise to take three consecutive measures, discarding the first of these and using the average of the last two to give the final MPOD value. However, this does seem a waste, particularly as the raw data showed that for many subjects the first MPOD value was very consistent with the following two. This requires further evaluation (see chapter 3).

As explained above, there was no question that the C-only test strategy provided more consistent MPOD data than the CP strategy, so completing the central test alone would be ideal if it produces the same – or a reliably correctable – MPOD as using the central and peripheral tests. Unfortunately, this was not necessarily the case. C-only MPOD was a little higher than CP MPOD for most, but by no means all, individuals, and while there was certainly a good level of agreement between the two, the mean bias towards higher C-only values was not consistent (see table 2.5). The research group who developed the MPS provided MPS data on 5616 eyes and reported a CP/C-only correlation of 0.92 (Makridaki et al. 2009), which is very similar to the correlations found in the current study, particularly when using the average of all three measurements (table 2.6). They also reported that the 95 percent limits of agreement were ±0.13, which are also very similar to those found here. There was a slight bias towards higher C-only MPOD in the Makridaki study, but not as much. This may be a reflection of the far greater number of eyes tested. Overall, the results are very alike, and the same conclusions are drawn; the C-only strategy is useful but should not replace the CP strategy unless a patient has trouble with the peripheral test. By completing both elements, any pessimist/optimist variations should be largely accounted for. For example, if a subject likes to be absolutely sure of flicker before pressing the response button, they are likely to do this for the central and the peripheral test. If only the central test is completed, though, this would result in a different MPOD than if they were to do both parts of the test, on account of their more conservative than normal approach. That said, for the purposes of monitoring MPOD over time, in response to supplementation for example, the central test alone could suffice, because the emphasis is on whether there is any change in MPOD, rather than whether the MPOD is a completely accurate representation of the subject's actual MP.

The statistical analyses looking at interocular MPOD for the 25 participants suggested that right and left MPOD were similar enough to be interchangeable, therefore negating the need to measure MPOD in both eyes of an individual. This confirms the findings from several earlier studies using other HFP instruments (Hammond and Fuld 1992; Beatty et

al. 2001; Lam et al. 2005; Neelam et al. 2006; Iannaccone et al. 2007; Schalch et al. 2007). While most studies comparing right-left MPOD have used paired t-tests and/or Pearson correlations to look at a group as a whole, two have used independent t-tests to look at individual subject differences (Hammond and Fuld 1992; Landrum et al. 1997). They report finding one or two individuals with interocular MPOD differences, so although the vast majority of individuals appear to have similar MPOD in each eye, there are exceptions. With this in mind, in the preliminary analyses done early on in the research period, independent t-tests were conducted for every subject using their three right and left eye MPOD measurements. In hindsight, this was probably an inappropriate choice of analysis considering the paucity of measures. Nevertheless, the results were interesting; only two subjects had a statistically significant interocular difference and both of these subjects were anisomyopic, with a right-left BVS refractive error difference of at least 1.50 DS. Moreover, each eye with the lower MPOD had the higher degree of myopia. Two other subjects had a refractive error difference of 1.50 DS (both myopic), and it seemed interesting to note that while not reaching statistical significance, the eyes with the lower MPOD again had the higher degree of myopia.

The ancillary studies provided some further interesting information, although it should perhaps be acknowledged that the low subject numbers – which also included some individuals who were already familiar with the MPS – were a limitation for these studies, although similar numbers of subjects have been used in other HFP repeatability investigations (e.g., Wooten et al. 1999; Hammond and Caruso-Avery 2000; Mellerio et al. 2002; Koh et al. 2004; Tang et al. 2004; Hammond and Wooten 2005). For the first ancillary study, the overall finding was that no more than three consecutive measures are necessary for CP MPOD, and no more than two for C-only MPOD. Interestingly, this applied even to a subject who clearly found the peripheral test difficult (evident from the subject's comments on the task and from several of their generated curves), thus implying that even when a patient's data seems particularly variable, there is little point in attempting lots of repeat measures, provided that they do fully comprehend the task in hand. The second ancillary study found that for CP and C-only MPOD, the inter-session CR reached its lowest level by the time the average of three repeat readings had been taken.

In conclusion, it appears that for accurate MPS-based MPOD values in a single session, measurements should be repeated three times, with either the average of all three readings being calculated or the average of the last two. Doing the latter may result in the dismissal of perfectly acceptable data, however. The C-only MPOD should not ordinarily

be a replacement for CP MPOD, but only one eye need be assessed, although both eyes may be necessary if the subject has an anisometropic refractive error.

2.5 Summary

This chapter has introduced a novel MPOD measuring instrument, the MPS 9000. Information on how to use it to its full potential has been established, showing that three measures of the central and peripheral tests are needed. However, the problem with having to repeat the test three times, albeit in only one eye, is that it is not particularly practical, especially in a time-pressured clinical setting. In the next chapter, an attempt is made to address this drawback, while also improving the overall reliability of the technique.

CHAPTER 3: IMPROVING THE REPEATABILITY OF THE MPS 9000

From the exploratory studies described in the previous chapter, it seemed that three within-session measurements were required on the MPS for consistent MPOD values to be achieved, but following this procedure on everyone would be undeniably time-consuming, especially where MPOD values in both eyes are required (approximately 20 minutes per eye, on average). In this chapter, the curves and MPOD values from the original 25-subject study are re-examined to find out whether repeatability of the MPS can be improved further, and preferably with a shorter average test time.

3.1 Background and rationale

The MPS is predominantly marketed towards clinicians working in optometric practice. However, the studies described in chapter 2 demonstrated that three measurements were required for an accurate one-off MP measure, which may be too time-consuming for many clinicians and patients, even if technical support staff (e.g., optical assistants) are assigned to oversee the tests. In this secondary analysis of the original data, the aim was to determine if the repeatability of the MPS could be stabilised in fewer than three measures, thus making it more suitable for observing MPOD in the clinical (and research) environment.

3.2 Methods

SUBJECTS

The data from the twenty-five subjects described in the earlier main repeatability study (section 2.2 in chapter 2) was reanalysed; no new subjects were recruited.

POST DATA COLLECTION SECONDARY ANALYSIS

As described in chapter 2, as subjects respond to flicker, the MPS generates a curve that is visible on a computer screen. It therefore provides a way for the operator to assess whether curves adhere to the expected V-shape (figure 3.1), and thus gives an indication as to the accuracy of the MPOD value produced. Many of the curves generated by the participants did not produce a perfect V-shape with a clearly defined minimum, but they were considered acceptable by the operator at the time, based on knowledge acquired from the product manual ('MPOD QuantifEYE Reference Guide and Technician Training'). In this study the influence on repeatability of data from these less than optimal curves was assessed. Hence, further careful observation of all subjects' curves took place; MPOD values generated from curves without a clear-cut minimum area were *removed* (see figure 3.2 for an example), and MPOD values generated from curves with

questionable minima were *adjusted* (see figures 3.2 to 3.4 and table 3.1). It should be noted that the product literature advises that MPOD values from graphs that are "scattered significantly" should not be relied upon as accurate, but the visual examples of unacceptable graphs are far removed from anything resembling a V-curve, and therefore it was difficult to know what the cut-off point for significant scatter was, particularly as very few subjects exhibited consistently 'perfect' curves. During collection of the data, the operator did discard and repeat any tests that were judged (subjectively) to be of unacceptable quality at the time.
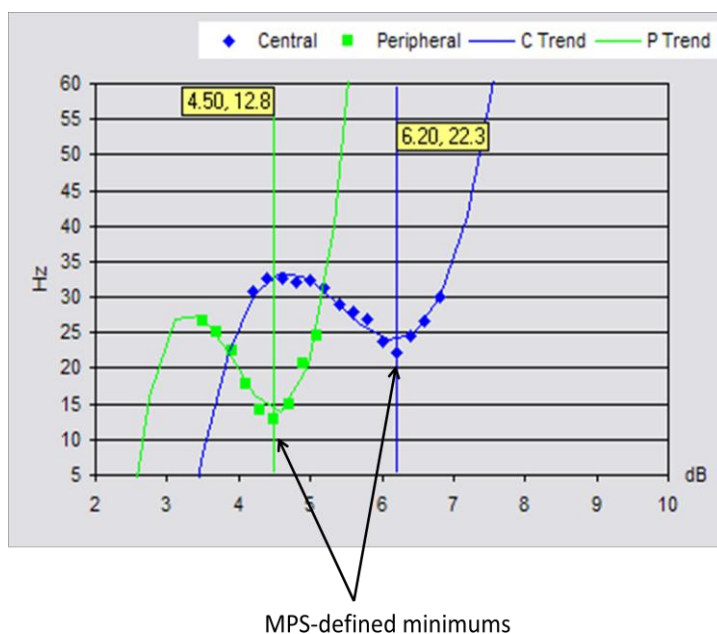
Figure 3.1 Example of an optimal central curve and an optimal peripheral curve – both V-shaped with well-defined minima.
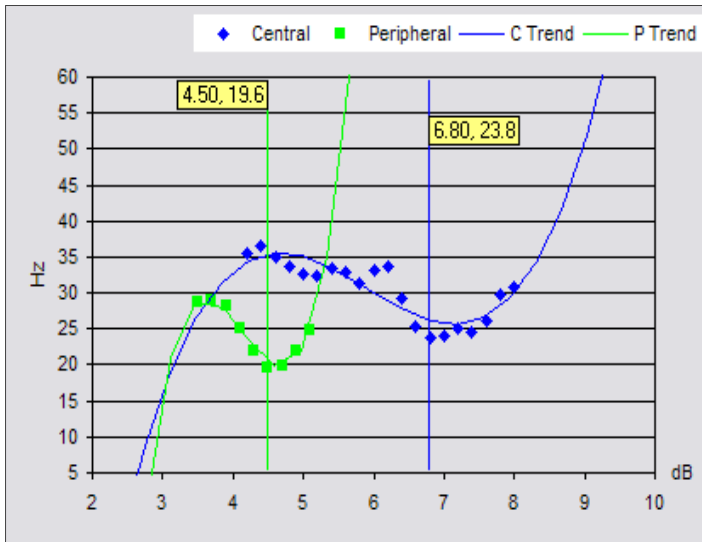
Figure 3.2 Example of a less than optimal central curve (diamond points – right curve) and a peripheral curve (square points – left curve) with two possible minima.

The central curve is less than optimal because it has several points all with similar flicker frequencies to the computer-chosen exact minimum at 6.80 dB, rather than an isolated, definite minimum. An MPOD value generated by a curve such as this would be *removed* from the repeatability analysis. The peripheral curve has an exact minimum at 4.50 dB but, judging by eye, its right adjacent point has a very similar flicker frequency that could also be argued to be a minimum (at 4.70 dB). An MPOD value generated by a curve such as this would be *adjusted*, by recalculation using a peripheral minimum of 4.60 dB, i.e., the mean of these two points.
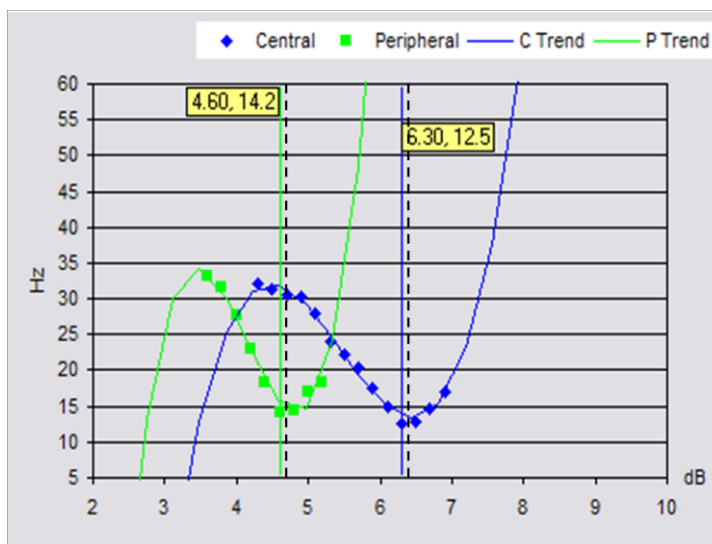
Figure 3.3 Example of a central and peripheral curve, both with two possible minimum points (see figure 3.2 for details).

The dashed lines indicate the adjusted minima (dB): 4.70 for the peripheral curve and 6.40 for the central curve.

62
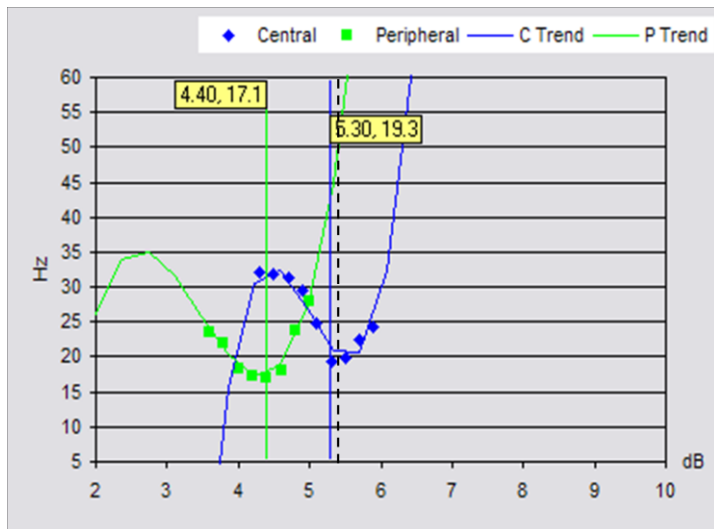
Figure 3.4 Example of a central curve with a questionable minimum.

There is a larger than usual drop in flicker frequency between the minimum and the previous point. On repeat testing, experience dictates that points like this normally prove to be anomalies resulting from a subject's lapse in concentration, with the true minimum occurring at the next point (5.50 dB in this case). The adjustments, however, would be cautious, with the dashed line indicating an adjustment to 5.40 dB and not 5.50 dB.

Difference: C min – P min	MPOD	Difference: C min – P min	MPOD
0.1	0.02	2.1	0.5
0.2	0.05	2.2	0.53
0.3	0.07	2.3	0.55
0.4	0.1	2.4	0.58
0.5	0.12	2.5	0.6
0.6	0.14	2.6	0.62
0.7	0.17	2.7	0.65
0.8	0.19	2.8	0.67
0.9	0.22	2.9	0.7
1.0	0.24	3.0	0.72
1.1	0.26	3.1	0.74
1.2	0.29	3.2	0.77
1.3	0.31	3.3	0.79
1.4	0.34	3.4	0.82
1.5	0.36	3.5	0.84
1.6	0.38	3.6	0.86
1.7	0.41	3.7	0.89
1.8	0.43	3.8	0.91
1.9	0.46	3.9	0.94
2.0	0.48	4.0	0.96

Table 3.1 Conversion table for MPS-based MPOD.

New MPOD values, following adjustment (by eye) of minimum points on the curves, were calculated as follows:

Difference between central (C) minimum and peripheral (P) minimum = X

X = MPOD, found by referring to the table above.

Example: C min = 6.1 dB, P min = 4.7 dB; 6.1 – 4.7 = 1.4; 1.4 = MPOD value of 0.34.

This conversion table was produced after much practice on the MPS.

MASKING

To avoid any potential bias, each curve was considered separately without looking at the same participant's other MPOD values.

STATISTICS

Microsoft Excel and IBM SPSS were used for data analysis. Simple calculations were used to work out mean individual standard deviations and coefficients of variation, and repeatability was determined using Bland-Altman analysis and plots (Bland and Altman 1986). The differences between the resulting CRs before and after data

removal/adjustment were assessed by a one-way repeated measures ANOVA (each data set was normally distributed).

3.3 Results

The mean individual standard deviations of the MPOD values are given in table 3.2 for each test condition. In other words – taking 'CP RE' as an example – the standard deviation of each individual's three CP RE MPOD values was worked out, followed by calculation of the mean of all 25 standard deviations. After *adjustment* of MPOD values as appropriate (minima adjustment), the standard deviations were slightly lower in all categories. Note that three MPOD values were obtained for each test condition and no data was *removed* for this analysis. Also shown are the mean individual coefficients of variation, calculated from the aforementioned individual standard deviations, and divided by the mean of an individual's three MPOD values. Again, the figures were lower after data adjustment.

Test condition	Mean SD between measures 1, 2 and 3	Mean SD after minima adjustment	Mean CV between measures 1, 2 and 3	Mean CV after minima adjustment
CP RE	0.063 ± 0.047	0.060 ± 0.045	20.0 ± 12.6	18.6 ± 11.7
CP LE	0.047 ± 0.030	0.041 ± 0.028	14.9 ± 9.3	13.2 ± 8.6
C-only RE	0.038 ± 0.027	0.034 ± 0.027	11.0 ± 6.9	9.8 ± 6.9
C-only LE	0.024 ± 0.021	0.023 ± 0.019	6.3 ± 5.4	6.1 ± 4.9

Table 3.2 The means of the individual standard deviations and the individual coefficients of variation (%) of three repeated MPOD measurements in the 25 subjects, before and after minima adjustment, demonstrated for each of the four test conditions.

The within-session CR values for MPOD, before and after data removal and minima adjustment, are shown in table 3.3. A one-way repeated measures ANOVA was conducted to analyse the original CR values (O), the CR values after data removal (R), and the CR values after data removal and adjustment (RA). Mauchly's test indicated that the assumption of sphericity was not met, and as a result, degrees of freedom were corrected using the Greenhouse-Geisser estimate of sphericity. There was a statistically significant improvement in repeatability ($F_{(1.2, 8.4)} = 11.09$, $p=0.008$). Pairwise comparisons (Bonferroni corrected) indicated a statistically significant difference between O and RA ($p=0.017$), and between R and RA ($p=0.045$), but not between O and R ($p=0.095$). Figures 3.5 and 3.6 illustrate in graphical format (Bland-Altman plots) the repeatability for the various data sets.

Comparison	Coefficient of repeatability			Number of MPOD values from a total of 50 *removed*	Remainder of MPOD values with minima *adjusted*
	Original (O)	After data removal (R)	After data removal & adjustment of minima (RA)		
CP R1, R2	0.25	0.16	0.16	11 (n=10)	7 (n=7)
CP R2, R3	0.17	0.15	0.12	7 (n=5)	17 (n=15)
CP L1, L2	0.18	0.15	0.13	6 (n=5)	18 (n=16)
CP L2, L3	0.14	0.13	0.12	4 (n=3)	18 (n=16)
C-only R1, R2	0.13	0.11	0.10	3 (n=3)	5 (n=4)
C-only R2, R3	0.11	0.08	0.07	5 (n=5)	9 (n=7)
C-only L1, L2	0.08	0.08	0.08	2 (n=2)	6 (n=5)
C-only L2, L3	0.09	0.08	0.07	1 (n=1)	9 (n=8)

Table 3.3 Coefficients of repeatability for the various data sets, before and after data removal and minima adjustment.

n = number of subjects (out of 25).
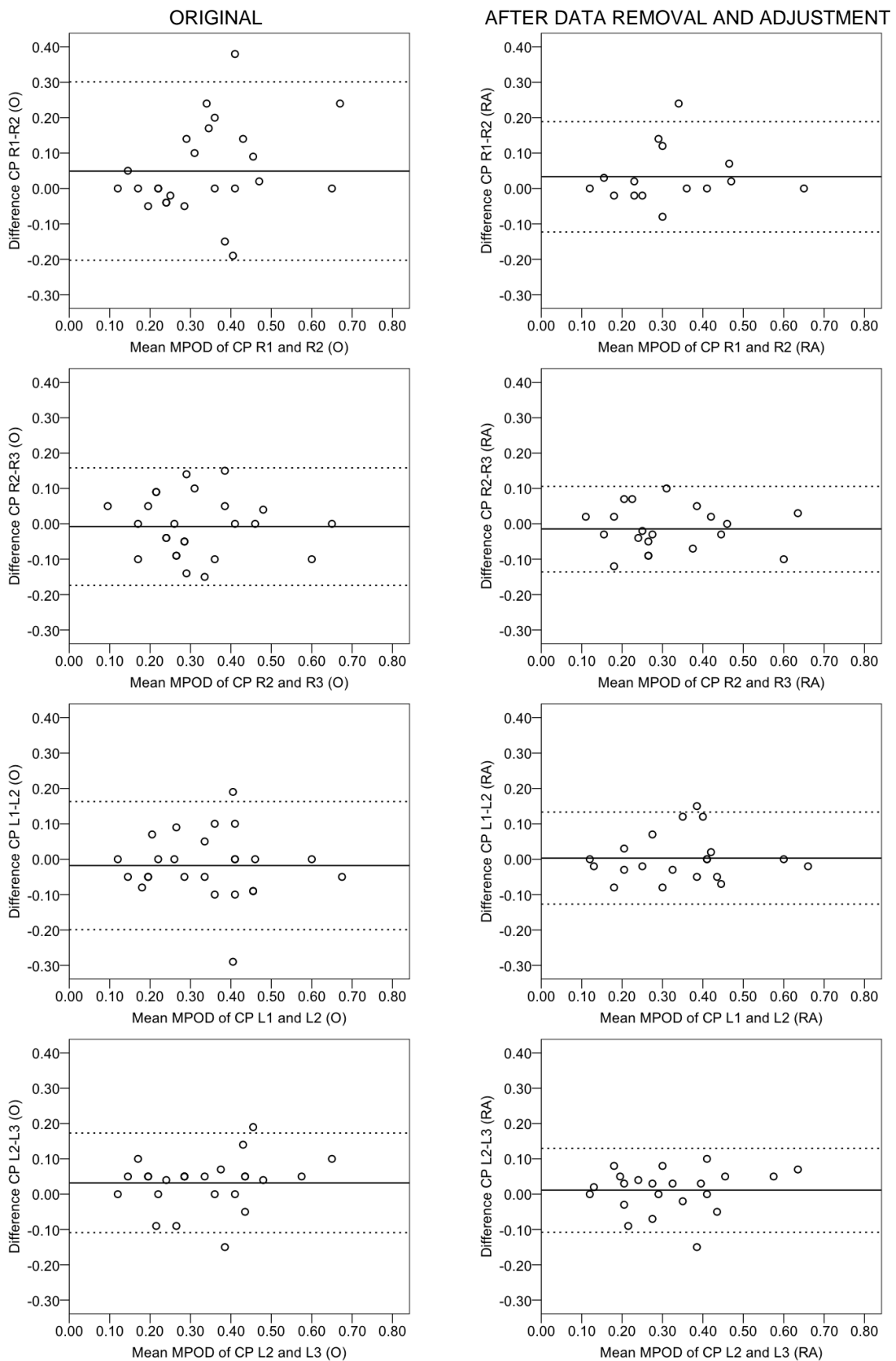
Figure 3.5 Bland-Altman plots representing the difference in CP MPOD readings between measures, compared with the mean of both measures.

The solid line represents the mean difference, and the dashed lines represent the 95% confidence limits. NB There are several overlapping points in each plot.
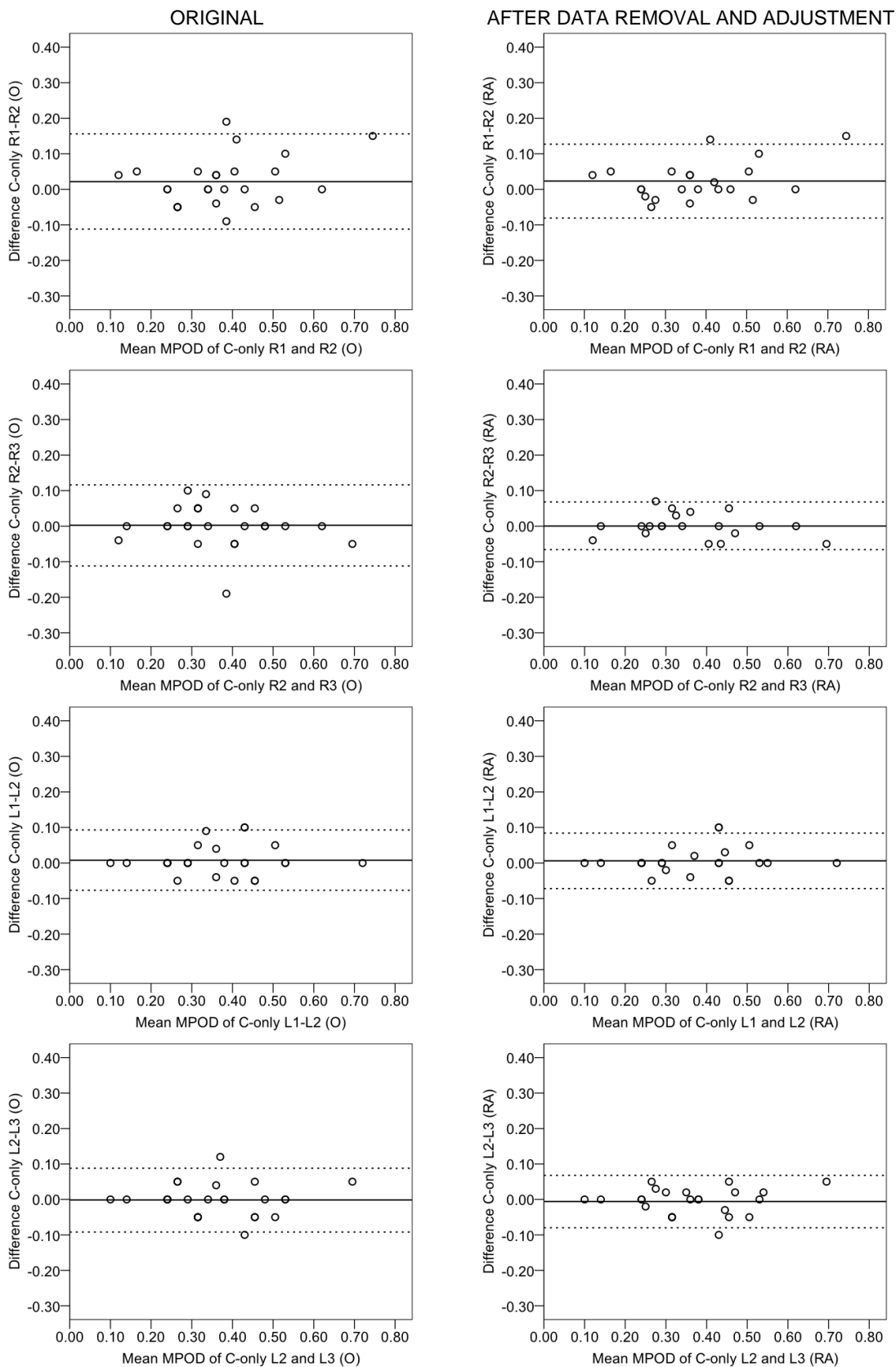
Figure 3.6 Bland-Altman plots representing the difference in C-only MPOD readings between measures, compared with the mean of both measures.

The solid line represents the mean difference, and the dashed lines represent the 95% confidence limits. NB There are several overlapping points in each plot.

The original raw MPOD difference data was also considered. It was calculated that with the CP MPOD data, between 64 percent (R1 vs R2) and 80 percent (L2 vs L3) of participants' MPOD values were within 0.09 of each other from one test to the next. With the C-only MPOD data, between 84 percent (R1 vs R2) and 92 percent (R2 vs R3, L1 vs L2, and L2 vs L3) were within 0.09 of each other from one test to the next. These reasonably high percentages imply that the CR values may be influenced by individuals who simply struggle to perform the flicker test well. Indeed, taking into account the removed MPOD values, plus the adjusted values, the above percentages increased to 80-91% and 86-100%, for CP MPOD and C-only MPOD respectively. To explore this a little further, table 3.4 is a more subject-specific breakdown of where and how much data was removed. It shows that few subjects had more than one central minimum value or one peripheral minimum value removed, thus indicating that most subjects achieved at least two optimal central and peripheral curves per eye (albeit with some later adjustments), and this was seemingly irrespective of whether subjects had some previous experience of using the MPS or not. One subject, in particular, clearly struggled with the peripheral MPS task, resulting in the removal of five of their six peripheral curves.

Number of minimum values removed:		0	1	2	3	4	5	6
Central (max 6 values removed)	Total number of subjects	18	5	2	0	0	0	0
	Number/percentage subjects with previous experience	7/78	1/11	1/11	0/0	0/0	0/0	0/0
	Number/percentage subjects with NO previous experience	11/69	4/25	1/6	0/0	0/0	0/0	0/0
Peripheral (max 6 values removed)	Total number of subjects	15	7	2	0	0	1	0
	Number/percentage subjects with previous experience	4/44	3/33	2/22	0/0	0/0	0/0	0/0
	Number/percentage subjects with NO previous experience	11/69	4/25	0/0	0/0	0/0	1/6	0/0
Total (max 12 values removed)	Total number of subjects	12	7	3	2	0	0	1
	Number/percentage subjects with previous experience	4/44	2/22	1/11	2/22	0/0	0/0	0/0
	Number/percentage subjects with NO previous experience	8/50	5/31	2/13	0/0	0/0	0/0	1/6

Table 3.4 The breakdown of how many minimum values were removed in the secondary analysis, and how many subjects were involved in these removals.

Note that for the three central and peripheral tests carried out on each subject in each eye, there were six central and six peripheral minimum values per subject.

3.4 Discussion

This second-look study aimed to find ways of improving the repeatability of the MPS, as well as the time required to achieve any such improved repeatability, by examining in detail all the MPOD data curves generated by the 25 subjects from the original exploratory study (chapter 2). Subsequent removal and adjustment of certain curves took place, and the effect of doing so was assessed by means of various statistical indicators.

In all four test conditions, minima adjustment alone successfully improved (i.e., reduced) the mean individual standard deviations and coefficients of variation of the three repeated MPOD measurements. This was encouraging, as it demonstrated that the adjustment technique (described in figures 3.2 to 3.4) could be beneficial.

Accurate analysis of test-retest data is achieved using the CR, and in this respect, the results indicated that a statistically significant improvement in the CR could be obtained through the removal and adjustment technique. The weakest CR was between the first and second RE tests (0.25), using the central plus peripheral-based MPOD. The CR was reduced to 0.16 following removal of less than optimal data, although this involved a fairly high number of such removals (11 out of 50 MPOD values). Nevertheless, the techniques of removal and adjustment did improve the within-session repeatability of the MPS.

Further examination of the data revealed that the majority of participants actually produced serial MPOD values within 0.09 of each other and, moreover, that after adjustment (if appropriate), all but a few subjects produced at least two optimal central and peripheral curves per eye. From all the data and experience gathered so far, an MPOD reading that is repeatable to within 0.09 seems like an acceptable limit of accuracy for a psychophysical task of this nature. Combined with the points already discussed, this leads to the following protocol suggestion for use in future studies: For reliable results, each subject should perform the central and peripheral test twice, even if the first curves are considered to be 'perfect'. If there is a ≥0.4 dB difference between equivalent minimum readings (central or peripheral), or if one or more of the generated curves are less than optimal in appearance (and not amenable to adjustment), then a third central and/or peripheral test (as appropriate) should be completed. When a curve exhibits a poorly defined minimum (see figure 3.2), it should not be included in the final calculation of MPOD, but adjustment of a computer-produced minimum reading is acceptable when a curve has two or three very similar minimum points, or an anomalous minimum (see figures 3.2 to 3.4). This method will lead to most subjects having at least two good quality central and peripheral curves, and from these the accepted minima can

be averaged, followed by calculation of MPOD using the earlier established table (table 3.1). At first glance this protocol may appear confusing. It is therefore explained in more detail, with examples, below.

The reason for choosing a difference of 0.4 dB as the cut-off point at which a repeat test should be completed is because this equates to a 0.09 or 0.1 difference in MPOD (see table 3.1). It could have been decided to use a ≥0.1 difference in MPOD readings as the point at which to repeat a test. The problem with this, however, is that it requires both tests to be repeated (central and peripheral), which may be unnecessary. By concentrating on the individual curves (and their minimum values), the testing time can be reduced.

Example 1. Subject A

Test	Minimum C (dB)	Minimum P (dB)	MPOD	Quality of curves	Removals/Adjustments
1	6.1	4.7	0.34	Good C. Good P.	None.
2	6.6	4.7	0.46	Good C. Good P.	None.
3	6.2	-	-	Good C.	None.

Here, the first four curves from tests 1 and 2 are all of good quality, but the MPOD results show a difference of 0.12. However, as the two peripheral (P) minima are identical, repeating the peripheral test seems unnecessary. The MPOD difference originates from the 0.5 dB difference between the two central (C) minima. Therefore, only the central test need be repeated a third time. MPOD is then calculated as follows:

Average minimum C = (6.1+6.6+6.2)/3 = 6.3. Average minimum P = 4.7.

6.3 – 4.7 = 1.6, which corresponds to an MPOD of 0.38 (using table 3.1).

Example 2. Subject B

Test	Minimum C (dB)	Minimum P (dB)	MPOD	Quality of curves	Removals/Adjustments
1	6.6	4.7	0.46	Good C. Good P.	Adjust C to 6.5 (2 mins).
2	6.4	4.9	0.36	Good C. Poor P.	Remove P.
3	-	4.5	-	Good P.	None.

Here, the second P curve is of poor quality and is therefore removed and repeated. The first C curve has two possible minima and is therefore adjusted to 6.5, but it does not require repeating a third time. MPOD is then calculated as follows:

Average minimum C = (6.5+6.4)/2 = 6.45. Average minimum P = (4.7+4.5)/2 = 4.6.

6.45 – 4.6 = 1.85, which corresponds to an MPOD of 0.445 (using table 3.1).

Example 3. Subject C

Test	Minimum C (dB)	Minimum P (dB)	MPOD	Quality of curves	Removals/Adjustments
1	5.8	4.4	0.34	Good C. Good P.	None.
2	5.5	4.6	0.22	Good C. Good P.	Adjust C to 5.6 (2 mins).

Here, the MPOD results show a difference of 0.12. However, observation of the two C and two P minima reveals that they are within 0.4 dB of each other; the difference in MPOD stems from the fact that in test 2, the C minimum decreased by 0.3 dB (and by 0.2 dB with adjustment), while the P minimum increased by 0.2 dB, so in truth (given that all four curves are of good quality) there is no need to repeat the tests a third time. MPOD is calculated as follows:

Average minimum C = (5.8+5.6)/2 = 5.7. Average minimum P = (4.4+4.6)/2 = 4.5.

5.7 – 4.5 = 1.2, which corresponds to an MPOD of 0.29 (using table 3.1).

Note that even with adjustment of the second C minimum to 5.6 (resulting in an MPOD of 0.24), the MPOD difference would still have been 0.1. Without paying attention to the individual C and P minima, this would have dictated that a third test be completed. This therefore emphasizes the importance of observing the C and P minima separately.

In a clinical setting, this protocol, regardless of any improved patient test time, would probably be considered too convoluted, and the calculations themselves too time-consuming. It might therefore be more appropriate to use a slightly different protocol, albeit resulting in one or two unnecessary tests: Each patient should perform the central and peripheral test twice, even if the first curves are considered to be 'perfect'. If there is a ≥0.1 difference between MPOD readings, or if one or more of the generated curves are less than optimal in appearance, then a third central and peripheral test should be completed. The average of the two or three MPOD values should give an accurate one-off measure of MP, unless many of the curves are poor or the MPOD values constantly variable, in which case it would be worth paying attention to the central curves and C-only MPOD readings to see whether the results are more consistent.

In conclusion, although there is still an element of examiner subjectivity involved, following either of these protocols, the first one in particular, will improve within-session repeatability, and should in turn mean that smaller MPOD changes over time (estimated at ≥0.09) can be classed as clinically significant, unless the curves of a patient are especially poor or their MPOD values variable. It will also make the testing time quicker, because the originally suggested 'three central and three peripheral tests for all' (see

section 2.5 in chapter 2) will be unnecessary for many individuals. For an optometrist practice or similar, the time required will be akin to that of a comprehensive visual field test.

3.5 Summary

This chapter has provided further information on using the MPS to its optimal capability, repeatability-wise and time-wise, and evidence-based testing protocols have subsequently been introduced that will make the instrument more useful in the clinical and research environment. In the chapters that follow, the more research-suitable protocol will be used to study MPOD in a variety of subject groups and situations.

CHAPTER 4: ANISOMETROPIA AND MACULAR PIGMENT OPTICAL DENSITY

Over the course of the preliminary work aimed at establishing how best to use the MPS (chapters 2 and 3), it was noted that the two subjects with the largest interocular MPOD difference were also considerably anisometropic; both were myopic with the higher refractive error eye having the lower MPOD. In this chapter, this potentially interesting finding is explored in detail.

4.1 Background and rationale

Anisometropia, the condition in which one eye has a different refractive error from the other eye, has not been examined with respect to interocular MP levels in any published research. Refractive error in general has been mentioned briefly (Ciulla et al. 2001a; Curran-Celentano et al. 2001), but no link with MP appears to have been found, and Neelam et al. (2006) looked in detail at a number of ocular biometric parameters (e.g., axial length, anterior chamber depth), but again there were no associations with MPOD. In an exploratory study for the present research, though, there was an indication that a link between anisometropia and MPOD may exist (see section 2.4 in chapter 2); while the vast majority of participants showed no statistically significant difference between right and left eye MPOD, two did, and of the background details recorded (age, gender, refractive error, race), it was noted that the only apparent distinguishing factor for these two individuals was an interocular refractive error difference of at least 1.50 DS. Both were myopic, and each subject's eye with the lower MPOD had the higher level of myopia. Two other participants were anisometropic to the same degree, and they also showed a difference in right-left MPOD, and followed the same higher myopia, lower MPOD pattern, but the differences did not reach statistical significance.

The investigation described here aimed to explore this potential anisometropia-MPOD link, with two main purposes in mind. Firstly, to establish whether MPOD ought to be measured in both eyes of an individual with a sizeable amount of anisometropia, and secondly, to see whether an association of lower MPOD with higher myopia (or lower hyperopia) exists beyond the two subjects discussed above. Such a result would perhaps be unexpected given the proposed protective effect of MP against AMD; if a hypothesis were to be suggested, it would likely anticipate a higher MPOD being associated with a higher level of myopia (or lower hyperopia), because AMD has been linked (albeit weakly) to hyperopia, not myopia, in many studies (Maltzman et al. 1979; Delaney and Oates 1982; Hyman et al. 1983; Sandberg et al. 1993; Chaine et al. 1998; AREDS 2000; Ikram et al. 2003; Xu et al. 2006; Fraser-Bell et al. 2010; Lavanya et al. 2010; Tao and

Jonas 2010; Xu et al. 2010). However, if the opposite were to be found, it could simply be that in a longer (i.e., more myopic) eye, the MP is stretched further apart, meaning that the foveal area of MPOD measured with the MPS is not a true reflection of the amount of MP in the central retina for these subjects. That said, the aforementioned study by Neelam et al. (2006) used an HFP device with a target the same size as used with the MPS, but they found no axial length association with MPOD. Nevertheless, observing MPOD in both eyes of a group of anisometropic individuals offered a unique opportunity, in that any between-subject variables that could unwittingly influence a study looking at MPOD and refractive error in general (e.g., diet, smoking, iris colour) were controlled for because the comparison was made within the same subject.

4.2 Methods

SUBJECTS

Participants were predominantly identified by stored autorefraction data of undergraduate optometry students. Permission to view the clinical eye records (held within the university's optometry clinic) of potential subjects was granted, and from these the individuals considered most suitable for the study were contacted by email. Suitable subjects were those with an interocular BVS difference of greater than 1.00 DS, astigmatism only up to a level that would be correctable by a daily disposable toric contact lens (CL), and VA better than 6/9.5, i.e., only mild amblyopia, if any. Ten subjects (5 males, 5 females) were eventually recruited. They were all in good general and ocular health, with ages ranging from 19 to 42 years (mean±SD: 27.2±6.9 years). Two of the subjects were those mentioned above as having demonstrated interocular MPOD differences in the MPS exploration study. The other two subjects with an appropriate level of anisometropia (from that study) were unavailable to take part in the current investigation.

Aston University's Ethics Committee approved the study. All subjects signed an informed consent form, and all procedures adhered to the tenets of the Declaration of Helsinki.

REFRACTIVE STATUS

Refractive error was determined with an open view autorefractor, the Shin-Nippon SRW-5000 (Ryusyo Industrial Co. Ltd., Osaka, Japan), which has been shown to have very good validity and repeatability (Mallen et al. 2001). Five measures per eye were taken and averaged, with the resulting prescription being used to calculate the BVS (spherical power plus half the cylindrical power), which was between +4.00 DS and -10.50 DS (all eyes together) for the ten participants.

OCULAR DIMENSIONS

Axial length (AL), corneal curvature (i.e., keratometry – K) and anterior chamber depth (ACD) were determined with a partial coherence laser interferometer, the Zeiss IOLMaster (Carl Zeiss, Jena, Germany), which is a non-contact technique that has been shown to have very good validity and repeatability (Vogel et al. 2001; Connors et al. 2002; Santodomingo-Rubido et al. 2002; Kielhorn et al. 2003; Sheng et al. 2004). The averages of five AL measures, three K measures, and one ACD measure (five readings taken automatically in one measure) were calculated.

CONTACT LENSES

To avoid any interocular magnification differences caused by spectacle lenses, CLs were worn for measurement of MPOD. Experienced CL wearers inserted either their own lenses or daily disposable lenses of appropriate power. The investigator (OH) inserted daily disposable lenses of appropriate power for inexperienced CL wearers. Visual acuity was then measured under standard testing conditions using a logMAR letter chart; all eyes had VAs of 0.1 logMAR (Snellen 6/7.5) or better, except for one eye of one subject (VA 0.2, 6/9.5).

MPS 9000

For measurement of MPOD using the MPS, each subject received verbal instructions and completed a short practice test, after which the prior established protocol was used (chapter 3). Right and left eyes were assessed alternately; the right eye was always tested first. The averages of the central minima and peripheral minima were used to calculate MPOD. Using this technique, all subjects (and eyes) had two to three good quality central and peripheral curves to derive MPOD from.

The total procedure time was between 45 and 60 minutes.

SAMPLE SIZE

A sample size of seven to twelve subjects was chosen on the basis of G*Power 3.1 power analysis comparing two dependent means (two-tailed) and looking for 80 percent power at the five percent alpha significance level. An effect size of 1.3 was calculated on the basis of an estimated significant mean difference in MPOD of 0.08 between groups, a correlation of 0.9, and a standard deviation of 0.14 in both groups. This led to a sample

size of seven. Adjusting the potential correlation to 0.8, the effect size was 0.9, resulting in a sample size of 12.

Microsoft Excel and IBM SPSS were used for data analysis. Mean values were compared using paired-samples t-tests (all data sets – including their differences – were normally distributed). Pearson's r correlations were used to quantify the associations between MPOD, BVS, AL, ACD and K.

4.3 Results

Table 4.1 is a summary of means for MPOD and the refractive parameters assessed in this study of ten anisometropic individuals. The means of the right and left eyes are shown, along with the means of the most myopic(/least hyperopic) and least myopic(/most hyperopic) eyes. The table also shows the significance results of paired-samples t-tests comparing the means. As expected, when comparing the most myopic eye with the least myopic eye, there were statistically significant differences in BVS and AL. In addition, there was a statistically significant difference for ACD, but not for K readings. It is clear from the table that interocular MPOD was not significantly different despite the interocular refractive error differences. Table 4.2 shows the mean differences for MPOD, BVS, AL, etc., along with the maximum and minimum differences. It can be seen that the averaged autorefractor results confirmed the anisometropic nature of the participants, with the mean anisometropic difference being 2.38 DS, and the minimum difference being 1.25 DS. All subjects met the refractive error criteria set out in the original recruitment process.

Variable	Mean (±SD) RE	Mean (±SD) LE	Significance (p-value)	Mean (±SD) 'myopic' eye	Mean (±SD) 'hyperopic' eye	Significance (p-value)
MPOD	0.45 ± 0.18	0.43 ± 0.16	0.584	0.44 ± 0.17	0.44 ± 0.17	0.901
BVS (DS)	-2.68 ± 4.54	-2.80 ± 4.71	0.882	-3.93 ± 4.42	-1.55 ± 4.49	<0.0005
AL (mm)	24.6 ± 1.73	24.7 ± 1.80	0.960	25.0 ± 1.72	24.3 ± 1.72	<0.0005
ACD (mm)	3.60 ± 0.31	3.57 ± 0.33	0.284	3.62 ± 0.30	3.55 ± 0.34	0.012
K (mm)	7.78 ± 0.27	7.77 ± 0.26	0.797	7.75 ± 0.26	7.79 ± 0.27	0.137

Table 4.1 Interocular comparisons of mean MPOD and various refractive variables for ten anisometropic subjects.

Paired-samples t-tests were used to assess the statistical significance of the differences between eyes.

BVS = best vision sphere. AL = axial length. ACD = anterior chamber depth. K = corneal curvature (the average of the two principle meridians). 'Myopic' eye = the eye with the highest level of myopia or the lowest level of hyperopia. 'Hyperopic' eye = the eye with the lowest level of myopia or the highest level of hyperopia.

Variable	Mean difference (±SD) 'myopic' versus 'hyperopic' eye	Maximum difference	Minimum difference
MPOD	0.003 ± 0.062	0.1	0.0
BVS (DS)	-2.38 ± 0.68	3.75	1.25
AL (mm)	0.76 ± 0.29	1.13	0.35
ACD (mm)	0.067 ± 0.068	0.220	0.010
K (mm)	-0.038 ± 0.074	0.155	0.010

Table 4.2 The average, maximum and minimum differences for MPOD and the various refractive variables for the ten participants.

The relationships between all the variables were also analysed (table 4.3). Unsurprisingly, the most myopic and least myopic eyes were all highly correlated in terms of MPOD, BVS, AL, ACD and K ($p < 0.0005$ in all cases), and statistically significant inter-relationships between BVS, AL and ACD were demonstrated. With respect to MPOD, there appeared to be a link with BVS and AL; increasing myopia and axial length were positively correlated with MPOD (figure 4.1). However, it should be borne in mind that with so few study subjects, the latter finding may be a misleading coincidence or may be influenced by some confounding variable(s).

	MPOD myo	MPOD hyp	BVS myo	BVS hyp	AL myo	AL hyp	ACD myo	ACD hyp	K myo	K hyp
MPOD myo		0.936*	-0.675^		0.672^		0.485		0.026	
MPOD hyp	0.936*			-0.657^		0.788*		0.566		0.114
BVS myo	-0.675^			0.988*	-0.909*		-0.852*		0.476	
BVS hyp		-0.657^	0.988*			-0.912*		-0.754^		0.517
AL myo	0.672^		0.909*			0.986*	0.881*		-0.131	
AL hyp		0.788*		-0.912*	0.986*			0.811*		-0.218
ACD myo	0.485		-0.852*		0.881*			0.984*	-0.408	
ACD hyp		0.566		-0.754^		0.811*	0.984*			-0.481
K myo	0.026		0.476		-0.131		-0.408			0.964*
K hyp		0.114		0.517		-0.218		-0.481	0.964*	

Table 4.3 Pearson correlation matrix analysing the relationships between MPOD, BVS, AL, ACD and K. Grey cells = correlations not applicable for analysis.

*p<0.01 ^p<0.05

myo = the eye with the highest level of myopia or the lowest level of hyperopia.

hyp = the eye with the lowest level of myopia or the highest level of hyperopia.
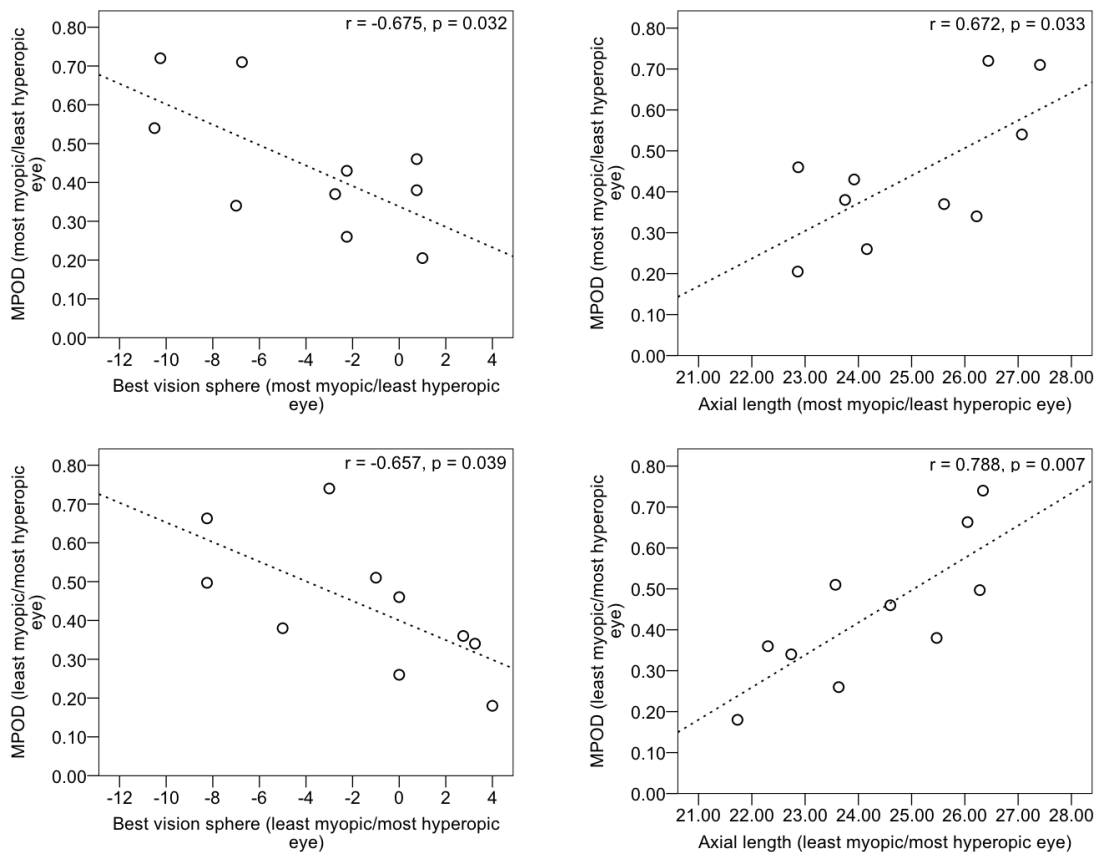
Figure 4.1 The relationship between MPOD and best vision sphere (DS), and between MPOD and axial length (mm). 'r' = Pearson's r.

4.4 Discussion

This study of anisometropia and MPOD was designed to explore whether an earlier finding of a right-left MPOD difference in two subjects with anisometropia, was merely an unusual result, or if the pattern would carry through to a group of anisometropic individuals.

The mean MPOD for ten subjects with anisometropia was 0.45 ± 0.18 in the right eye and 0.43 ± 0.16 in the left eye ($p>0.05$). These values are higher than the means determined in an earlier study (see table 2.1 in chapter 2), and may reflect differences in the sample characteristics. For instance, there were a higher proportion of males in the present study (50% vs 16%), and a higher proportion of Asian, as opposed to Caucasian, subjects (50% vs 14%). Males have been found to have significantly higher ($p<0.05$) MPOD than females in nine MP studies (Hammond et al. 1996a; Wooten et al. 1999; Hammond and Caruso-Avery 2000; Berendschot et al. 2002a; Broekmans et al. 2002; Mellerio et al. 2002; Lam et al. 2005; Nolan et al. 2007b; Bartlett et al. 2010c), whereas the opposite has been found in only one MP study (Nolan et al. 2010), although it is worth pointing out

that no statistical difference has been found in the majority of studies (see table 1.1 in chapter 1). The mean MPOD for males in this study was 0.46±0.18, and for females was 0.42±0.16 (using both eyes in the analysis), but the difference was not statistically significant (Mann-Whitney U test, $p > 0.05$). In terms of race and MPOD, no studies have explored a (South) Asian population sample, but there have been findings of other non-white subjects having higher mean MPODs than white subjects (Wolf-Schnurrbusch et al. 2007; Nolan et al. 2008; Sasamoto et al. 2010). The mean MPOD for Asians in this study was 0.47±0.14, and for Caucasians was 0.41±0.20 (using both eyes in the analysis), but the difference was not statistically significant (independent t-test, $p > 0.05$). However, in both cases (gender and race) it is likely that the sample size was too small to have sufficient power to reach statistical significance, so the results do not necessarily rule these factors out as possible contributors to the higher mean MPOD.

Comparing MPOD in the more myopic (or less hyperopic) eyes with MPOD in the less myopic (or more hyperopic) eyes, resulted in means of 0.44±0.17 for both, so there was no overall significant interocular MPOD difference ($p > 0.05$), despite significant differences in BVS, AL and ACD. It would therefore seem that the original reason for performing this study was driven by a purely coincidental finding in the earlier study; while the two anisometropic subjects from that study maintained interocular MPOD differences of 0.08 and 0.09 in the present one, all but one of the eight previously untested subjects did not have differences as big as this, and the one individual that did followed an opposite pattern – they were hyperopic and the more hyperopic eye had a lower MPOD by 0.1.

Although a conclusion of no relation between MPOD and anisometropia has been made, there was a statistically significant correlation between BVS and MPOD, and between AL and MPOD. Against the original preliminary finding of lower MPOD in the more myopic eye, the results suggested that increasing myopia is associated with higher MPOD. However, in light of the much larger (n=180) study by Neelam et al. (2006), who found no link at all between AL and MPOD, this result should be interpreted with caution. With only ten subjects, the present finding may well be influenced by other unknown variables, as per the higher mean MPOD discussed above. Nonetheless, it might be interesting to explore MPOD in two groups of opposite refractive error, such as subjects with myopic refractive errors greater than -5.00 DS, and subjects with hyperopic refractive errors greater than +2.00 DS. Only 14 (16.5%) of Neelam and colleagues' study population had myopic refractive errors greater than -5.00 DS, and although 46 (25.8%) were hyperopic by more than +0.50 DS, no further information on the levels of hyperopia was reported.

As a result, any MPOD differences between two opposite refractive error groups (such as ≤-5.00 vs ≥+2.00) may have been missed. Unfortunately, after examining all the gathered data from this current body of research (i.e., not just data from the anisometropia study), there are too few subjects meeting the hyperopic criteria (even if reducing the criteria to ≥+1.00 DS) to make any meaningful statistical comparisons at this stage.

If it were to be concluded that some level of association between MPOD and refractive error exists, what might be the cause of that? Myopia is well documented as being associated with accelerated nuclear sclerosis and yellowing of the crystalline lens (e.g., Wensor et al. 1999; Wong et al. 2000; Wong et al. 2001; Chen et al. 2003; Chang et al. 2005; Praveen et al. 2008). Therefore the lens of someone with myopia might be expected to absorb more blue light than the lens of an emmetropic or hyperopic individual. Consequently, the MP in the retina might have a lighter workload, with L and Z being depleted less readily than in the retina of the emmetropic/hyperopic eye. However, if this were the case, then one might expect to see an increase in MPOD with age, on account of the increase in lens yellowing that occurs with age (Pokorny et al. 1987; van de Kraats and van Norren 2007), but so far there is no consistent proof of an age-MPOD relationship (see table 1.1 in chapter 1). Nolan et al. (2009), though, postulated that the increased oxidant load that occurs in the retina with age (Beatty et al. 2000b) might counteract any potential advantage of increased blue light absorption by the yellowing lens, so the myopia-higher MPOD theory is not necessarily ruled out. All the same, it is perhaps unlikely that lens yellowing would have had such a big influence in the fairly young cohort of subjects from the present study. Indeed, observation of the peripheral minimum values for each subject – the peripheral minimum essentially being a basic measure of lens yellowing – does not reveal any apparent refractive error based differences between subjects. Moreover, if the opposite had been found, i.e., if increasing myopia was associated with lower MPOD, it could be argued that a thinner retina, the result of a longer eye (AL), leads to lower MPOD, either because the MP is stretched further apart, meaning that the measured foveal area is not a true reflection of MPOD in the central retina, or because of previous findings of foveal thickness correlating positively with MPOD (Aleman et al. 2001; Duncan et al. 2002; Liew et al. 2006; Aleman et al. 2007; van der Veen et al. 2009c; Sandberg et al. 2010; Sasamoto et al. 2010).

In conclusion, firstly, no relationship between anisometropia and MPOD was found. This negative finding is helpful in that it confirms the need to only measure MPOD in one eye, even when there is a considerable interocular refractive error difference. Rarely, there will be interocular MPOD differences, but these are not predictable from any information

gathered so far (in this study or otherwise), and the right-left difference is unlikely to be more than 0.1 'density units'. Secondly, a positive and statistically significant correlation was found between MPOD and increasing myopia/AL, but this result may have been inadvertently caused by other variables influencing MPOD in the small study population.

4.5 Summary

This chapter has described an assessment of MPS-based MPOD in both eyes of individuals with considerable anisometropia. An earlier theory of anisometropia-induced interocular MPOD differences was disproved, but a link between refractive error and MPOD could not be ruled out altogether. The possibility of other factors, such as gender and race, influencing MPOD was raised, and it was noted that no studies have examined MPOD in South Asians. This leads onto the next chapter, in which MPOD in a group of adults of South Asian origin is explored for the first time.

CHAPTER 5: MACULAR PIGMENT OPTICAL DENSITY IN YOUNG ADULTS OF SOUTH ASIAN ORIGIN

In the preceding chapter it was confirmed that an MPOD measurement in one eye is a good reflection of MPOD in the other eye, even when there is a marked difference in refractive error. As a result, measurement of MPOD in one eye alone will be the norm in the studies that follow, unless there is a particular reason for measurement in both eyes. In this next chapter, an aspect of the rarely mentioned issue of ethnicity and MPOD is dealt with, by examination of MPOD in a group of young adults of South Asian origin.

Please note that in this chapter, the words ethnicity, ethnic group and race are used synonymously, though it is recognized that their definitions are not entirely the same. Similarly, the term 'South Asian' is generally shortened to 'Asian', and is not intended to encompass any other Asian groups, such as East Asians or South-East Asians. Finally, the terms 'Caucasian' and 'white' are used interchangeably.

5.1 Background and rationale

Evidence suggests that ethnicity has a role to play in AMD, with white people having the highest prevalence of the disease, and black people the lowest prevalence (Sommer et al. 1991; Rahmani et al. 1996; Klein et al. 1999; Frank et al. 2000; Muñoz et al. 2000; Klein et al. 2003; Congdon et al. 2004; Friedman et al. 2004a; Friedman et al. 2004b; Leske et al. 2006; Duan et al. 2007; Klein et al. 2011; VanderBeek et al. 2011). Other ethnic groups that have been studied include East Asians (Chinese/Japanese), South-East Asians (Malays), Hispanics, and Latinos (Chang et al. 1999; Rodriguez et al. 2002; Iwase et al. 2006; Li et al. 2006; Sivaprasad et al. 2006; Yasuda M et al. 2009; Varma et al. 2010), and in general the AMD prevalence in these groups has been found to lie somewhere between blacks and whites, i.e., lower than in white people but higher than in black people. A North Indian and South Indian population has also been studied, and the prevalence was reported as being comparable to Western countries (Krishnaiah et. al. 2005; Gupta et al. 2007; Krishnaiah et al. 2009). However, there have been no direct comparisons with other ethnicities, although a study is underway in Singapore that will compare the epidemiology of eye diseases in South Asians, Chinese and Malays (Lavanye et al. 2009). In a meta-analysis study, Kawasaki et al. (2010) pooled prevalence data from studies on Japanese, Chinese, Malay and Indian populations. On combining these four Asian groups, their findings were that early AMD prevalence was lower than in white populations, but late AMD prevalence was similar.

In the effort to find out whether MP, in high levels, can protect against AMD, it has been common practice in many studies to explore MPOD with respect to factors that are believed to have some level of involvement with the development and progression of AMD (see table 1.1 in chapter 1). Yet the relationship, if any, between race and MPOD has rarely been mentioned, largely because the vast majority of participants in MPOD studies have been Caucasian. The only dedicated study to examine racial differences and MPOD was by Wolf-Schnurrbusch et al. (2007), who compared MPOD (using fundus AF) in a group of 51 African and 67 white non-Hispanic subjects, aged 35 to 49 years. The mean MPOD for the African subjects (0.59±0.14) was significantly higher than for the white subjects (0.36±0.13, p<0.0001). Conversely, Iannaccone et al. (2007), in an older group (69-86 years) of 35 black and 148 white subjects, reported MPOD means (using HFP) of 0.22±0.23 and 0.37±0.19, for the black and white subjects respectively. These differences were also statistically significant (p=0.0002). Nolan et al. (2008), though, agreed with Wolf-Schnurrbusch and colleagues, with their HFP-based MPOD study comprising 18 non-white (Indian, 'Asian', Hispanic and black) and 41 white subjects, aged 18 to 60 years. Mean MPOD was significantly higher in the non-white group (0.55±0.28) than the white group (0.34±0.13, p<0.01). Two other studies found no statistically significant MPOD ethnicity differences (Hammond et al. 1996b; Bernstein et al. 2002), but the numbers of non-white subjects (7-8% of total samples) were very low compared with white subjects so there may well have been a lack of statistical power involved.

Outside of ethnic comparisons, there have been MP investigations on non-Caucasian population samples, but they are few in number, and variations in instrumentation (including within the same technique), methods and population backgrounds make comparisons between ethnicities inappropriate. Chen et al. (2001) used FR to measure MPOD in a group of East Asians; Tang et al. (2004) and Lam et al. (2005) used two different HFP devices to measure MPOD in separate Chinese groups; Obana et al. (2008) used RRS to measure MPOD in a group of Japanese subjects; and Sasamoto et al. (2010) used AF to measure MPOD in another group of Japanese subjects.

Similar to AMD prevalence studies, there is an absence of South Asian ethnicity in all prior MPOD work, which was therefore the motivation behind the present investigation. The aims here were to assess the distribution of MPOD in a healthy group of young adults of South Asian origin, to investigate whether any dietary factors or personal characteristics were related to inter-subject variations in MPOD, and to compare mean MPOD in the Asian group with mean MPOD in a Caucasian group.

5.2 Methods

SUBJECTS

One hundred and twenty Asian subjects were recruited by email and by word-of-mouth, and consisted mainly of undergraduate optometry students from Aston University. There were 104 British Asians, 14 Canadian Asians, and two African Asians. They were all in good health, with ages ranging from 18 to 30 years (mean±SD: 21.3±2.6 years). Visual acuity was measured under standard testing conditions using a logMAR letter chart; all participants had VAs of 0.14 logMAR (Snellen $6/7.5^{-2}$) or better in the eye being tested. Exclusion criteria were: age younger than 18 years; VA worse than 0.2 logMAR (Snellen 6/9.5); and the presence of any ocular disease in the eye being tested.

In addition to the Asian subjects, MPOD data was collected on 53 Caucasian subjects. Most of this data was taken from the other studies in this body of research, but 19 subjects were recruited specifically for the present study. There were 47 British Caucasians, two Canadian Caucasians, one Australian Caucasian, one German Caucasian, one Irish Caucasian, and one Polish Caucasian. All 53 participants were in good health, with ages ranging from 18 to 61 years (mean±SD: 27.6±9.3 years). Visual acuity was measured under standard testing conditions using a logMAR letter chart; all participants had VAs of 0.14 logMAR (Snellen $6/7.5^{-2}$) or better in the eye being tested.

Aston University's Ethics Committee approved the study. All subjects signed an informed consent form, and all procedures adhered to the tenets of the Declaration of Helsinki.

LIFESTYLE QUESTIONNAIRE

A questionnaire was constructed for the Asian participants to complete. Questions were primarily focused on the various physical, ocular, lifestyle, dietary and environmental factors that may be associated with MPOD or AMD. For example, height and weight (for body mass index calculation), iris colour, smoking history, fruit and vegetable consumption, sunlight exposure. A copy of the questionnaire is provided in appendix 5.

FOOD DIARY

The Asian participants were also asked to complete a three-day food diary, in which they recorded everything they ate and drank over the course of three days (two weekdays and one weekend day). A copy of the food diary is provided in appendix 6. A nutritional database and analysis package (WISP, Tinuviel Software, UK) was used to assess the nutritional content of each subject's three-day food intake. The database did not include L

or Z values, but it did allow for additional 'custom' nutrients. Information on L/Z (combined) was therefore obtained from the USDA (United States Department of Agriculture) national nutrient database (USDA 2010). A careful, systematic approach was taken. To start, the basic foods in each diary were searched for their L/Z content (e.g., fruits, vegetables, cereals, breads, pastas, meats, dairy products, confectionaries, beverages), followed by a L/Z search for the breakdown of extra food components making up a meal recipe (e.g., herbs, spices, salt, sauces, flour). Nothing was presumed to have zero L/Z so every food was searched for. The L/Z levels were entered into the WISP database under the appropriate heading, e.g., boiled, raw (see figure 5.1). During this process, standard recipes for certain meals were created (e.g., pasta dishes, soups, curries, pasties). Not all of these were needed specifically, because the WISP database contained many pre-entered standard meals, but recipes did need to be created in order to establish L/Z contents. Finally, the food intakes for each subject were entered (see figure 5.2).
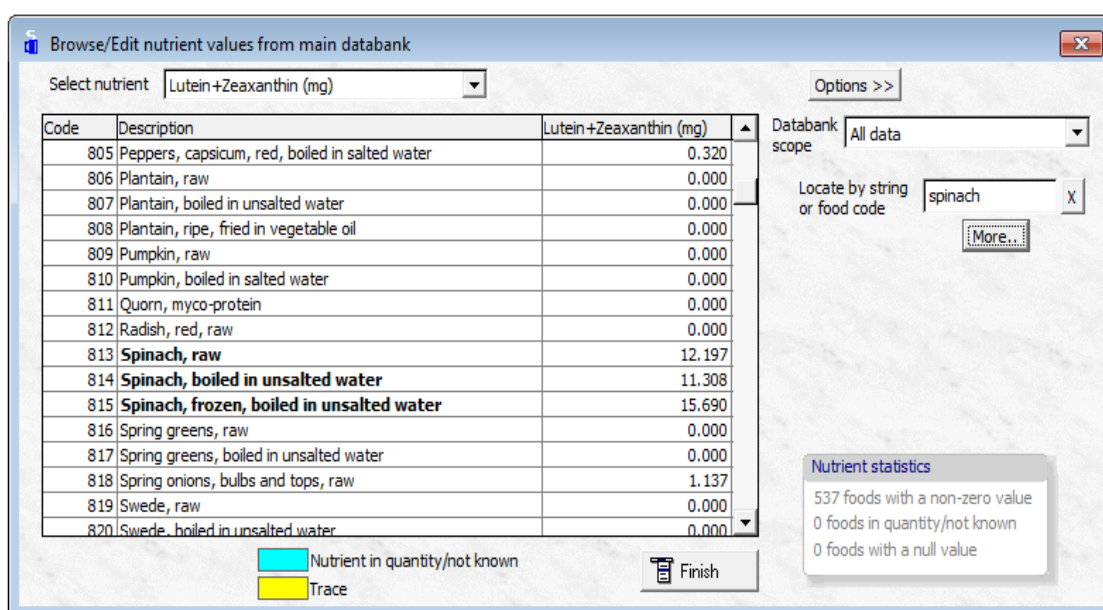
Figure 5.1 An example of manually-entered L and Z content into the WISP nutrient database.

Figure 5.2 An example of a daily food intake record for one subject, using the WISP nutritional analysis software.

MPS 9000

For measurement of MPOD using the MPS, each subject received verbal instructions and completed a short practice test, after which the prior established protocol was used (chapter 3) to measure MPOD in one eye (the right eye unless it was unsuitable, e.g., due to amblyopia). The averages of the central minima and peripheral minima were used to calculate MPOD. Using this technique, the vast majority of participants had two to three good quality central and peripheral curves to derive MPOD from. Three Asian subjects and one Caucasian subject were removed from the analyses due to continually poor curve quality and/or highly variable MPOD readings.

All subjects were tested by the same investigator (OH). The total MPS testing time was between 15 and 30 minutes (includes time taken to go through any questionnaire/food diary queries).

SAMPLE SIZE

Originally, the aim was to recruit approximately 200 Asian subjects, which would be similar to other studies examining MPOD in population samples (e.g., Hammond and Caruso-Avery 2000; Iannaccone et al. 2007). This target was not reached, but the final numbers were still in line with a number of other MPOD studies that each examined a host of variables and their possible associations with MPOD (e.g., Mellerio et al. 2002;

Burke et al. 2005; Wolf-Schnurrbusch et al. 2007; Nolan et al. 2010). In addition, G*Power 3.1 was used. An effect size of 0.57 was calculated on the basis of an estimated significant mean difference in MPOD of 0.08 between two groups, both with standard deviations of 0.14. For a two-tailed independent-samples t-test, this corresponded to a sample size of 100, with 50 subjects per group, for 80 percent power at the five percent alpha significance level.

STATISTICS

Microsoft Excel, IBM SPSS and the WISP nutritional analysis package (Tinuviel Software) were used for data analysis. Mean values were mainly compared using independent-samples t-tests or a non-parametric equivalent, as appropriate in terms of normality. Pearson's r or Spearman's rho correlations were used to quantify associations between continuous variables.

5.3 Results

LIFESTYLE QUESTIONNAIRE

Of the 117 Asian participants with successful MPOD measurements, 100 fully completed the lifestyle questionnaire, and one subject partially completed it. Macular pigment measurements had been taken on ten Asian subjects in the other MPOD studies, so these measurements were included in the analyses where applicable, but as they had not been required to complete the questionnaire, only limited information was available. The same was true for the remaining six subjects who failed to complete the questionnaire. Table 5.1 is a summary of the information gathered for the Asian sample.

Continuous data		Categorical data	
Variable	**Mean ± SD**	**Variable**	**N (%)**
Age (years)	21.3 ± 2.6 (n=117)	Gender (n=117)	Male: 44 (37.6%)
			Female: 73 (62.4%)
Body mass index	All: 21.9 ± 4.0 (n=100) Male: 23.8 ± 4.6 (n=38) Female: 20.8 ± 3.1 (n=62)	Asian background (n=106)	Indian: 75 (70.8%)
			Pakistani: 22 (20.8%)
			Bangladeshi: 3 (2.8%)
			Sri Lankan: 4 (3.8%)
			Other: 2 (1.9%)
Vegetable servings per week	7.0 ± 5.2 (n=100)	Iris colour (n=106)	Dark brown: 61 (57.5%)
			Brown: 37 (34.9%)
			Light brown: 1 (0.9%)
			Hazel: 5 (4.7%)
			Green: 2 (1.9%)

Fruit servings per week	6.6 ± 5.8 (n=100)
Eggs (including yolks) per week	All: 1.8 ± 2.1 (n=100) Yes group: 2.6 ± 2.0
Oily fish servings per week	All: 0.7 ± 1.1 (n=101) Yes group: 1.7 ± 1.1
Alcohol units per week	All: 3.8 ± 8.2 (n=101) Yes group: 9.9 ± 10.7
Hours spent outside per week	A/W: 8.7 ± 5.8 (n=100) S/S: 17.0 ± 10.0 (n=100)
BVS (spectacle prescription)	-2.08 ± 2.82 DS (n=110)

Smoking status (n=102)	Current: 7 (6.9%) Former: 3 (2.9%) Never: 92 (90.2%)
Dietary background (n=101)	Meat-eater: 72 (71.3%) Part-vegetarian: 8 (7.9%) Vegetarian: 21 (20.8%)
Egg intake (n=100)	Yes: 71 (71%) No: 29 (29%)
Oily fish intake (n=101)	Yes: 41 (40.6%) No: 60 (59.4%)
Alcohol intake (n=101)	Yes: 39 (38.6%) No: 62 (61.4%)
Regular exercise (n=101)	Yes: 44 (43.6%) No: 57 (56.4%)
Strong sunlight exposure(n=101)	Yes: 24 (23.8%) No: 77 (76.2%)
Sunbed use (n=101)	Yes: 1 (1.0%) No: 100 (99.0%)
Skin sun sensitivity(n=101)	Yes: 12 (11.9%) No: 89 (88.1%)
Sunglasses use (n=101)	Yes: 19 (18.8%) Part-time: 39 (38.6%) No: 43 (42.6%)
Glasses and CL wear (n=105)*	Glasses/UV CLs: 39 (37.1%) None/non-UV CLs: 62 (59.0%) Mixture: 4 (3.8%)
Family history of AMD (n=101)	Yes: 2 (2.0%) No: 99 (98.0%)

Table 5.1 A summary of the physical, ocular, lifestyle, dietary and environmental data collected on the Asian subject group.

n/N = number of subjects. A/W = autumn/winter. S/S = spring/summer. CL = contact lens. UV CLs = ultraviolet-filtering CLs. AMD = age-related macular degeneration.

'Part-vegetarian' refers to someone who eats one form of meat only, e.g., fish.

For sunglasses use, yes = always or most of the time, part-time = sometimes or occasionally, and no = very rarely or never.

*Glasses and UV-filtering CLs were grouped together because of their UV protection – all plastic spectacle lens materials absorb up to at least 370 nm (Wilkinson 1999).

Information on vitamin and supplement use was also acquired from the questionnaire, but it is not included here as very few subjects took either, and of those that did, no vitamins/supplements included L or Z.

The mean MPOD of all 117 Asian subjects was 0.43±0.14. Table 5.2 is a summary table of the analyses conducted to assess whether any MPOD differences existed for the various physical, ocular, lifestyle, dietary and environmental factors examined in the questionnaire. Some variables were not analysed because of the very low subject numbers in one or more of the groups involved. These variables were: Asian backgrounds other than Indian and Pakistani, smoking status, sunbed use, and family history of AMD. For the same reason, some variables were adjusted: iris colours other than dark brown were combined together to make a larger group size that could then be compared with dark brown; part-vegetarians were excluded from the dietary background analysis; and the 'mixture' group was excluded from the glasses and CL wear analysis.

Variable	Mean (±SD) MPOD	Statistic value	Significance (p) value
Gender	Male: 0.47 ± 0.13 Female: 0.41 ± 0.14	U = 1134.5	0.008
Asian background	Indian: 0.44 ± 0.15 Pakistani: 0.42 ± 0.12	t = 0.806	0.422
Iris colour	Dark brown: 0.46 ± 0.15 Other (n=45): 0.41 ± 0.13	t = 1.778	0.078
Dietary background	Meat-eater: 0.43 ± 0.13 Vegetarian: 0.46 ± 0.16	t = -1.001	0.319
Egg intake	Yes: 0.43 ± 0.13 No: 0.46 ± 0.17	t = -1.101	0.273
Vegetable + Fruit + Egg intake per week	≤15 servings (n=59): 0.44 ± 0.13 >15 servings (n=41): 0.44 ± 0.15	t = 0.023	0.982
Oily fish intake^	Yes: 0.44 ± 0.14 No: 0.43 ± 0.14	t = 0.356	0.723
Alcohol intake	Yes: 0.47 ± 0.16 No: 0.42 ± 0.13	t = 1.845	0.068
Regular exercise	Yes: 0.43 ± 0.13 No: 0.44 ± 0.15	t = -0.368	0.714
Outdoor daylight exposure – A/W	≤7 hours (n=56): 0.43 ± 0.14 >7 hours (n=44): 0.44 ± 0.15	t = -0.219	0.827
Outdoor daylight exposure – S/S	≤15 hours (n=52): 0.43 ± 0.13 >15 hours (n=48): 0.44 ± 0.15	t = -0.332	0.740
Strong sunlight exposure	Yes: 0.43 ± 0.13 No: 0.44 ± 0.15	t = -0.400	0.690
Skin sun sensitivity	Yes: 0.46 ± 0.11 No: 0.43 ± 0.14	t = 0.562	0.575
Light exposure score*	Minimal (0, n=35): 0.43 ± 0.14 Moderate (1-2, n=57): 0.44 ± 0.14 Heavy (3-4, n=8): 0.39 ± 0.14	F = 0.500	0.608

Light exposure score (2 groups)*	Minimum (0-1, n=58): 0.43 ± 0.14	t = -0.853	0.396
	Maximum (2-4, n=42): 0.45 ±0.14		
Sunglasses use	Yes: 0.40 ± 0.16	F = 0.858	0.427
	Part-time: 0.43 ± 0.13		
	No: 0.45 ± 0.14		
Sunglasses use (2 groups)	Yes/part time (n=58): 0.42 ± 0.14	t = -1.025	0.308
	No: 0.45 ± 0.14		
Glasses and CL wear	Glasses/UV CLs: 0.47 ± 0.15	t = 1.911	0.059
	None/non-UV CLs: 0.42 ± 0.14		

Table 5.2 Mean MPODs and comparisons for the variables examined in the Asian subject group.

Unless stated in the table here, please refer to table 5.1 for subject numbers.

't' refers to an independent-samples t-test, which was used when each data set was normally distributed. Otherwise, the Mann-Whitney U test ('U') was used. For variables containing three groups, a one-way between-groups ANOVA ('F') was used (each data set was normally distributed). For all t-tests and ANOVAs, the assumption of homogeneity of variance was met.

^Oily fish intake was also analysed by including in the 'yes' group subjects who took fish oil supplements; there was no difference in the overall outcome.

*The light exposure score was based on an idea from Mellerio et al. (2002), in which points were assigned for various aspects of light exposure. Here, the points were assigned as follows. Zero points for: ≤7 hours autumn/winter outdoor daylight exposure; ≤15 hours spring/summer outdoor daylight exposure; an answer of 'no' to strong sunlight exposure; an answer of 'no' to sunbed use. One point for: >7 hours autumn/winter outdoor daylight exposure; >15 hours spring/summer outdoor daylight exposure; an answer of 'yes' to strong sunlight exposure; an answer of 'yes' to sunbed use. The results were categorized into minimal (0 points), moderate (1-2 points) and heavy (3-4 points) light exposure. Because of the small subject numbers in the latter group, subjects were also re-assigned into one of two groups, minimum (0-1 points) and maximum (2-4 points).

Of all the variables examined, only gender demonstrated a statistically significant difference in MPOD, with the males having a higher average MPOD than the females (p=0.008). Figure 5.3 is a frequency distribution of MP densities for both gender groups. It illustrates the high proportion of males that had MPODs of 0.41 or higher (72% compared with 50% of females). The possible influence of the differing sample sizes (44 males vs 73 females) was also explored; a random sample of 44 females was selected (using the website 'random.org'); the male-female difference remained highly significant (U=613.0, p=0.003).
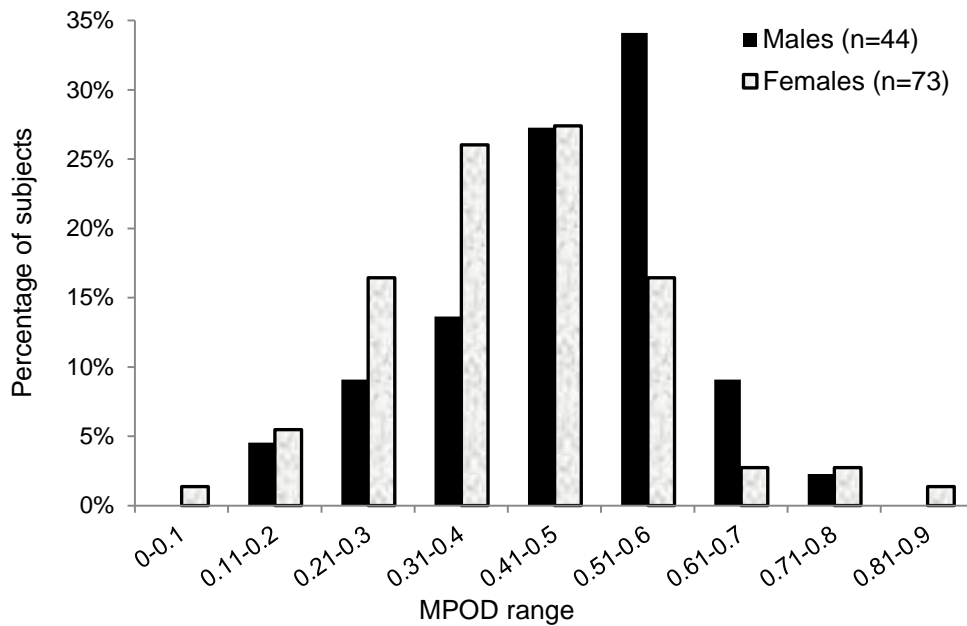
Figure 5.3 The frequency distribution of MPOD for males and females in the Asian subject group.

In addition to looking for differences in mean MPOD between the various groups, the possibility of any MPOD associations was explored for the continuous data. Using Pearson's r or Spearman's rho (dependent on normality), and scatter graph observations, virtually no statistically significant correlations between MPOD and any variable (body mass index, vegetable intake, fruit intake, vegetables/fruits combined, egg intake, vegetables/fruits/eggs combined, oily fish intake, alcohol intake, hours spent outside, and best vision sphere) were found, even when analysing males and females separately (in view of the MPOD gender difference). The only significant correlation to emerge was a positive association between MPOD and alcohol intake (rho=0.229, p=0.021). This association remained significant for females (rho=0.330, p=0.009) but not for males (rho=0.108, p=0.511). However, observation of the scatter graphs indicated that the large proportion of subjects who did not drink alcohol (see table 5.1) might have influenced the correlations. Indeed, separating the group as a whole into drinkers of alcohol and non-drinkers resulted in no relationship for alcohol units and MPOD (rho=0.045, p=0.782). With the same reasoning, this method was applied to egg intake and oily fish intake. Removing the individuals who did not consume any oily fish did not alter the (insignificant) outcome. Removing the individuals who did not consume any eggs, though, did alter the outcome, and a statistically significant positive correlation between egg intake and MPOD emerged (rho=0.287, p=0.015; see figure 5.4).
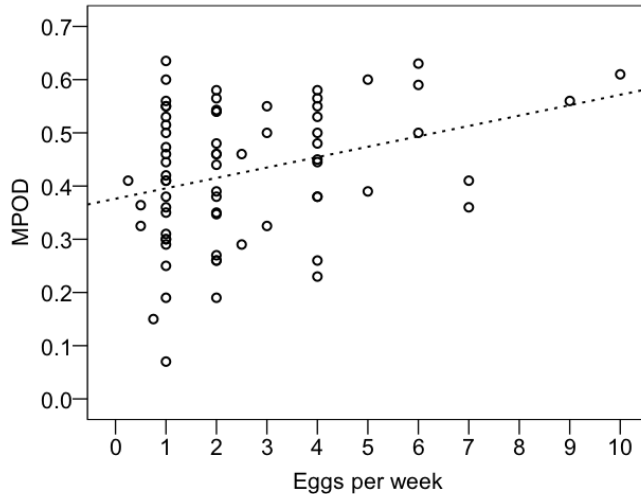
Figure 5.4 The relationship between MPOD and the number of eggs consumed per week (Spearman's rho=0.287, p=0.015).

FOOD DIARY

Of the 100 participants who fully completed the questionnaire, 50 also completed the three-day food diary. Four out of the remaining 50 subjects failed to produce the food diary on request, but the other 46 were not asked to complete the diary for three reasons. Firstly, their MPOD measurements were made at the beginning of an academic year, so the student volunteers had just returned to university from their family homes, and in view of the anticipated change in their diets, it was felt that their food diaries may not be an accurate reflection of any nutrient-MPOD associations (or lack thereof), given that the rate of L and Z turnover in the retina has not been conclusively established – supplementation and dietary modification studies have shown a lot of variability with respect to how quickly or slowly MPOD changes occur in response to dietary modification or supplement use (e.g., Landrum et al. 1997; Bone et al. 2003; Stringham and Hammond 2008; Connolly et al. 2010; Nolan et al. 2011). Secondly, the food diary aspect of the study had proved something of a barrier to subject recruitment so far, and by removing this requirement, it was hoped that more individuals would be willing to participate, thus offering a better chance (power-wise) of establishing some statistically significant MPOD links with the variables examined in the questionnaire. Finally, a total of 45 food diaries had already been received, and from observation it seemed highly unlikely that MPOD in the Asian subjects was going to be related to their dietary intakes. Further to these reasons, two previous studies have obtained nutrient intakes for 45 and 50 subjects, and both of them reported statistically significant relationships with MPOD (Werner et al. 2000; Wenzel et al. 2007a), so the lower numbers here (compared with the

questionnaire data) should not have been a barrier to establishing such relationships if they existed.

Table 5.3 is a summary of questionnaire-collected and food diary-collected information that was considered pertinent to the ensuing analyses. Gender, Asian background and dietary background were reconsidered with respect to MPOD in this smaller group of subjects. The same results were encountered, namely, a higher mean MPOD in males than females (p=0.027), but no significant differences for Indian Asians compared with Pakistani Asians, or for meat-eaters compared with vegetarians (p>0.05). These same three variables were also taken into account for body mass index (BMI) and vegetable/fruit/egg intake, because it seemed reasonable to expect that their diets may be different from one another, with subsequently differing BMI and so on. The results revealed a higher mean BMI for males than females (p=0.001), but no other statistically significant differences. Consequently, Asian and dietary background was not considered in some of the specifically-chosen food diary variables given in the lower half of the table. Of these variables, energy, carbohydrates and total fat showed statistically significant differences between males and females, thus instilling some confidence in the credibility of the food diary data, given the BMI gender differences. Dietary L (combined with Z) was selected because of its assumed and reported association with MP (see table 1.1 in chapter 1); it was compared by gender (because of the differences in MPOD and energy values), by Asian background (because of potentially different types of vegetable intake etc.), by dietary background, and by vegetable/fruit/egg combined intake. Despite the male-female difference in MPOD, no such difference existed for L/Z intake. Conversely, vegetarians had a higher L/Z intake than meat-eaters (p=0.023), despite there being no difference in their mean MPOD values. No other significant differences were identified.

Continuous data		Categorical data	
Variable	Mean ± SD	Variable	N (%)
From questionnaire/MPOD measurements		From questionnaire	
MPOD	All: 0.44 ± 0.14 Male: 0.50 ± 0.10 Female: 0.41 ± 0.15* Indian: 0.44 ± 0.16 Pakistani: 0.43 ± 0.12 Meat-eater: 0.44 ± 0.12 Vegetarian: 0.45 ± 0.20	Gender	Male: 17 (34%) Female: 33 (66%)
Body mass index	All: 21.6 ± 4.0 Male: 24.8 ± 4.6	Asian background	Indian: 32 (64%) Pakistani: 14 (28%)

		Bangladeshi: 1 (2%)
		Sri Lankan: 2 (4%)
		Other: 1 (2%)

Vegetable + Fruit + Egg intake per week	All: 15.1 ± 8.5	Dietary background	Meat-eater: 35 (70%)
	Male: 16.1 ± 10.5		Part-vegetarian: 4 (8%)
	Female: 14.5 ± 7.3		Vegetarian: 11 (22%)
	Indian: 15.8 ± 9.7		
	Pakistani: 14.1 ± 5.3		
	Meat-eater: 14.5 ± 6.7		
	Vegetarian: 19.9 ± 11.8		

From food diary (nutrients per day)		VFE group	≤15 servings: 29 (58%)
			>15 servings: 21 (42%)

Energy (kilocalories)	All: 1950 ± 615
	Male: 2435 ± 659
	Female: 1699 ± 414*

Carbohydrate (grams)	All: 248.4 ± 80.3
	Male: 298.6 ± 97.5
	Female: 222.5 ± 55.6*

Total fat (grams)	All: 78.2 ± 29.6
	Male: 98.7 ± 27.6
	Female: 67.7 ± 25.0*

Lutein + Zeaxanthin (milligrams)	All: 1.31 ± 2.46
	Male: 0.63 ± 0.34
	Female: 1.66 ± 2.97
	Indian: 1.51 ± 2.97
	Pakistani: 1.12 ± 1.19
	Meat-eater: 0.94 ± 0.97
	Vegetarian: 2.76 ± 4.84*
	VFE ≤15: 0.99 ± 1.05
	VFE >15: 1.74 ± 3.59

Table 5.3 Summary data for the 50 Asian subjects with completed questionnaires and food diaries.

VFE = vegetable+fruit+egg.

*$p < 0.03$ (Comparison by independent-samples t-tests or Mann-Whitney U tests, e.g., male vs female mean MPOD: $t = 2.285$, $p = 0.027$ – see table A7.1 in appendix 7 for full details.)

Besides the food diary nutrients listed in table 5.3, the food diary analysis software provided data on many other nutrients. All these nutrients and their means are listed in appendix 7 (table A7.2), but the nutrients that are commonly labelled on food and drink

items in the UK are given in table 5.4, alongside the means found in the subject group here, and the guideline daily amounts as recommended by the Food and Drink Federation (FDF 2010). With the exception of protein, the averages for the subjects appear to compare well with the guideline amounts (which is to be expected given the healthy BMI average of this Asian sample), therefore implying that the food diary analysis was an accurate method of assessing nutrient intake.

Nutrient	Mean (±SD) from food diaries	Guideline daily amount
Energy (kilocalories)	1950 ± 615	2000
Protein (grams)	75.8 ± 32.9	45
Carbohydrate (grams)	248.4 ± 80.3	270
Sugars (grams)	96.9 ± 42.4	90
Fat (grams)	78.2 ± 29.6	70
Saturated fat (grams)	26.5 ± 10.5	20
Fibre (grams)	17.1 ± 7.1	24
Sodium (grams)	2.66 ± 1.03	2.4

Table 5.4 Mean nutrient values per day, derived from analysis of the 50 food diaries, plus the recommended daily allowances for each nutrient.

Correlation analysis was used to explore the possibility of any MPOD-nutrient associations. Using Pearson's r or Spearman's rho (dependent on normality), and scatter graph observations, no significant correlations between MPOD and any nutrient were found, including L and Z. In view of the gender difference in MPOD, the correlations were repeated for males and females separately. The only significant correlation to emerge was between male MPOD and both sodium (r=-0.609, p=0.01) and chloride (r =-0.690, p=0.002), i.e., sodium chloride (salt). In other words, as sodium chloride intake increases, MPOD decreases (figure 5.5).
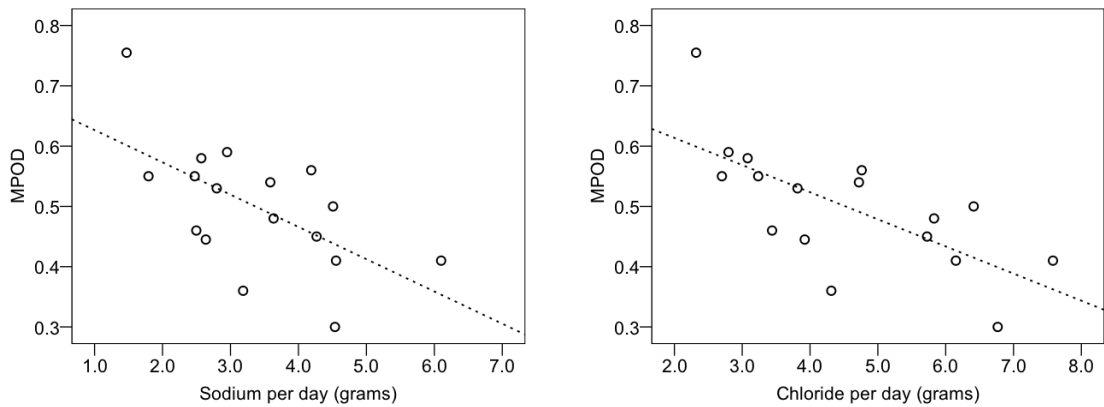
Figure 5.5 The relationship between MPOD and sodium chloride intake per day in male subjects (r=-0.609, p=0.01 for sodium, and r=-0.690, p=0.002 for chloride).

The possibility of any BMI-nutrient associations was also explored via Pearson and Spearman correlations, and scatter graph observation; no significant correlations were found, even when comparing males and females separately (on account of the BMI gender difference).

ASIAN VERSUS CAUCASIAN MPOD COMPARISONS

The total number of Caucasian subjects was 52, and their mean MPOD was 0.33±0.13. As established earlier, there were 117 Asian subjects in total, and their mean MPOD was 0.43±0.14. These means are significantly different (p<0.0005), as demonstrated in table 5.5, which is a breakdown of the information that was available for both study groups, and the results of all subsequent analyses. Figure 5.6 is a frequency distribution of MP densities for both racial groups. Because the number of Asian subjects was more than double that of the Caucasian subjects, the possible influence of differing sample sizes was explored by a random selection of 52 Asian participants (using the website 'random.org'); the mean Asian MPOD remained higher than the Caucasian mean, and the difference was still highly significant (see table 5.5). However, it was also confirmed that the Caucasian group was older than the Asian group, on average, and moreover, that a significant decline in MPOD with age was present in this particular sample (figure 5.7), which could therefore be the cause of the differing MPOD means between the two races. Although there was still a difference in the mean ages (p=0.01), limiting the Caucasian subjects to the same age range as the Asian subjects (18-30 years) removed the MPOD-age correlation, yet a statistically significant difference between the two MPOD means (p=0.002) was maintained. The age range limitation gave rise to an even bigger difference in sample size, though, so a further random selection of the Asian participants took place. Again, the MPOD means remained significantly different

(p=0.007). Finally, MPOD in the Caucasian subject group was examined for any gender differences. In contrast to the Asian subject group, the mean MPOD in males was lower than the mean MPOD in females, but this difference was not statistically significant.

Variable	Group (n)	Mean ± SD	Statistic
MPOD (full samples)	Asian (117)	0.43 ± 0.14	U = 1705, p < 0.0005
	Caucasian (52)	0.33 ± 0.13	
MPOD (equal sample sizes)	Asian (52)	0.45 ± 0.13	U = 661, p < 0.0005
	Caucasian (52)	0.33 ± 0.13	
Age, years (full samples)	Asian (117)	21.3 ± 2.6	U = 1657, p < 0.0005
	Caucasian (52)	27.6 ± 9.3	
MPOD & Age (full samples)	Asian (117)	N/A	rho = -0.105, p = 0.260
	Caucasian (52)	N/A	rho = -0.357, p = 0.009
MPOD & Age (18-30 years)	Asian (117)	N/A	rho = -0.105, p = 0.260
	Caucasian (39)	N/A	rho = -0.135, p = 0.413
Age (18-30 years)	Asian (117)	21.3 ± 2.6	U = 1657, p = 0.01
	Caucasian (39)	23.2 ± 3.6	
MPOD (18-30 years)	Asian (117)	0.43 ± 0.14	t = 3.175, p = 0.002
	Caucasian (39)	0.35 ± 0.14	
MPOD (18-30 years, equal sample sizes)	Asian (39)	0.43 ± 0.12	t = 2.785, p = 0.007
	Caucasian (39)	0.35 ± 0.14	
Caucasian MPOD	Male (15)	0.30 ± 0.14	U = 234.5, p = 0.385
	Female (37)	0.34 ± 0.13	

Table 5.5 Comparisons between the Asian and Caucasian subject groups.

'U' refers to a Mann-Whitney U test, which was used when one or both data sets were not normally distributed. Otherwise, an independent-samples t-test was used (the assumption of homogeneity of variance was met in both cases). 'rho' refers to a Spearman's rho correlation, which was used because one or both data sets were not normally distributed.
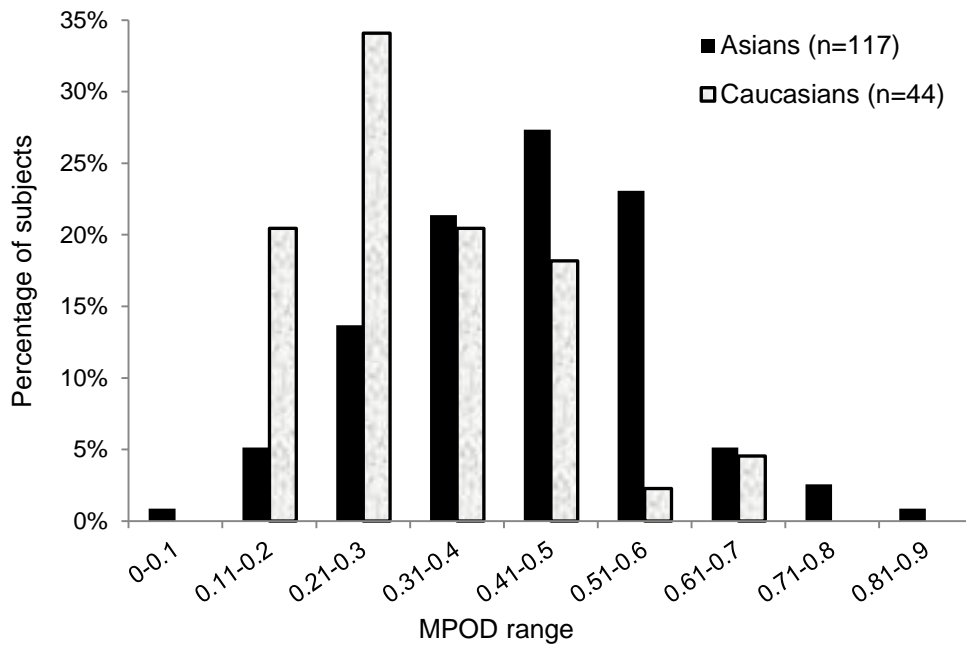
Figure 5.6 The frequency distribution of MPOD in the Asian and Caucasian subjects.
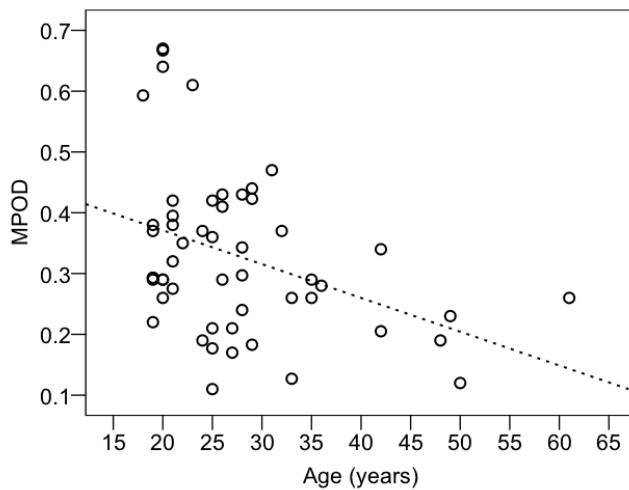
Figure 5.7 The relationship between age and MPOD for the Caucasian subjects (Spearman's rho=-0.357, p=0.009).

5.4 Discussion

With no previously published research on South Asians, current knowledge regarding MPOD in this particular racial group was negligible at best. The present investigation addressed this issue by obtaining MPOD measurements in a South Asian population sample, evaluating those measurements against a variety of personal and dietary characteristics obtained from a purposely-designed questionnaire and a three-day food diary, and finally, comparing them with mean MPOD measurements from a Caucasian group.

For 117 young Asian subjects, the mean MPOD was 0.43±0.14, which is higher than all previously published MPS-based MP averages (Makridaki et al. 2009; van der Veen et al. 2009a; van der Veen et al. 2009c; Bartlett et al. 2010c; de Kinkelder et al. 2011). The male subjects averaged a higher mean MPOD than the female subjects, and the difference was statistically significant. As stated in chapter 4, this finding is in common with nine other MPOD studies (Hammond et al. 1996a; Wooten et al. 1999; Hammond and Caruso-Avery 2000; Berendschot et al. 2002a; Broekmans et al. 2002; Mellerio et al. 2002; Lam et al. 2005; Nolan et al. 2007b; Bartlett et al. 2010c), while the opposite has been reported in only one study to date (Nolan et al. 2010). Several reasons have been put forward for why this difference may exist. Firstly, L in adipose tissue has been shown to correlate positively with MPOD in men, but negatively or not at all in women (Johnson et al. 2000; Broekmans et al. 2002). In another study, a female group of volunteers had statistically significant higher adipose L levels than a male group, while the males had statistically significant higher MP levels than the females (Berendschot et al. 2002a). It therefore seems sensible to suggest that competition between adipose tissue and retinal tissue may exist in females, and indeed this has been the opinion of others (Johnson et al. 2000; Broekmans et al. 2002). Hammond et al. (1996a) also hypothesized that retinal L and Z are metabolized differently between sexes, Berendschot et al. (2002a) proposed that gender differences might arise as a result of differences in the transport of L and Z to the retina, while Curran-Celentano et al. (2001) and Nolan et al. (2007b) thought that hormonal differences could be the cause. Nolan and colleagues also suggested that the higher body fat of women might also be a reason. Nevertheless, and whatever the reason might be, it should not be forgotten that the majority of studies have not found any gender differences (see table 1.1 in chapter 1), and even in the present study, the effect size (r-value 0.25) was only small to medium, thus suggesting that there is still a great deal of unexplained variation in what determines an individual's MPOD.

Aside from the gender-related difference, MPOD in the present study was unrelated to virtually all of the physical, ocular, lifestyle, dietary and environmental factors determined from the questionnaire. Most notably lacking any association – in the context of findings from other MPOD investigations – was fruit and vegetable intake, and BMI. In view of the dietary sources of L and Z (Sommerburg et al. 1998; Perry et al. 2009; USDA 2010), it would not be unexpected for there to be a positive relationship between MPOD and consumption of fruits and vegetables, and two (out of six) previous studies have found such a relationship (Mellerio et al. 2002; Burke et al. 2005). One of these obtained their significance between a 'low consumer' fruit and vegetable group, and a 'very high

consumer' fruit and vegetable group (Burke et al. 2005) (there were four groups in total). The low group averaged less than three servings per day, while the very high group averaged more than five servings per day. Considering that the average combined consumption of fruits and vegetables in this study was less than two servings per day, it is perhaps unsurprising that no link with MPOD was found. On the other hand, Mellerio et al. (2002) combined fruit and vegetable intake with egg intake, and compared MPOD between a group of subjects consuming 16 or more servings per week (n=62) and a group of subjects consuming 15 or less servings per week (n=62). Despite these two groups being extremely similar to the present investigation in terms of servings and subject numbers, and notwithstanding the fact that both studies equate to a considerably lower average intake than the low consumer group in Burke et al.'s study (even with eggs included), Mellerio et al. obtained a highly significant difference in mean MPOD between the two categories (p<0.001). Nevertheless, it could be that the contrasting finding of the current study with that of Mellerio's was to do with the types of fruit and vegetable being eaten (and hence the levels of L/Z being consumed).

Although there have been several studies, like the current one, that have not found any association between BMI and MPOD, an equal number of studies have reported a statistically significant relationship (see table 1.1 in chapter 1), with increasing BMI being linked to decreasing MPOD. However, not all of these significant relationships have stemmed from correlation analysis. For example, there wasn't a significant linear correlation for subjects in the above-mentioned study by Burke et al. (2005), but there was a significant difference when the subjects were split into two BMI groups (<27 and ≥27). Similarly, Hammond et al. (2002) reported that their correlation was led by participants with BMIs greater than 29; the correlation became insignificant after removing these individuals. As a result, it could be that the lack of any significant relationship in the present study reflects the fact that very few subjects were overweight or obese (n=15 [15%] with a BMI ≥25, and n=4 [4%] with a BMI ≥30). Still, splitting the Asian study group as per Burke et al. did not reveal any statistically significant MPOD differences. In fact, the mean MPOD of the group with BMIs greater than 27 was higher than the mean MPOD of the group with BMIs less than 27, although this was probably because 75 percent of the former group were male (with their accompanying higher MP levels), compared with 33 percent in the latter group.

Of the many other variables that were unrelated to MPOD in this study, it is noteworthy that although there was a weak correlation between increasing hyperopia and decreasing

MPOD (Spearman's rho=-0.114), this correlation was not statistically significant (p=0.235), unlike the anisometropia study described in the preceding chapter.

After delving a little deeper, a significant correlation did come to light between MPOD and the number of eggs consumed per week (Spearman's rho=0.287, p=0.015). This is a plausible relationship because eggs are known to be a highly bioavailable source of L (Handelman et al. 1999; Chung et al. 2004) and have also been shown to increase MPOD in a fairly modest (six eggs per week) intervention study (Wenzel et al. 2006b).

The final observation for the questionnaire was an approaching-significance (p=0.059) MPOD difference between individuals who wore spectacles or ultraviolet (UV)-filtering CLs full time (MPOD 0.47±0.15) and those who wore no spectacles or non-UV-filtering CLs (MPOD 0.42±0.14). Unlike BMI, the differences in the means here were not apparently driven by a higher proportion of males in the spectacle/UV CL group (36% males vs 64% females) than in the opposite group (42% males vs 58% females). This appears to be the first MPOD study to explore these refractive correction variables, so there are no comparisons to be drawn in this case, although the reasoning behind any association, should it truly exist, would presumably have something to do with UV light. Light exposure, particularly UV and blue light, is damaging to the retina (e.g., Ham et al. 1976; Ham et al. 1978; Ham et al. 1980; Ham et al. 1982), and it has been shown to cause the production of free-radicals, thus leading to oxidative stress and possibly AMD (Algvere et al. 2006). From population-based studies, there is some evidence to suggest that chronic light exposure is a risk factor for AMD (e.g., Taylor et al. 1992; Tomany et al. 2004; Hirakawa et al. 2008). So, taking all this into account, with less UV light reaching the retina as a result of absorption by spectacles (see note under table 5.1) or UV CLs, there may be less light-induced oxidative stress occurring in the retina, thus permitting an augmentation of MP by reducing its antioxidant-function workload. That said, there was no sign of any MPOD relationship with questionnaire-reported sunglasses use or light exposure, but this is not particularly surprising. With respect to sunglasses, they are mostly limited to wear in bright conditions (and the questionnaire only asked about their use in bright conditions) so the UV protection is intermittent. Spectacles and CLs, though, would be worn far more often because of the need to see clearly (and the questionnaire asked specifically about the frequency of spectacle/CL wear and their use in public), so chronic exposure to UV light would be less than with sunglasses. For light exposure, an approach similar to Mellerio et al. (2002) was chosen, because they found a significant (negative) correlation between MPOD and light. However, in the present study, the questions on time spent outdoors were far and above the ones that participants struggled

with the most, so the lack of any significant correlation may in part be a reflection of the overall difficulties in establishing an individual's light exposure.

For the 50 Asian subjects with completed three-day food diaries, the WISP nutritional analysis package produced daily nutrient averages that were in agreement with recommended daily intakes (table 5.4), so in view of the healthy average BMI of the participants, it seems reasonable to assume that the food diary inputs were accurate. Furthermore, published L and Z mean daily intakes from MPOD studies have varied from 1.1 mg (Ciulla et al. 2001a) up to 3.7 mg (Beatty et al. 2001), so the average of 1.3 mg per day in this study lends support to the reliability and accuracy of manually inputting L/Z levels using the USDA nutrient database (USDA 2010).

On average, the vegetarians in this part of the study had a statistically significant higher average intake of L/Z than the meat-eaters. This was not altogether unexpected, but it was interesting that there was no concurrent higher average MPOD for the vegetarians. In the same vein, the males in this part of the study did not have a higher intake of L/Z despite maintaining a higher average MPOD than the females. In fact, although not statistically significant, the female L/Z mean was more than double that of the male. This kind of outcome has been reported before (Hammond et al. 1996a; Curran-Celentano et al. 2001; Burke et al. 2005), and it ties in with the theories for male-female MPOD differences discussed earlier. However, the L/Z-MPOD insignificance for dietary background and gender was more likely driven by the lack of any overall correlations between MPOD and L/Z, which was against the findings of some investigations (e.g., Bone et al. 2000; Werner et al. 2000; Ciulla et al. 2001a; Curran-Celentano et al. 2001; Mares et al. 2006; Wenzel et al. 2007a), and in accordance with several others (e.g., Hammond et al. 1996a; Beatty et al. 2001; Kirby et al. 2010). Aside from a genuine lack of any L/Z-MPOD correlation, it is possible that the cause was rooted in a problem with the method used to collect dietary information; in the life of a student, a three-day food diary may not be very representative of overall diet, so in hindsight a detailed food frequency questionnaire would probably have been a better option. This theory is in part supported by the fact that BMI was unrelated to any of the major dietary components that might be expected to influence it (i.e., energy, fat, carbohydrate).

The only nutrients that did demonstrate a relationship with MPOD were sodium and chloride (i.e., salt) in males. The correlation was inverse and strong (see figure 5.5), but in view of the food diary issue mentioned in the previous paragraph, this result should

probably be interpreted with caution, particularly as salt has never been mentioned before in terms of MP.

Nutrients aside from L and Z that have been noted to have some level of association with MPOD in earlier studies are fat, fibre and iron. Positive and significant associations have been reported between dietary fat intake and MPOD in males, and the exact opposite for females (Hammond et al. 1996a), although in most other studies (Hammond et al. 1995; Werner et al. 2000; Ciulla et al. 2001a; Curran-Celentano et al. 2001; Nolan et al. 2004; Nolan et al. 2007b), including an all-female one (Mares et al. 2006), there have been no significant associations. Mares et al. (2006) did find, however, that polyunsaturated fat intake, in combination with dietary L/Z and fibre, accounted for three percent of the variation in MPOD in their study of 1698 women; higher intakes of these nutrients resulted in higher MPOD. Ciulla et al. (2001a) also reported a positive and significant link with fibre, while Hammond et al. (1996a) obtained a significant, positive relationship between MPOD and iron intake in men.

Comparing the Asian MPOD mean with the Caucasian MPOD mean revealed highly significant differences; MPOD in the Asian participants was higher than in the Caucasian participants, and this was the case regardless of various sample size and age restrictions (table 5.5). Even though there was still a lot of MP overlap between the two races (figure 5.6), and hence a lot of variation left unexplained by ethnicity (the effect size, r, was 0.35, i.e., a medium effect), the difference was clear. So what might be the cause? Going by the low fruit and vegetable consumption of the Asian group, it seems unlikely that this difference would be the result of dietary factors. It also seems unlikely that gender differences between the groups would be a factor, because the ratio of males to females in each group was similar, and comparison tests demonstrated significant MPOD differences even when each gender was considered separately (U=120, p<0.0005 for males, and U=890, p=0.004 for females). It therefore seems reasonable to assume that the cause lies elsewhere.

Various aspects of foveal architecture have been associated with MPOD (see table 1.1 in chapter 1). For instance, Nolan et al. (2008) found MPOD to be positively and significantly correlated with foveal width (r=0.41, p=0.001), and moreover, that the foveas of their non-white subjects (Indian/Asian/Hispanic/black) were significantly wider than the foveas of their white subjects (p<0.05). Consequently, it is possible that ethnic variations in foveal architecture could be a reason for the disparity in MPOD, at least in part. From this point of view, it would have been useful to know the spatial profile of MP for the study

participants, something that is becoming increasingly popular and beneficial in MP studies (Chen et al. 2001; Robson et al. 2003; Berendschot and van Norren 2006; Wenzel et al. 2007b; Trieschmann et al. 2008), including race-related ones (Wolf-Schnurrbusch et al. 2007; Nolan et al. 2008).

Another likely candidate for what might cause differences in MPOD between these two races is iris colour, or more specifically, melanin. Like MP, melanin absorbs short-wavelength light (mainly UV rather than blue) and has antioxidant properties. In the iris, its function is predominantly the former, and in the RPE and choroid its function is predominantly the latter (Hu et al. 2008). Returning to parallels between AMD and MPOD, light irides, which by virtue of their colour contain less melanin than dark irides, have frequently been identified as a risk factor for AMD (e.g., Hyman et al. 1983; Weiter et al. 1985; Sandberg et al. 1994; Mitchell et al. 1998; Frank et al. 2000; Nicolas et al. 2003) and a risk factor for low MP (Hammond et al. 1996b; Wooten et al. 1999; Hammond and Caruso-Avery 2000; Ciulla et al. 2001a; Mellerio et al. 2002; Kirby et al. 2010; Sandberg et al. 2010), so in an ethnic group where the vast majority of irides are dark, it seems logical that they might have higher levels of MP (and a lower risk for AMD) than an ethnic group with a mixture of light and dark iris colours. On account of the expected high propensity of dark irides in the Asian sample, an eye colour related MPOD difference was not anticipated. All the same, the group were split into a 'dark brown' and an 'other' category, and although not reaching statistical significance, there did appear to be a trend towards the darker brown eyes having higher MPODs (p=0.078). The spread of MPOD in both groups is illustrated in figure 5.8. Iris colour in this study, as in others (Hammond and Caruso-Avery 2000; Ciulla et al. 2001a; Mellerio et al. 2002), was self-reported and not verified, so the fact that a difference may exist between two such subtle iris colour groups lends support to the theory of melanin, or indeed a genetic unknown that has a common inheritance with melanin, being a cause of MPOD differences between racial groups. In order to rule out the possibility of a greater male dominance in the dark brown than the 'other' iris group (with subsequent higher MPOD being the result of gender rather than eye colour), the means were compared separately by independent t-tests (the assumption of normality was met by all groups). For males, the difference became more insignificant – dark brown mean (n=24): 0.47±0.12, 'other' mean (n=16): 0.48±0.13 (t=-0.379, p=0.707). For females, however, the difference became statistically significant – dark brown mean (n=37): 0.45±0.16, 'other' mean (n=29): 0.37±0.12 (t=2.365, p=0.021). To investigate this further, table 5.6 shows the individual MPOD breakdown of the more unusual iris colours in the 'other' category (unusual, that is, for an Asian population), so brown irides, which accounted for the largest percentage eye colour

in this group, have been omitted. Although the small numbers are probably unsuitable for statistical analysis, it is striking that all but one MPOD value is well below the overall average of 0.43±0.14 and below the 'other' iris colour group's average of 0.41±0.13. The exception to the rule is one of only two males in this subgroup, so it is possible that the higher MPOD is related to his gender. It therefore seems highly plausible that iris colour is a cause of the racial differences in mean MPOD observed in this study. By filtering out more light than lighter coloured eyes, dark eyes could reduce the amount of harmful wavelengths reaching the retina (Hammond et al 1996b; Hu et al. 2008), and as a result, there might be a lowering of oxidative stress and less depletion of MP than in lighter eyes (by removing some of the antioxidant workload). Furthermore, an additional benefit might come from the choroid, whose melanin levels are correlated with iris melanin (Wakamatsu et al. 2008) and are known to vary between races (Weiter et al. 1986). As stated above, choroidal melanin is an antioxidant, so with higher levels of it, there may again be less work for MP, although the antioxidant activity of melanin in the choroid is clearly in a different location to MP. Another possibility, as suggested by Hammond et al. (1996b), is that melanin and MP deposition are co-inherited traits. An interesting investigation that could truly establish whether MPOD is related to iris colour/melanin would be a study that measured MPOD in individuals with simple iris heterochromia.
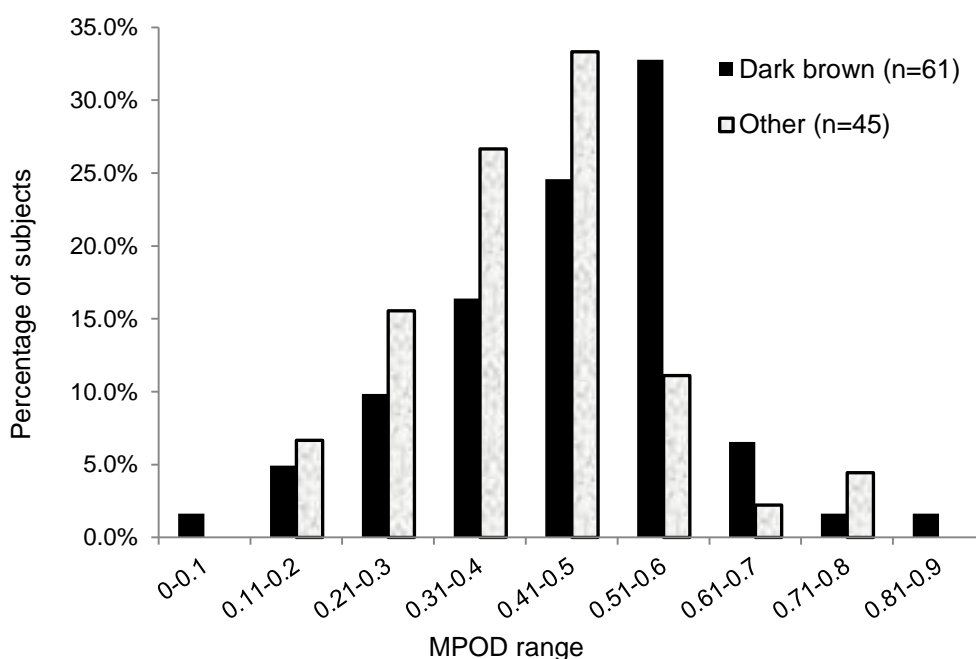
Figure 5.8 The frequency distribution of MPOD for individuals with dark brown eyes and other-coloured eyes (brown, light brown, hazel, green) in the Asian subject group.

Iris colour	MPOD	Gender
Light brown	0.36	Male
Hazel	0.58	Male
Hazel	0.26	Female
Hazel	0.30	Female
Hazel	0.35	Female
Hazel	0.38	Female
Green	0.31	Female
Green	0.36	Female

Table 5.6 The individual MPOD values of the eight Asian subjects with iris colours other than dark brown or brown.

A final rationale for racial differences in MPOD could lie in ethnicity-variant genetics besides melanin, but the possibilities in this respect are highly diverse. For example, there might be ethnic variations in how effectively L and Z are absorbed, transported or deposited, or there could be specific genes/alleles that lead to higher or lower MPOD, or indeed the first example could be the result of the second example. Twin studies on MPOD have demonstrated that there is definitely a genetic component to individual MP levels (Hammond et al. 1995; Liew et al. 2005), and a spousal-based MPOD study appeared to confirm this also – despite statistically significant correlations for dietary and blood serum levels of L and Z between married couples, MPOD between husbands and wives was not correlated at all (Wenzel et al. 2007a). Recently, and for the first time, a specific gene and its respective genotypes were examined as part of an MPOD study; Loane et al. (2010a) compared MPOD in three different genotype groups of the apolipoprotein E gene, a gene that codes for lipoproteins that may carry L and Z in serum. It was found that subjects in one of the genotype groups – which contained at least one specific allele – had significantly higher MPOD than subjects in the other two groups – which were both without the specific allele. (As described by Loane et al., the allele in question, Apo ε4, has also been shown to reduce the risk for AMD.) This confirms again the genetic aspect of MP, which is not unexpected given the still large amount of unexplained inter-subject MPOD variation in the current study and many previous ones.

In conclusion, MPOD in a group of young adults of South Asian origin was largely unrelated to any physical, ocular, lifestyle, dietary or environmental factors. Males and females, however, did have significantly different MP levels, and compared with a Caucasian group of adults, MPOD was significantly higher in the Asian group, which in

turn could mean a decreased risk for AMD in this race. As discussed in the introduction, though, current information on AMD prevalence in South Asians, particularly with respect to comparisons with Caucasians, is limited.

5.5 Summary

This chapter has explored for the first time the range of MP in a South Asian population sample. In the next chapter, observation turns to intervention, with a novel attempt to increase MPOD.

CHAPTER 6: THE EFFECT OF BLUE-LIGHT-FILTERING CONTACT LENSES ON MACULAR PIGMENT OPTICAL DENSITY: AN EXPLORATORY STUDY

In each of the earlier chapters, the MPOD measurements have been a one-visit assessment, because that was all that was required for the nature of the research being conducted. However, the key findings from the previous chapter were that, in general, South Asians have higher MPOD values than Caucasians, and that male Asians have higher MPOD values than female Asians. In other research a number of additional variables have been associated with differences in MPOD. So what can be done for those individuals who have reduced MPOD compared with others, and thus a potentially increased risk for AMD? Dietary intervention is the standard method by which researchers have aimed to increase MPOD, but the study described in this chapter steps away from convention and introduces a fresh attempt at increasing MPOD.

6.1 Background and rationale

In the preceding chapter, cumulative exposure to sunlight as a risk factor for AMD was mentioned briefly. Like many of the proposed causes of AMD, the reasoning behind the hypothesis is interwoven with several other hypotheses as well. Firstly there is the widely quoted work of Ham and colleagues who reported a series of monkey-based experiments that showed a harmful effect to the retina of short-wavelength light (UV, violet and blue) compared with longer wavelengths of light (Ham et al. 1976; Ham et al. 1978; Ham et al. 1980; Ham et al. 1982). As a result, there might be a directly damaging effect of this type of light over time in the human eye. In combination with this reason, or unaccompanied, it could be that light in general (and short-wavelength light in particular) contributes to oxidative stress in the retina (Beatty et al. 2000b; Algvere et al. 2006), oxidative stress being one of the major pathogenesis theories of AMD (Beatty et al. 2000b). Both these theories then go hand in hand with the next thread in the AMD-light exposure story, whereby cataract surgery (and lens replacement with a UV-filtering intraocular lens) has been connected on some level with the development or progression of AMD (Bockelbrink et al. 2008), the theory being that after cataract surgery, the blue light absorption previously carried out by the aging, yellowing crystalline lens can no longer happen, so the retina experiences an influx of blue light (Bockelbrink et al. 2008) and everything that may or may not go along with that as described above. Add to all this the potential contribution of MP, and things become even more interesting, which is what led to the original idea for the current study.

There has been a great deal of interest surrounding the use of yellow (i.e., blue-light-filtering) intraocular lenses (IOLs), and the possibility that they may confer some protection against AMD by filtering out blue light in addition to UV light (Cuthbertson et al. 2009; Edwards and Gibson 2010; Henderson and Grimes 2010; Davison et al. 2011). Several thorough, contemporary reviews have advocated the use of these IOLs, concluding that there are no clinically-significant disadvantages over UV-only IOLs (Cuthbertson et al. 2009; Henderson and Grimes 2010; Davison et al. 2011), and that besides the potential for AMD protection, they may actually offer other advantages, such as improvements to glare disability (Hammond et al. 2010; Gray et al. 2011). Although at the present time there have been no clinical studies examining whether yellow IOLs decrease the risk for, or slow the progression of, AMD, Davison et al. made the seemingly valid statement that the large number and range of studies conducted are enough to support the use of yellow IOLs, in the same way that UV-blocking IOLs were introduced 'without definitive clinical trial' (Davison et al. 2011).

With a yellowing natural lens or a yellow artificial lens filtering out some of the blue light before it reaches the retina, there could be an expected augmentation in MP, or at least less depletion of MP, because it is being assisted by either lens type. This was alluded to in a previous chapter when discussing the possibility of myopic refractive error being associated with higher MPOD (section 4.4 in chapter 4). In that chapter it was also pointed out the relationship between age and MP is unresolved. This might well be down to a complicated balance between increased lens yellowing with age (and the subsequent possibility of an augmentation in MP), and increased oxidative stress with age (Nolan et al. 2009; Beatty et al. 2000b) (and the subsequent possibility of a reduction in MP), both of which would be expected to vary to some degree from person to person. So what if a blue filter was introduced to a younger, less lens-yellowed eye, such that it would mimic the absorption characteristics of an older, more lens-yellowed eye? Without the equivalent oxidant load of an older eye, it is theoretically plausible that the MP of that younger eye would increase. However, in the absence of any pathology or refractive requirement, clear lens exchange for a blue-filtering IOL would be improbable and unethical. The wearing of yellow spectacle lenses is an option, but they would perhaps be considered cosmetically unappealing. Yellow CLs, on the other hand, would be more discreet than spectacles, and they could offer further protection to the retina by filtering light from all directions (Kwok et al. 2003; Walsh and Bergmanson 2011), assuming, that is, that any spectacles worn would be of a non-wraparound style.

The aim of this exploratory study was to examine the effect, if any, on MPOD of wearing a yellow (blue-light-filtering) CL over the course of one year. If found to increase MPOD (or even if not), more extensive trials could see these lenses eventually being offered as a protective measure to individuals most at risk of AMD, such as those with a family history of the disease.

6.2 Methods

SUBJECTS

A university-wide recruitment email was sent to staff at Aston, and a separate email was sent to all current undergraduate optometry students at Aston. The emails specified that the study was for experienced soft CL wearers only. Interest from 22 people was received. The exclusion criteria for this study included toric and multifocal CL wearers, so six of the 22 potential volunteers were unsuitable on these grounds. In addition, two volunteers anticipated being unable to attend all the appointments, one did not wish to wear non-daily disposable CLs, one developed an eye infection before the first planned appointment and consequently decided against participating, and four failed to reply to follow-up emails. Further exclusion criteria (besides toric and multifocal lenses) were: age younger than 18 years or older than 60 years; VA worse than 0.2 logMAR (Snellen 6/9.5); and the presence of any relevant ocular disease. Eight individuals attended an initial appointment with investigator 1 – an eye and CL examination, and an MPOD assessment on the MPS. All were deemed suitable for inclusion and consented to take part in the study, but one volunteer (for reasons unknown) dropped out before the next appointment. The remaining seven subjects (1 male, 6 females) took part for the whole duration of the study. They were all in good general and ocular health, with a spectacle and CL VA of 0.14 logMAR (Snellen $6/7.5^{-2}$) or better in each eye (except for one subject's CL VA, which was corrected for her near vision).

Aston University's Ethics Committee approved the study. All subjects signed an informed consent form, and all procedures adhered to the tenets of the Declaration of Helsinki.

CHOICE OF CONTACT LENS AND YELLOW TINT

Several factors were taken into consideration when choosing which CL to use, including type, frequency of replacement, and whether or not have a lens with a high level of UV absorption. The decision was made to use a two-weekly disposable, daily wear silicone hydrogel lens with high UV-blocking properties (Acuvue Oasys). The UV element meant that the yellow CLs would be comparable to yellow IOLs, with UV light being absorbed as well as blue.

Several sample lenses were sent for tinting to an independent CL manufacturer with expertise in specialist CLs (Cantor and Nissel Ltd, Brackley, Northamptonshire, UK). The samples were returned with corresponding transmission curves. Two types of yellow tint were supplied. According to the curves provided in a review article by Cuthbertson et al. (2009) (figure 6.1), the transmission of the lighter tint approximated well to the transmission of a 50-year-old crystalline lens for the wavelength range 430 nm to 500 nm. This included a virtually identical transmission (48% and 50%) at 460 nm, i.e., the peak of MP absorption. Beyond 500 nm, the transmission of the CL increased rapidly, reaching 80 percent transmission by 505 nm, compared with approximately 73, 65 and 56 percent at 505 nm for a 30, 50 and 70-year-old crystalline lens respectively. The deeper tint transmitted less than 20 percent of light up to 490 nm, then transmission increased rapidly, reaching 80 percent by 530 nm (figure 6.2), which equated well to 80, 73 and 65 percent at 530 nm for a 30, 50 and 70-year-old crystalline lens respectively. Although it was recognized that most blue-light-filtering IOLs attempt to mimic the transmission of an eye with a lens age somewhere between 30 and 50 years old (see figure 6.1), because the subjects taking part in this study were anticipated to be a range of ages (up to 60 years old), there seemed to be little advantage in choosing the lighter tint with transmission characteristics equivalent to a reasonably young lens. The darker yellow tint was thus selected (see figure 6.2). Thought was given to the possibility of disruption to visual function, but preliminary investigations showed no differences in terms of contrast sensitivity (Pelli-Robson), colour vision (City and Ishihara tests, plus traffic light colour recognition), stereopsis or VA. There has been some debate as to whether yellow IOLs reduce scotopic sensitivity and/or disrupt circadian rhythm (Mainster and Turner 2010), but the abovementioned reviews of the literature, particularly the later ones with access to the most recent data, have overwhelmingly come to the conclusion that any effects are clinically insignificant (Cuthbertson et al. 2009; Henderson and Grimes 2010; Davison et al. 2011). Admittedly, the chosen tint did suppress more light than a blue-filtering IOL, but the decision had already been taken to fit only one eye with a yellow CL, so any effects would be reduced by this approach, and moreover, the CLs were unlikely to be worn for all waking hours. Fitting the contralateral eye with an untinted lens of the same brand and type also offered a simple way of controlling for any within or between subject variables that could influence MPOD, such as dietary changes over time.

Figure 6.1 The average light transmission of the human crystalline lens at age 30, 50 and 70 (demonstrated on every graph), compared with the light transmission of a variety of intraocular lenses, divided by category. (Reprinted from Cuthbertson et al. 2009.)
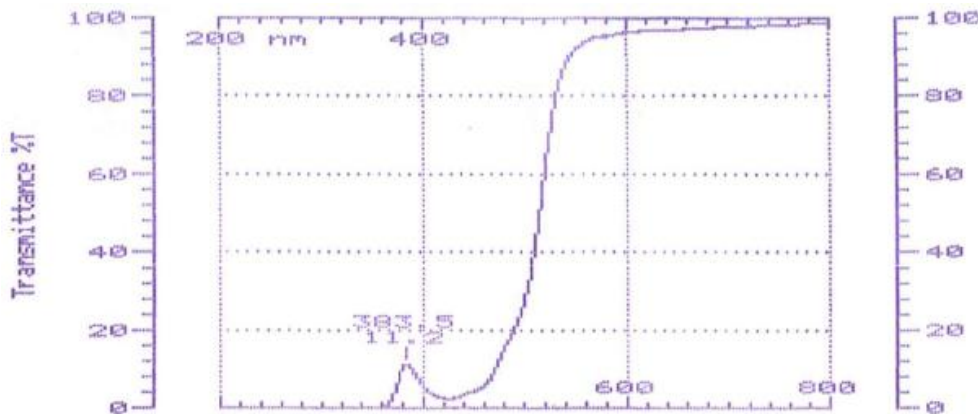
Figure 6.2 The transmission curve of the chosen yellow (blue-filtering) CL (top), and its appearance in situ (bottom). (Photograph courtesy of Miss Cecylia Jones.)

MPS 9000 AND CONTACT LENS CHECKS

Visit 1: Investigator 1 (OH) performed an eye examination and a CL examination (with the subject's current CLs), followed by a CL fitting with the new (Acuvue Oasys) CLs, and then an assessment of MPOD using the MPS. For the latter, each subject received verbal instructions and completed a short practice test, after which the prior established protocol was used (chapter 3). Right and left eyes were assessed alternately; the right eye was always tested first. The averages of the central minima and peripheral minima were used to calculate MPOD. For the purposes of masking (see below) and consistency, subjects wore their spectacles for MPOD measurements throughout the study period.

After visit one, the appropriate CLs were ordered and sent on for yellow tinting. Unfortunately, some substantial delays in receiving the first batches of lenses meant that the length of the study had to be reduced to a total of nine months (eight months wear time and a one month post-study check-up).

Visit 2: Investigator 2 (FE) carried out a CL collection with the clear and yellow-tinted CLs. With no problems evident, subjects were permitted to begin lens wear. They were instructed to wear their CLs at least five days a week, on a daily wear basis.

Visit 3: After one month of CL wear, investigator 2 carried out a CL check. The lenses were removed and investigator 1 used the MPS to assess MPOD in both eyes of each subject.

Visit 4: After three months of CL wear, a third MPOD assessment was performed (investigator 1).

Visit 5: After five months of CL wear, investigator 2 carried out another CL check. The lenses were removed and a fourth MPOD assessment was performed (investigator 1).

Visit 6: After eight months of CL wear, investigator 2 carried out a final CL check. The lenses were removed and a fifth MPOD assessment was performed (investigator 1). Subjects were asked to stop wearing the yellow CL from then on; any surplus yellow lenses were collected at this appointment.

Visit 7: One month after ceasing yellow CL wear, a sixth and final MPOD assessment was performed (investigator 1).

The total time per visit was normally between 30 and 60 minutes.

MASKING

The study was single-masked. For all subjects, investigator 1 (operator for the MPOD measurements) was unaware of which eye was wearing the blue-light-filtering CL; investigator 2 was responsible for sending the appropriate lenses for yellow tinting.

SAMPLE SIZE

As an exploratory study, it was difficult to estimate the sample size required. Nevertheless, on the basis of G*Power 3.1 calculations for two dependent means (one-tailed), a sample size of ten subjects was recommended for 80 percent power at the five percent alpha significance level. This was assuming an increase in mean MPOD of 0.08 between the first and fifth MPOD measurements.

STATISTICS

Microsoft Excel and IBM SPSS were used for data analysis. Perhaps unsurprisingly for a small subject group, the data sets did not meet the assumption of normality, so mean MPOD values over time were compared by non-parametric repeated measures analysis. The differences between data sets were all normally distributed, though, so paired-samples t-tests were used when comparing mean MPOD values between two visits.

6.3 Results

MEAN MPOD

Table 6.1 shows the mean MPOD of the seven participants at each of the visits where MP was measured. Also shown is the mean C-only MPOD (peripheral estimate provided by the MPS, based on subject age), because for the purposes of monitoring change in MPOD over time, this test is acceptable, and the test-retest repeatability is better than it is for the standard (centre and peripheral derived) MPOD (see chapter 7).

Measure	Time	Mean (±SD) MPOD		Mean (±SD) C-only MPOD	
		Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	Baseline	0.45 ± 0.20	0.40 ± 0.14	0.49 ± 0.19	0.48 ± 0.15
2	One month of CL wear	0.45 ± 0.18	0.43 ± 0.18	0.50 ± 0.18	0.47 ± 0.18
3	Three months of CL wear	0.43 ± 0.20	0.44 ± 0.19	0.49 ± 0.20	0.48 ± 0.19
4	Five months of CL wear	0.45 ± 0.21	0.43 ± 0.18	0.50 ± 0.19	0.48 ± 0.19
5	Eight months of CL wear	0.46 ± 0.21	0.42 ± 0.15	0.50 ± 0.21	0.46 ± 0.17
6	One month post CL wear	0.45 ± 0.17	0.39 ± 0.12	0.50 ± 0.19	0.45 ± 0.13

Table 6.1 Mean MPOD (and C-only MPOD) of the seven participants at each of the six MPS visits.

By observation only, there did not appear to be any noticeable differences in the mean MPODs, and repeated measures analysis confirmed that there was no statistically significant change from baseline to the end of the wearing period for the eyes wearing the yellow CL or the eyes wearing the normal CL (table 6.2). The same was true for C-only MPOD. Paired-samples t-tests were used to compare two visits at a time. Only the results for measure one (baseline) versus measure five (end of the wearing period) are shown in table 6.2, but the results of all the other combinations can be found in appendix 8 (table A8.1). Again, there were no statistically significant differences, apart from a borderline difference between MPOD measures five and six for the eyes wearing the normal CL (p=0.047). Figure 6.3 clearly illustrates the lack of MPOD change over time in all categories.

	Comparison	Statistic value	Significance (p) value
Yellow CL eye	MPOD measure 1 to 5	$\chi^2 = 2.928$	0.570
	C-only MPOD measure 1 to 5	$\chi^2 = 0.992$	0.911
	MPOD measure 1 vs 5	t = -0.617	0.560
	C-only MPOD measure 1 vs 5	t = -0.634	0.549
Normal CL eye	MPOD measure 1 to 5	$\chi^2 = 4.571$	0.334
	C-only MPOD measure 1 to 5	$\chi^2 = 5.190$	0.268
	MPOD measure 1 vs 5	t = -1.592	0.162
	C-only MPOD measure 1 vs 5	t = 1.641	0.152

Table 6.2 Statistical comparisons of the mean MPOD across all of the MPS visits combined, except the last (i.e., measure 1 through to measure 5), and between visits one (measure 1 – baseline) and five (measure 5 – end of the wearing period) specifically.
'χ^2' refers to Friedman's ANOVA test, and 't' refers to a paired-samples t-test. Both tests were appropriate for the distribution of the data sets involved.
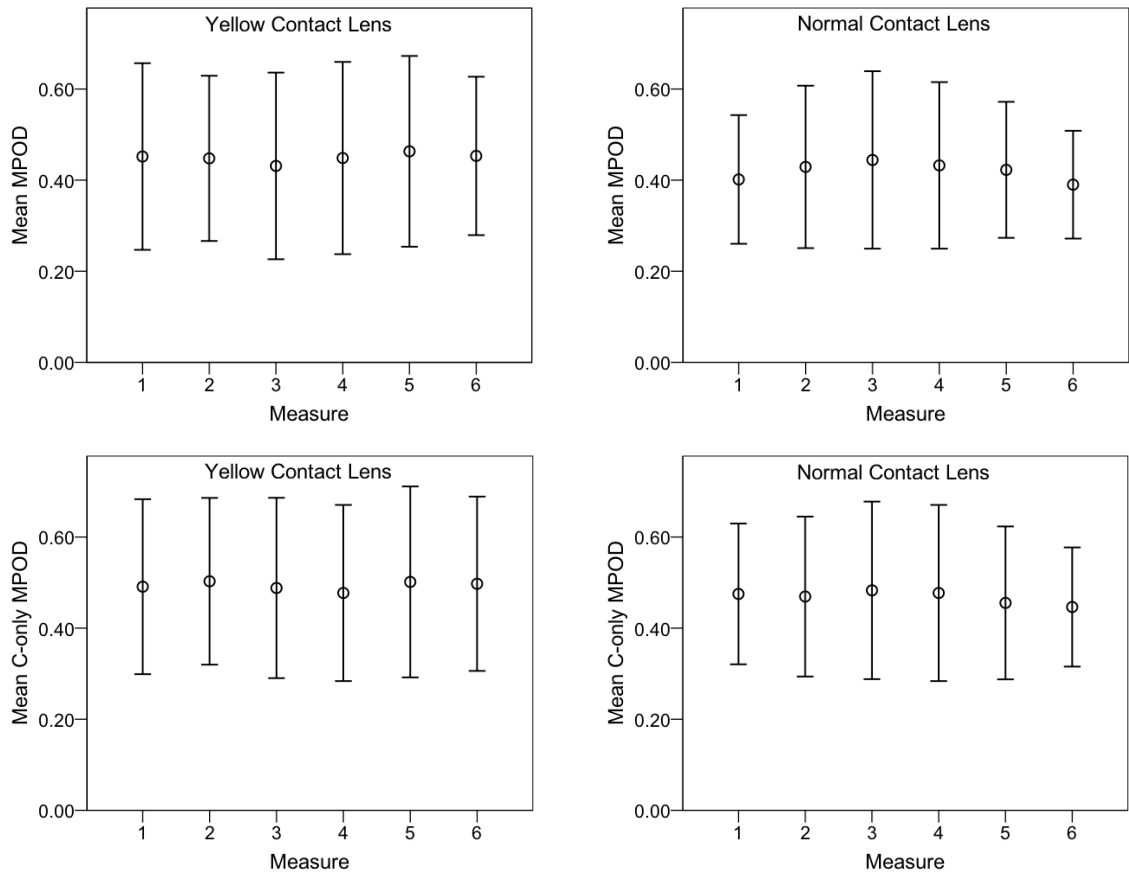
Figure 6.3 Mean MPOD (and C-only MPOD) at each MPS visit.

The error bars are ± 1 SD.

Measures: 1 = baseline; 2 = after one month of CL wear; 3 = after three months of CL wear; 4 = after five months of CL wear; 5 = after eight months of CL wear; 6 = one month post yellow CL wear.

INDIVIDUAL MPOD – SUBJECT 1

Background: 23-year-old Asian female with brown eyes.

Previous CLs: Silicone hydrogel, UV-blocking, two-weekly disposable, daily wear.

Study CLs: RE -3.25 DS, LE -3.00 DS. Worn for 8½ months between August 2010 and May 2011.

MPOD: See table 6.3 and figure 6.4.

MPS proficiency: Excellent – consistently optimal curves produced throughout the study.

Outcome (taking MPS proficiency into account): No change in MPOD.

Measure	MPOD		C-only MPOD	
	Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	0.33	0.34	0.38	0.38
2	0.29	0.29	0.36	0.36
3	0.30	0.34	0.37	0.38
4	0.34	0.34	0.38	0.36
5	0.36	0.31	0.38	0.35
6	0.36	0.33	0.41	0.38

Table 6.3 MPOD over time for subject 1.
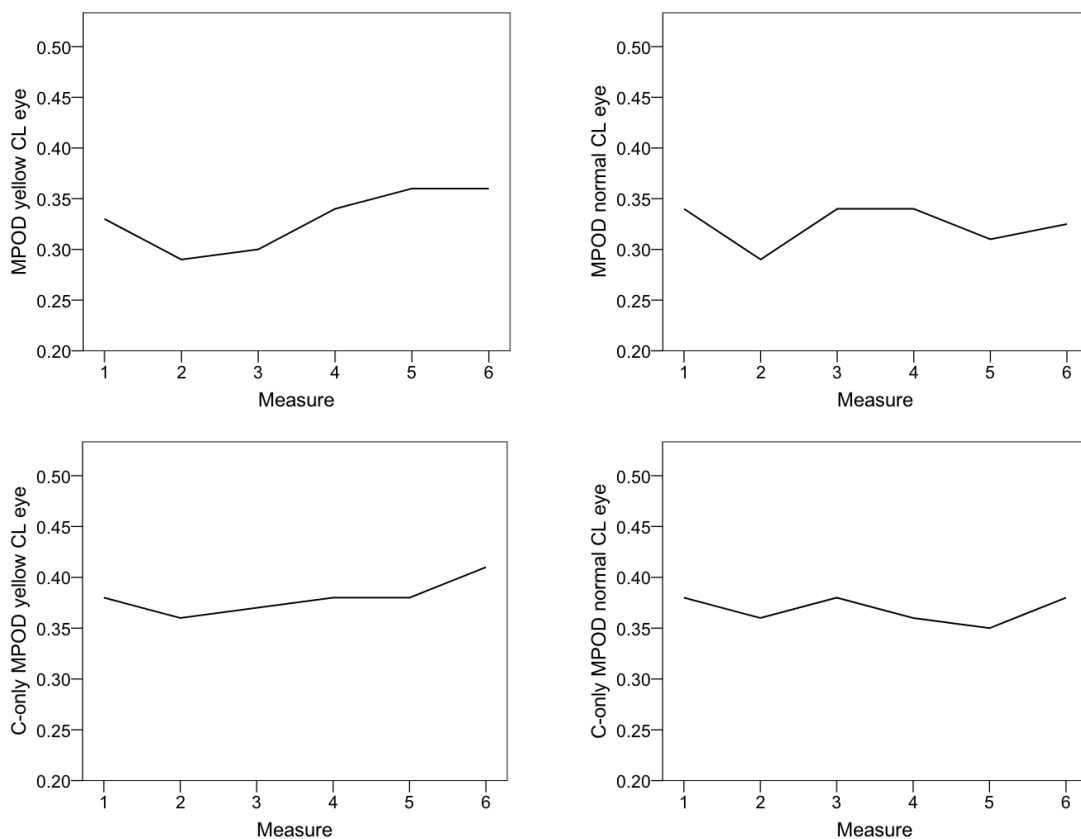
Figure 6.4 MPOD over time for subject 1.

INDIVIDUAL MPOD – SUBJECT 2

Background: 19-year-old Asian female with brown eyes.

Previous CLs: Non silicone hydrogel, non UV-blocking, monthly disposable, daily wear.

Study CLs: RE -4.00 DS, LE -3.50 DS. Worn for 8½ months between August 2010 and May 2011.

MPOD: See table 6.4 and figure 6.5.

MPS proficiency: Slightly variable – central and peripheral curves were mostly optimal, but sometimes not. Three tests were required for at least one eye at every visit.

Outcome (taking MPS proficiency into account): No change in MPOD.

Measure	MPOD		C-only MPOD	
	Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	0.39	0.30	0.42	0.45
2	0.42	0.38	0.45	0.41
3	0.34	0.40	0.40	0.41
4	0.38	0.36	0.42	0.42
5	0.47	0.35	0.49	0.37
6	0.41	0.32	0.41	0.37

Table 6.4 MPOD over time for subject 2.
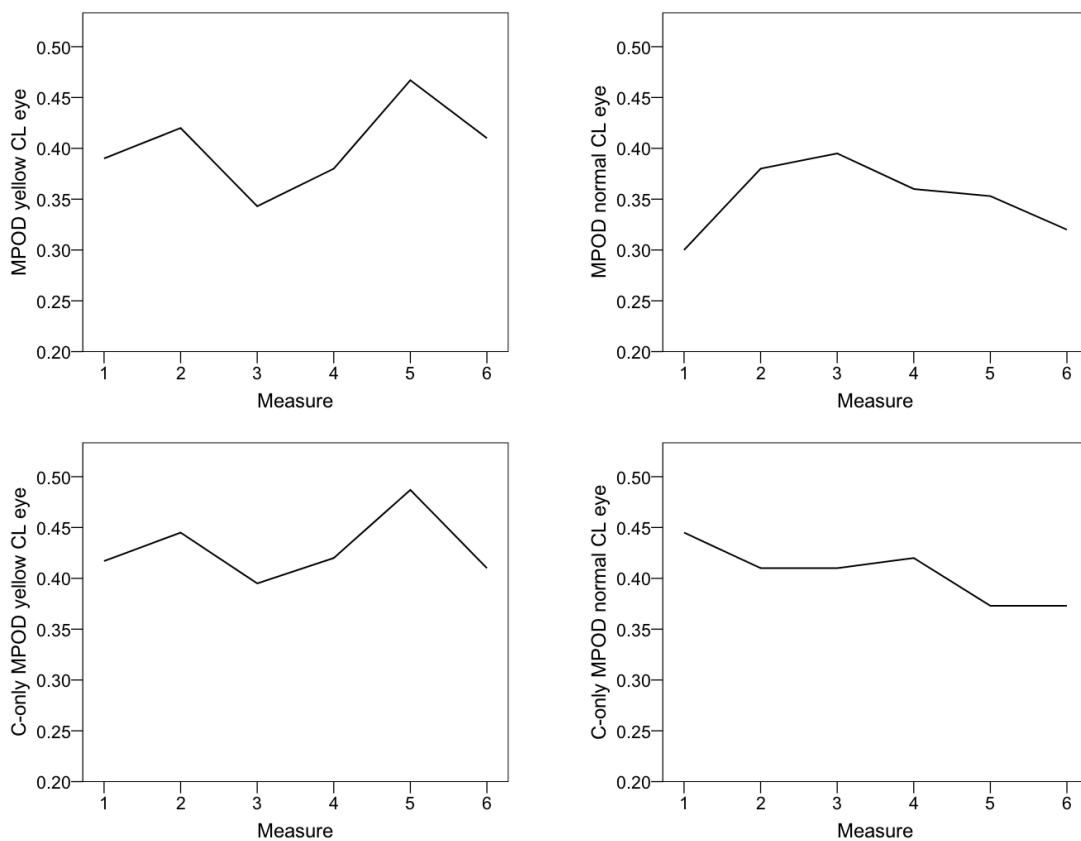
Figure 6.5 MPOD over time for subject 2.

INDIVIDUAL MPOD – SUBJECT 3

Background: 25-year-old Caucasian female with blue eyes.

Previous CLs: Non silicone hydrogel, non UV-blocking, daily disposable, daily wear.

Study CLs: RE -5.50 DS, LE -6.00 DS. Worn for 8 months between October 2010 and July 2011.

MPOD: See table 6.5 and figure 6.6.

MPS proficiency: Very good – virtually all optimal curves throughout the study.

Outcome (taking MPS proficiency into account): No change in MPOD.

| Measure | MPOD | | C-only MPOD | |
	Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	0.46	0.42	0.53	0.45
2	0.45	0.41	0.52	0.43
3	0.47	0.38	0.52	0.41
4	0.45	0.38	0.49	0.41
5	0.42	0.45	0.47	0.46
6	0.49	0.41	0.49	0.43

Table 6.5 MPOD over time for subject 3.
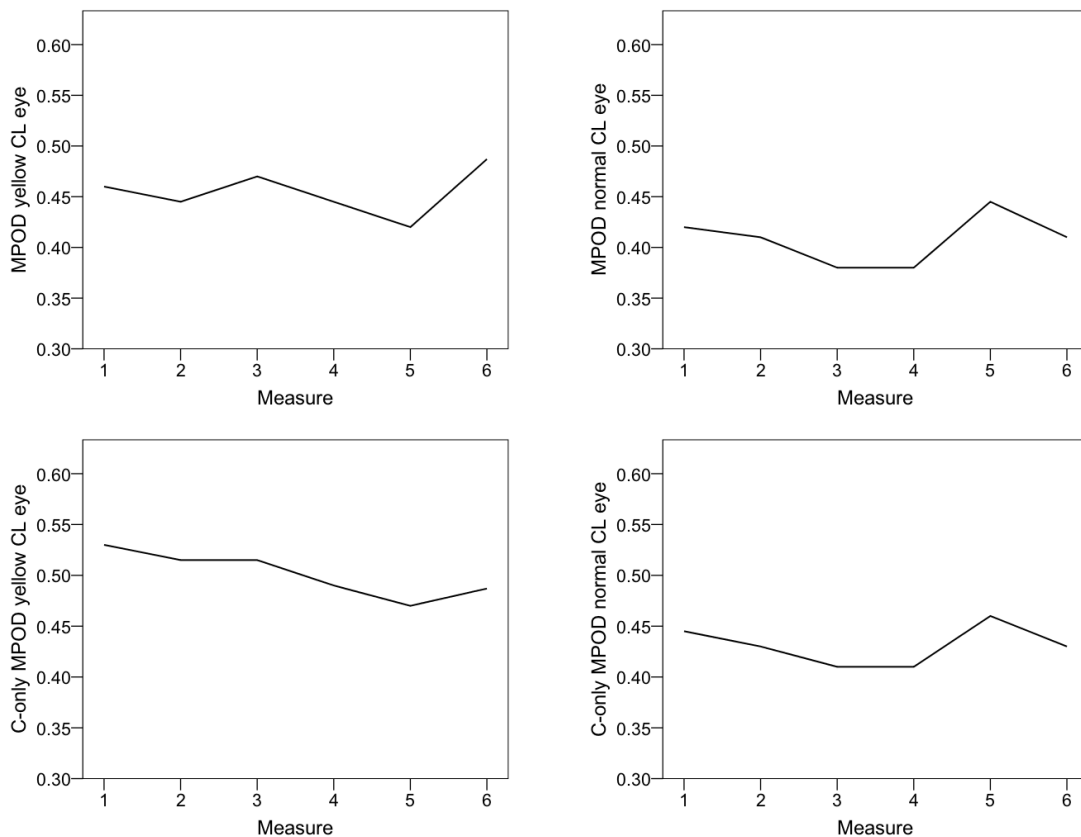
Figure 6.6 MPOD over time for subject 3.

INDIVIDUAL MPOD – SUBJECT 4

Background: 28-year-old Caucasian female with blue eyes.

Previous CLs: Non silicone hydrogel, part UV-blocking, daily disposable, intermittent daily wear.

Study CLs: RE -1.75 DS, LE -1.75 DS. Worn for 8 months between October 2010 and July 2011.

MPOD: See table 6.6 and figure 6.7.

MPS proficiency: Very good – virtually all optimal curves throughout the study.

Outcome (taking MPS proficiency into account): No change in MPOD.

Measure	MPOD		C-only MPOD	
	Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	0.34	0.35	0.37	0.41
2	0.34	0.38	0.41	0.41
3	0.33	0.39	0.35	0.42
4	0.30	0.36	0.36	0.38
5	0.30	0.35	0.37	0.41
6	0.31	0.32	0.38	0.41

Table 6.6 MPOD over time for subject 4.
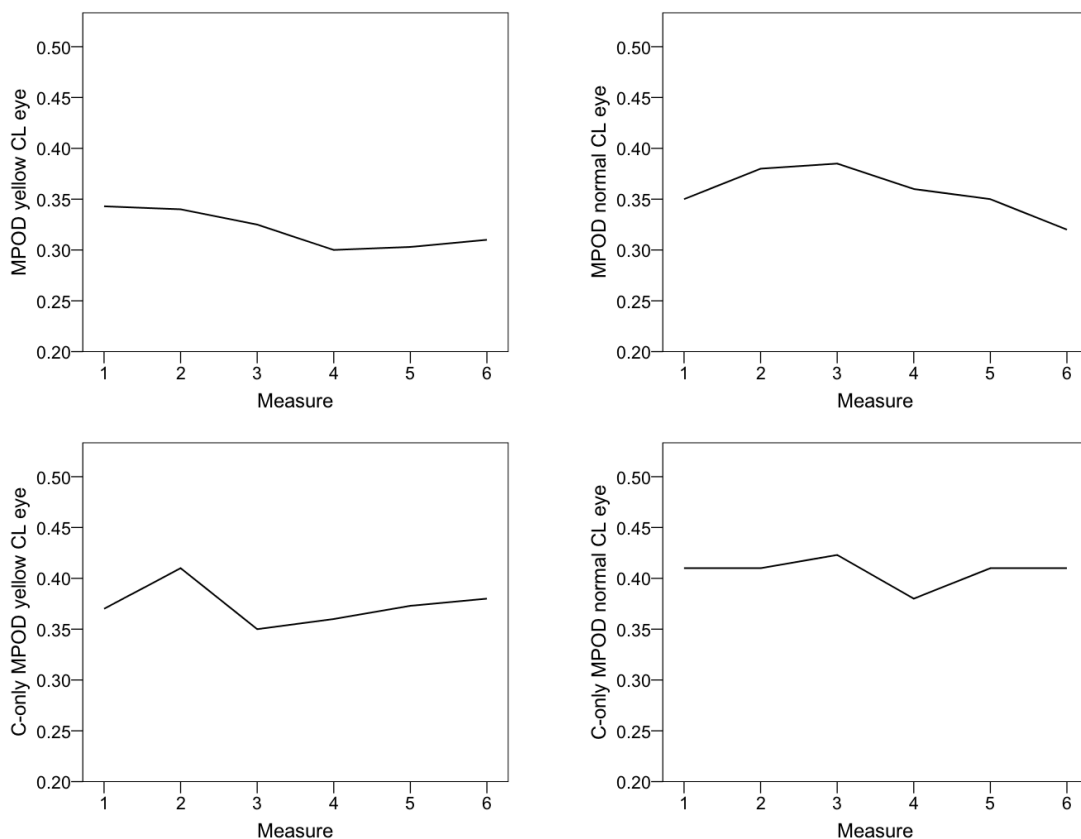
Figure 6.7 MPOD over time for subject 4.

Background: 28-year-old Caucasian female with blue eyes.

Previous CLs: Non silicone hydrogel, non UV-blocking, monthly disposable, daily wear.

Study CLs: RE -2.25 DS, LE -2.75 DS. Worn for 8 months between November 2010 and July 2011.

MPOD: See table 6.7 and figure 6.8.

MPS proficiency: Good – required three tests at a few visits but this normally resulted in at least two optimal central and peripheral curves.

Outcome (taking MPS proficiency into account): No change in MPOD.

	MPOD		C-only MPOD	
Measure	Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	0.31	0.24	0.31	0.29
2	0.37	0.25	0.37	0.29
3	0.26	0.25	0.34	0.29
4	0.28	0.23	0.33	0.29
5	0.29	0.29	0.31	0.26
6	0.30	0.29	0.33	0.31

Table 6.7 MPOD over time for subject 5.
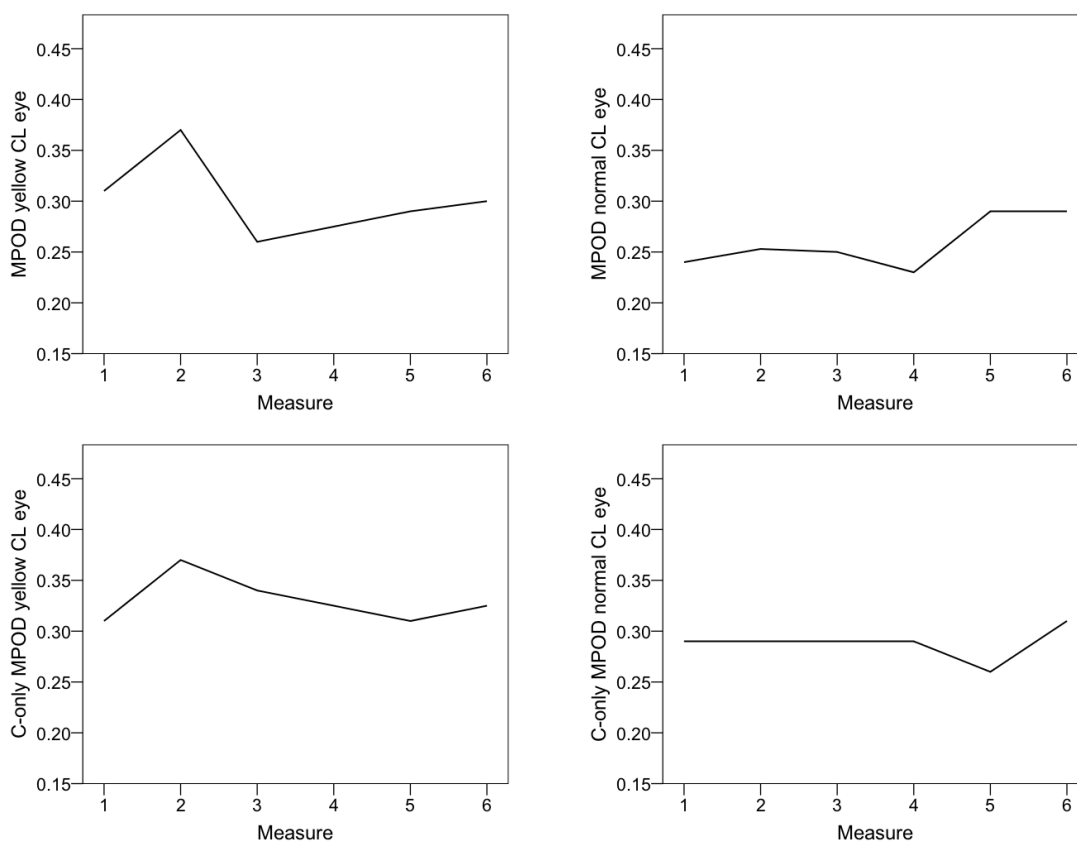
Figure 6.8 MPOD over time for subject 5.

123

INDIVIDUAL MPOD – SUBJECT 6

Background: 57-year-old Caucasian female with light brown eyes.

Previous CLs: Silicone hydrogel, non UV-blocking, monthly disposable, daily wear (monovision).

Study CLs: RE -0.50 DS (near), LE -2.75 DS (distance). Worn for 8 months between October 2010 and July 2011.

MPOD: See table 6.8 and figure 6.9.

MPS proficiency: Slightly variable – central and peripheral curves were sometimes optimal, sometimes not. Three tests were required for at least one eye at every visit.

Outcome (taking MPS proficiency into account): No change in MPOD.

| Measure | MPOD | | C-only MPOD | |
	Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	0.43	0.50	0.55	0.60
2	0.43	0.50	0.53	0.57
3	0.46	0.51	0.55	0.59
4	0.51	0.59	0.61	0.64
5	0.50	0.49	0.55	0.57
6	0.50	0.44	0.58	0.52

Table 6.8 MPOD over time for subject 6.
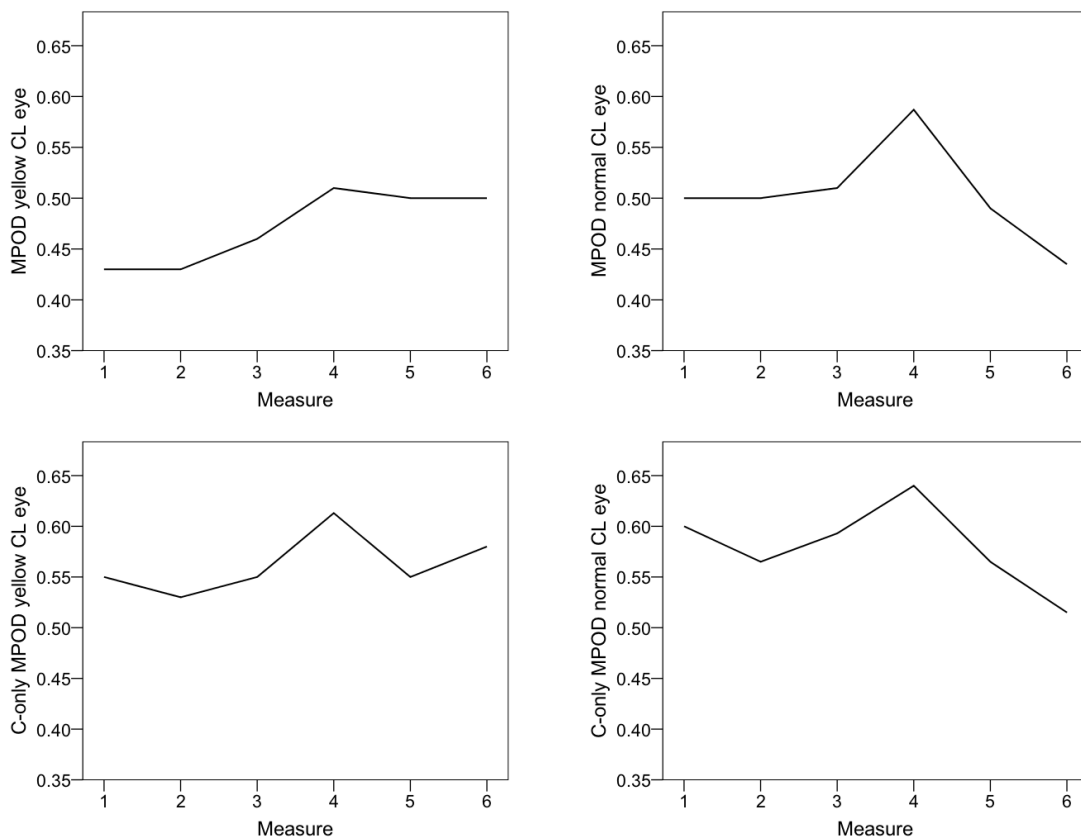
Figure 6.9 MPOD over time for subject 6.

INDIVIDUAL MPOD – SUBJECT 7

Background: 21-year-old Asian male with dark brown eyes.

Previous CLs: Non silicone hydrogel, non UV-blocking, daily disposable, daily wear.

Study CLs: RE -5.00 DS, LE -5.00 DS. Worn for 7½ months between November 2010 and July 2011.

MPOD: See table 6.9 and figure 6.10.

MPS proficiency: Variable – concentration levels were low for this subject, which was not helped by his very high levels of MP making every test rather long. As a result, some but nowhere near all curves were optimal. Three tests were required for at least one eye at every visit.

Outcome (taking MPS proficiency into account): No change in MPOD.

| Measure | MPOD | | C-only MPOD | |
	Yellow CL eye	Normal CL eye	Yellow CL eye	Normal CL eye
1	0.90	0.66	0.88	0.76
2	0.84	0.79	0.89	0.82
3	0.86	0.85	0.90	0.88
4	0.89	0.77	0.88	0.84
5	0.90	0.72	0.94	0.77
6	0.81	0.63	0.89	0.71

Table 6.9 MPOD over time for subject 7.
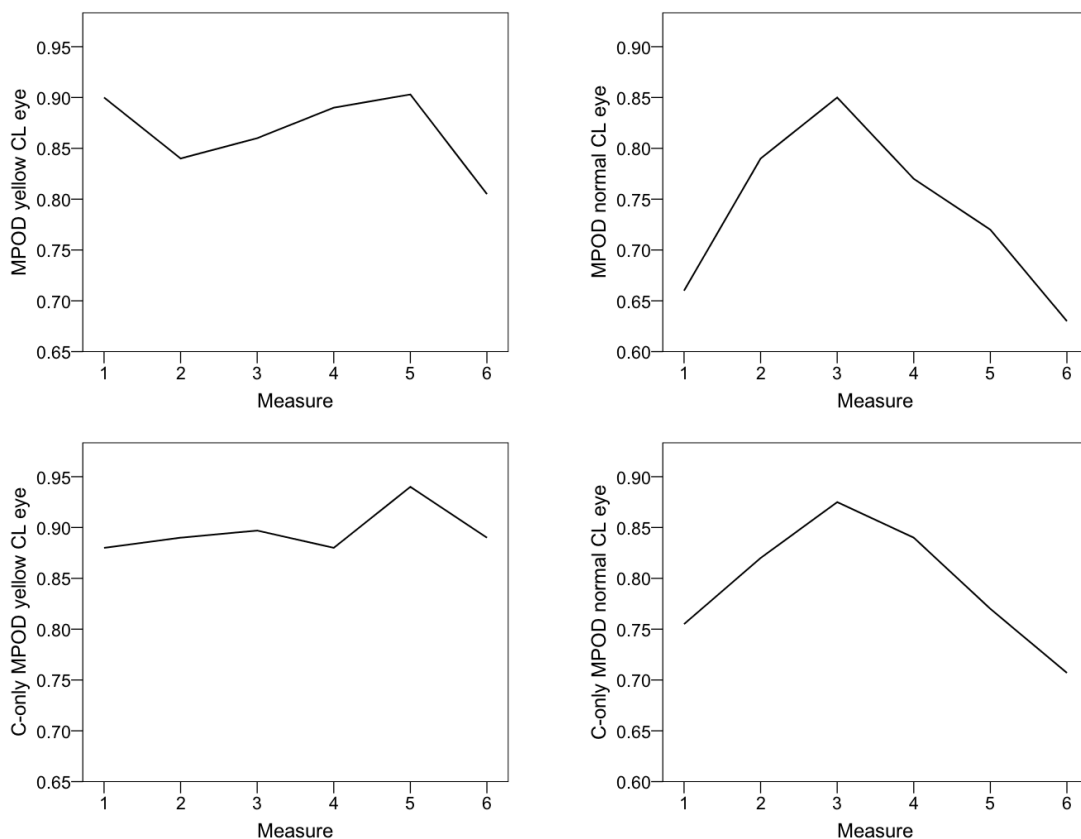
Figure 6.10 MPOD over time for subject 7.

6.4 Discussion

Cumulative exposure to light is a frequently cited but unsettled-upon risk factor for AMD (e.g., Taylor et al. 1992; Darzins et al. 1997; Tomany et al. 2004; Khan et al. 2006; Fletcher et al. 2008; Hirakawa et al. 2008). Any association is thought to arise more from damage caused by short-wavelength light such as UV and blue, rather than longer-wavelength light, although out of UV and blue, the latter is probably more likely because in adults most UV light is absorbed by the crystalline lens before it reaches the retina (Margrain et al. 2004). As an absorber of blue light, and as an antioxidant, MP may also have a place in the AMD-light exposure story. It was for this reason that the present study investigated whether MPOD increases by wearing a blue-light-filtering CL.

For eight (±0.3) months, seven subjects wore a yellow (UV and blue-filtering) CL in one of their eyes and a clear (UV-filtering) CL in their other eye. There was no apparent change in MPOD for the group as a whole or for any one individual. The hypothesis for this study was that there would be an increase in MPOD in the eye wearing the yellow CL, because the MP would have a reduced antioxidant and blue light absorption workload, and would therefore be depleted less, although the blue-filtering function of MP is probably a passive mechanism, so the lack of change in this respect is not unexpected. The results suggest that MP is stable in the retina, regardless of any change in the amount of light reaching it. This is in contrast to the results of Nolan et al. (2009), who published an interesting study during the planning stages of this experiment.

In Nolan's investigation MPOD was measured by HFP five times over the course of a year in a group of 24 (or 30 – contradictory numbers in the article) presbyopes (Nolan et al. 2009). All of the subjects were due to have cataract surgery at the time of the first measurement. Thirteen (or 16) of them had a standard IOL implanted and 11 (or 14) had a yellow IOL implanted. Measurements of MPOD were taken one week before surgery and one week after, followed by further measures three, six and twelve months later. The results indicated a statistically significant increase in MPOD over time for the yellow IOL group but not for the standard IOL group. This was against the hypothesis of the researchers involved, who expected to see no change in MPOD for the yellow IOL group, but rather a decrease in MPOD for the standard IOL group, on account of the anticipated increase in incident blue light for this group (Nolan et al. 2009). As stated in section 6.2, the implanted yellow IOL would be expected to absorb a lot more blue light than a standard IOL, but still considerably less than an age-yellowed cataractous lens (figure 6.1). The authors conceded that their results were difficult to explain, but they did offer

some plausible theories. However, looking more closely at the actual MPOD values provided in the paper for each subject at each visit, the findings are perhaps not quite as remarkable as the overall results indicate. Four of the 11 yellow IOL subjects demonstrated an increase in MPOD at each visit, with an average increase of 0.24 (range 0.1 to 0.33) from one week post-surgery to 12 months post-surgery. The remaining seven subjects' MPOD values tended to go up and down by a small amount over the course of the visits, much like the subjects in the present study. For two of these subjects, the final comparison (visit 5 minus visit 2) indicated an increase, yet one subject's MPOD appeared to have increased only because of an erroneous low reading at the second visit, to which all the other visits were then compared (subject 6 in table 2 of Nolan et al. 2009). The other subject's MPOD increase may also have been artificial, as their readings seemed rather variable and could therefore be the result of difficulties with the HFP task (subject 19 in table 2 of Nolan et al. 2009). Nevertheless, there does seem to have been a genuine rise in MPOD for some subjects in the yellow IOL group, and it is hard to reconcile the statistically significant results of Nolan et al. with the not statistically significant results of the present study, considering that here a filter was used that blocked out more blue light than that of a yellow IOL. Possible reasons for the discrepancy are now discussed.

There were a reasonably good variety of volunteers in this investigation, but ideally there would have been a few more males and a slightly wider age range; apart from one 57-year-old subject, the age range was limited to between 19 and 28 years old. Notably, it was only for this older subject that MPOD showed any consistent and sustained increase (top left graph of figure 6.9). While not wishing to draw any invented conclusions, this was in the eye wearing the yellow CL, so it is tempting to speculate that the results of this study might have been different with a sample of subjects closer in age to the subjects in Nolan et al.'s sample (Nolan et al. 2009). Given that any anticipated change in MPOD rested largely on the antioxidant function of MP, it might simply be that in a twenty-something adult, the oxidant load isn't yet enough to require any major contribution from MP, and for that reason there was no possibility of reducing its depletion. In chapter 1 (section 1.2) it was reported that MP has been located in the polyunsaturated fatty acid rich membranes of human perifoveal rods (Rapp et al. 2000), and also that these polyunsaturated fatty acids are highly susceptible to lipid peroxidation (and hence cell damage) by free radicals (Beatty et al. 2000b). However, lipid peroxidation in the central human retina is strongly correlated with age (De La Paz and Anderson 1992; Beatty et al. 2000b), which supports the reasoning for why there was no augmentation of MP in this particular group of subjects.

In dietary intervention studies, it has been noted several times that subjects with the lowest baseline MPOD often show the greatest rise in MPOD (Wenzel et al. 2006b; Aleman et al. 2007; Richer et al. 2007; Borel et al. 2011). With this in mind, it is interesting to note that the four subjects who appeared to demonstrated a real increase in MP in Nolan and colleagues' yellow IOL study had starting MPODs (the mean of one week pre-surgery to one week post-surgery) between 0.11 and 0.29, whereas the starting MPODs in the present study were between 0.31 and 0.90. It is therefore possible that an individual with a blue-light-filtering IOL or CL who has a low baseline MPOD may be more likely to see an increase in MP than someone with a higher baseline MPOD. This might then offer another explanation as to why there was no increase in the current investigation. That said, six of the remaining seven subjects in the IOL study had similar starting MPODs to the other four (between 0.03 and 0.29), so one could argue otherwise.

Other possible reasons come down to limitations of the study. Firstly, the frequency of CL wear could not be closely monitored. Secondly, the study duration may not have been long enough to produce a change in MPOD. Thirdly, the MPS is not able to assess the spatial distribution of MP. Addressing the first of these limitations, it should be noted that before beginning the study, all but one of the volunteers wore their regular CLs for at least six days of the week. The other volunteer had observed that in recent months she had been wearing her daily disposable lenses most days, and had therefore been planning on changing to a monthly CL. Consequently, this limitation is unlikely to be a reason for the insignificant findings. Study duration is a credible reason, particularly in view of the proposed lower age-related need for MP in an antioxidant capacity. All the same, the four subjects that demonstrated a realistic increase in MPOD in the yellow IOL study (Nolan et al. 2009) did so by the six-month check. With respect to the third limitation, it is worth returning again to Nolan and colleagues, who were able to measure the MP spatial distribution of their participants; the rise in MP was only significant in the central one degree of the fovea. As a result, it does not seem likely that any increases in MP were missed by not extending the area over which MP was measured. Furthermore, any augmentation of MP in the perifovea (where the peripheral 'control' test is carried out), which might have caused an underestimation of MP in the fovea, was negated by observing the C-only MPOD values as well as the actual MPOD values.

In conclusion, there was no increase in MPOD in this group of mainly young adults wearing a blue-light-filtering CL for eight months, but the possibility that such an increase might occur in an older group of adults cannot be excluded. If, in time, MP is proven to be

a true player in the defence against AMD, blue-blocking CLs could be an added option for a CL-wearing presbyope who, for example, has a close family history of this disease. The subjects in this study reported no problems specific to the yellow CL, and they adapted quickly to the altered appearance of their environment, so from this point of view there is no reason why this kind of lens could not be worn in place of a standard CL.

6.5 Summary

The study described in this chapter attempted to increase MPOD by an innovative CL method, and the results were a little disappointing. It is possible that variation in the abilities of individuals to accurately complete the psychophysical task of the MPS may in some small way have contributed to the insignificant findings, so having an objective MPOD measuring device would have been a welcome addition to the investigation. One such objective device did become available after beginning this study, but before it could be used as a possible replacement for the MPS, it needed to be evaluated in terms of its repeatability. The next chapter describes this evaluation of the new device, and in doing so, it also allows for an inter-session repeatability assessment of the MPS, using the protocol that has been used throughout the main studies described so far (chapters 4 to 6).

CHAPTER 7: CLINICAL ASSESSMENT OF THE MACULAR PIGMENT REFLECTOMETER

Macular pigment measurement in the preceding studies has been carried out using only the MPS, and as previous chapters have described, this instrument requires a considerable amount of time, as well as good concentration from the subject, to produce accurate and consistent results. However, over the course of the research period, another MPOD device was purchased, called the Macular Pigment Reflectometer (MPR), which provided the opportunity for fast, objective measurements. In this chapter, an assessment of the MPR is described.

7.1 Background and rationale

As the name suggests, the MPR is an MP device that employs the FR (fundus reflectometry) method of measurement. As such, it offers the advantages of objectivity (on the part of the subject) and speed, and unlike most reflectometry equipment, it does not require pupil dilation (van de Kraats et al. 2006). Furthermore, it is the first MPOD instrument to provide individual measures of L and Z, in addition to their combined value (van de Kraats et al. 2008).

To recap, FR is the quantitative measurement of light reflected from the fundus. In terms of MP measurement, there are two main approaches: comparison and spectral analysis. The comparison technique is akin to the HFP method; light reflected from the fovea is compared with light reflected from a non-foveal retinal area, using two wavelengths (one absorbed by MP and one not) or a spectrum of wavelengths (Berendschot and van Norren 2004). A spectral analysis, which is the method used by the MPR, uses a detailed optical model of the pathways of light in the eye (e.g., van de Kraats et al. 1996) to analyse a spectrum of light reflected from the retina.

So far, only the research group who developed the MPR has published data on its reliability. The aim of this study was therefore to provide an independent assessment of the MPR, by determining inter-session repeatability and inter-operator reproducibility, as well as comparing it with the MPS. This also allowed for an assessment of MPS inter-session repeatability, using the proposed protocol described in chapter 3.

7.2 Methods

SUBJECTS

Twenty-five subjects (10 males, 15 females) were recruited by email and by word-of-mouth, and consisted of staff and students from Aston University. They were all in good general health, with ages ranging from 19 to 52 years (mean±SD: 29.9±10.1 years). Refractive error (BVS) was between +3.00 DS and -8.75 DS (mean±SD: -1.50±3.00 DS). Visual acuity was measured under standard testing conditions using a logMAR letter chart; all participants had VAs of 0.14 logMAR (Snellen 6/7.5[-2]) or better in the eye being tested (the right eye for all but one of the participants). Exclusion criteria were: age younger than 18 years; VA worse than 0.2 logMAR (Snellen 6/9.5); and the presence of any ocular disease. A further two subjects (one male and one female, aged 24 and 28) took part in the MPS part of the study only. Thirteen of the subjects had prior experience with the MPS, and one with the MPR.

Aston University's Ethics Committee approved the study. All subjects signed an informed consent form, and all procedures adhered to the tenets of the Declaration of Helsinki.

MPS 9000

Due to the high light levels of the MPR producing bright afterimages, measurement of MPOD with the MPS was completed first, followed by measurement with the MPR. Each subject received verbal instructions and completed a short practice test, after which the prior established protocol was used (chapter 3). The averages of the central minima and peripheral minima were used to calculate MPOD. Using this technique, all subjects had two to three good quality central and peripheral curves to derive MPOD from, and no subjects needed to be removed from the analysis. Only one operator (OH) took the measurements on the MPS, with each subject repeating the test 7 to 14 days after the first session.

MACULAR PIGMENT REFLECTOMETER

The MPR (figure 7.1) is currently a research-based instrument produced in the Netherlands (University Medical Centre Utrecht). It uses a series of optical relay systems to focus a 30W halogen lamp onto a one-degree spot on the retina (figure 7.2). The reflected light is received by an optical fibre and sent to a spectrometer, which produces a spectral reflection output that is visible via a computer with the appropriate software (figure 7.3). Training was provided by one of the developers of the MPR, and the recommended protocol was adhered to throughout.
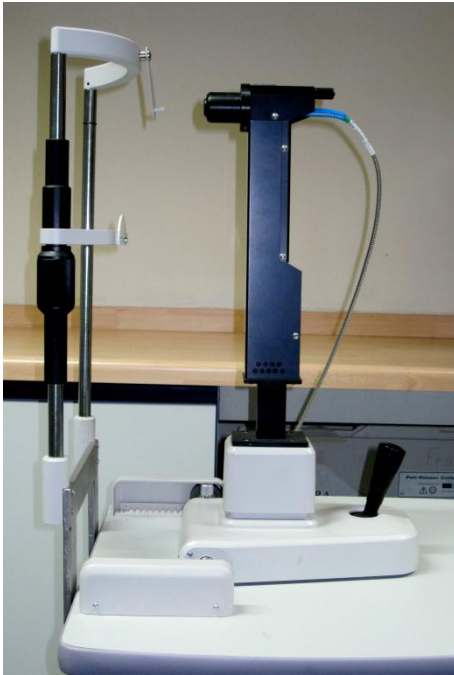
Figure 7.1 The Macular Pigment Reflectometer (MPR).

Aston University

Illustration removed for copyright restrictions

Figure 7.2 Schematic diagram of the MPR.

L1-L8 = lenses 1-8. M = mirror. Also shown (left) is the configuration of the illumination and detection beams. The semicircular illumination beam must be positioned in the upper half of a subject's pupil. This is to ensure that the detection beam, which is invisible, stays within the pupil. (Reprinted from van de Kraats et al. 2006.)
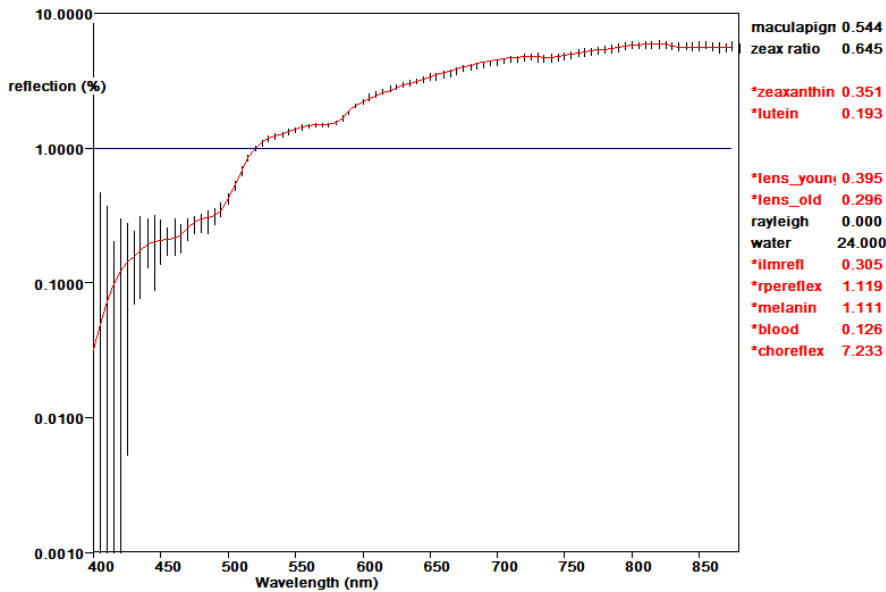
Figure 7.3 A typical spectral reflection output, with corresponding MPOD values to the upper right. It shows a high level of reflection in the long-wavelength region, with a gradual reduction due to absorption by blood and melanin, followed by further absorption (and lower reflection) by MP from around 520 to 440 nm. The lowest reflection from 440 to 400 nm is due to increasing absorption by the crystalline lens. The output is actually the mean of two successive one-second measurements, with the vertical error bars representing their standard deviations at the numerous wavelengths; the error bars are generally larger in the short-wavelength region than in the longer-wavelength region. The operators in this study saved outputs according to the following criteria: error bars that were as small as possible from about 500 nm upwards; reflections that looked high from about 550 nm upwards; and reflections that looked low from about 450 nm downwards.

At the beginning of each data collection session, the MPR was switched on and left to run for at least ten minutes, to ensure the lamp reached its maximum brightness. The rest of the procedure took place in a darkened room.

Firstly, the machine was calibrated using a 230 mm black, anodized tube with an inner white surface at the end of it ('white calibration'). This involved mounting the tube onto the device, setting a lens focusing dial to 'R' (reference, +2.00 DS), and using the computer software to begin measuring (30x1-second measurements). A single measurement was then taken, with the expectation of a one percent reflection (figure 7.4).
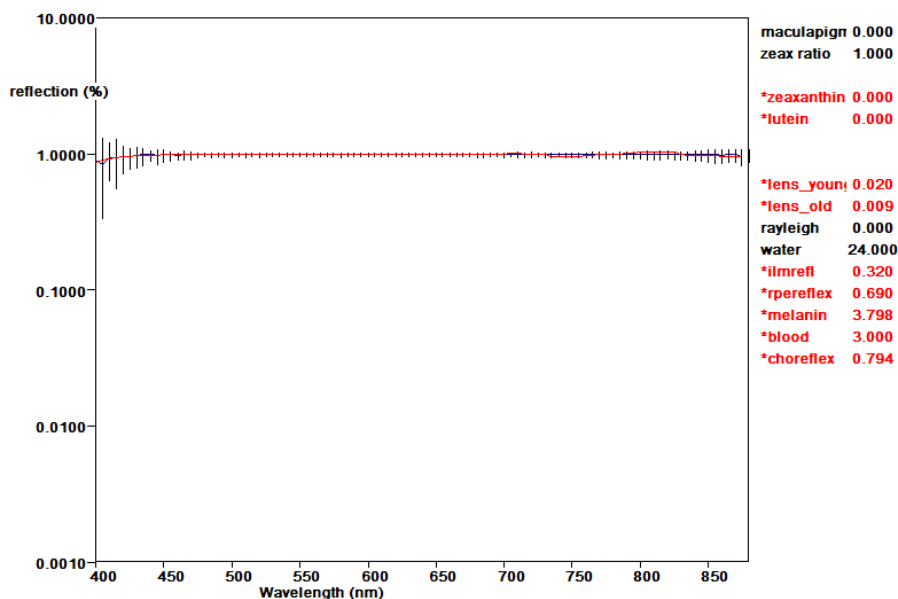
Figure 7.4 Single measure output following a white calibration. It shows a uniform one percent reflection.

After this initial setup (carried out once at the start of each data collection session), a 'dark calibration' was done, using a shorter, completely black tube, and with the lens focusing dial set to the individual subject's BVS distance refraction. In the dark calibration mode, a further 30x1-second measurements were automatically taken, followed by a single measurement. Each subject (without spectacles or CLs) was directed to place their chin onto a rest and their forehead onto a brow bar; they were asked to remain as still as possible. The light beam was aligned (using a joystick) into the upper half of their pupil, at a distance of one to two cm from the eye. The subject looked directly into the centre of the light spot while the operator monitored the continuously-produced reflection output and the light spot position in the pupil, making small joystick adjustments as necessary for a good output (see figure 7.3 for details). Five or six such outputs were saved by each operator for later analysis. This analysis involved choosing and averaging the best reflections according to the criteria given in figure 7.3. As a result, a minimum of two reflection outputs were accepted per subject. Two subjects were removed from the MPR analysis altogether, due to consistently poor outputs (one male, aged 19, with a BVS of -6.25 DS; one female, aged 35, with a BVS of -8.75 DS).

Data from the MPR was collected by two operators, PV and OH, in two sessions separated by 7 to 14 days. In the first session, PV took the measurements first (PV1), followed by OH (OH1). In the second session, OH took the measurements first (OH2), followed by PV (PV2). The reference measure, dark measure and lens focus dial were

common to both operators, i.e., these procedures were carried out by the first operator of the session, and were not repeated by the second.

MASKING

To avoid bias, neither operator looked at any prior MPOD results of any subjects (regardless whether collected by themselves or the other operator) before taking the measurements in either session.

SAMPLE SIZE

A sample size of 25 is in line with many studies examining the repeatability and agreement of MP measuring devices (e.g., Hammond et al. 1995; Ciulla et al. 2001b; de Kinkelder et al. 2011).

STATISTICS

Microsoft Excel and IBM SPSS were used for data analysis. Mean MPS-based MPOD for the two sessions was compared using a paired-samples t-test or its non-parametric equivalent, as appropriate in terms of normality. MPR-based MPOD for the two sessions, and between operators, was compared in the same way. Repeatability and reproducibility of each instrument was determined using Bland-Altman analysis and plots (Bland and Altman 1986), whereas Pearson's r correlations were used to quantify the association between MPS and MPR MPODs, on account of their different measurement principles.

7.3 Results

MEAN MPOD

Table 7.1 is a summary of the mean MPODs for the 23 final participants at the two sessions, and using the two devices. The means for L and Z are also given, as provided by the MPR.

Instrument	Mean (±SD) MPOD		Mean (±SD) L		Mean (±SD) Z	
	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2
MPS (OH)	0.324±0.148	0.328±0.146	N/A	N/A	N/A	N/A
MPR (PV)	0.590±0.202	0.571±0.222	0.287±0.172	0.276±0.113	0.304±0.133	0.296±0.139
MPR (OH)	0.562±0.165	0.564±0.215	0.251±0.112	0.269±0.123	0.311±0.126	0.295±0.151

Table 7.1 Mean MPODs for 23 subjects, as determined using the MPS and the MPR, along with the separate means for L and Z, where appropriate.
PV = operator PV. OH = operator OH. N/A = not applicable.

Paired-samples t-tests and Wilcoxon signed rank tests (as appropriate) comparing within-operator mean MPOD between the first and second sessions showed no statistically significant differences for the MPS or for the MPR (see table A9.1 in appendix 9 for details). The same was true when comparing L and Z separately. Moreover, comparing between-operator mean MPR-based MPOD at the first and second sessions (including L and Z separately) also showed no statistically significant differences. Finally, the four sets of MPR repeat data considered together (PV1, PV2, OH1, OH2) were not significantly different according to a one-way repeated measures ANOVA (F=0.373, p=0.774). Again, the same was true for L and Z values. These findings indicate an absence of any learning or fatigue effect with either instrument.

INSTRUMENT AGREEMENT

Paired-samples t-tests comparing the MPS-based mean MPODs with the MPR-based mean MPODs (i.e., MPS1 vs PV MPR1; MPS1 vs OH MPR1; MPS2 vs PV MPR2; MPS2 vs OH MPR2) yielded highly significant differences between the two instruments (p<0.0005 in all cases), with consistently higher MPOD obtained on the MPR. However, there was a statistically significant correlation between the instruments in all cases, with r-values ranging from 0.58 to 0.72 (figure 7.5).
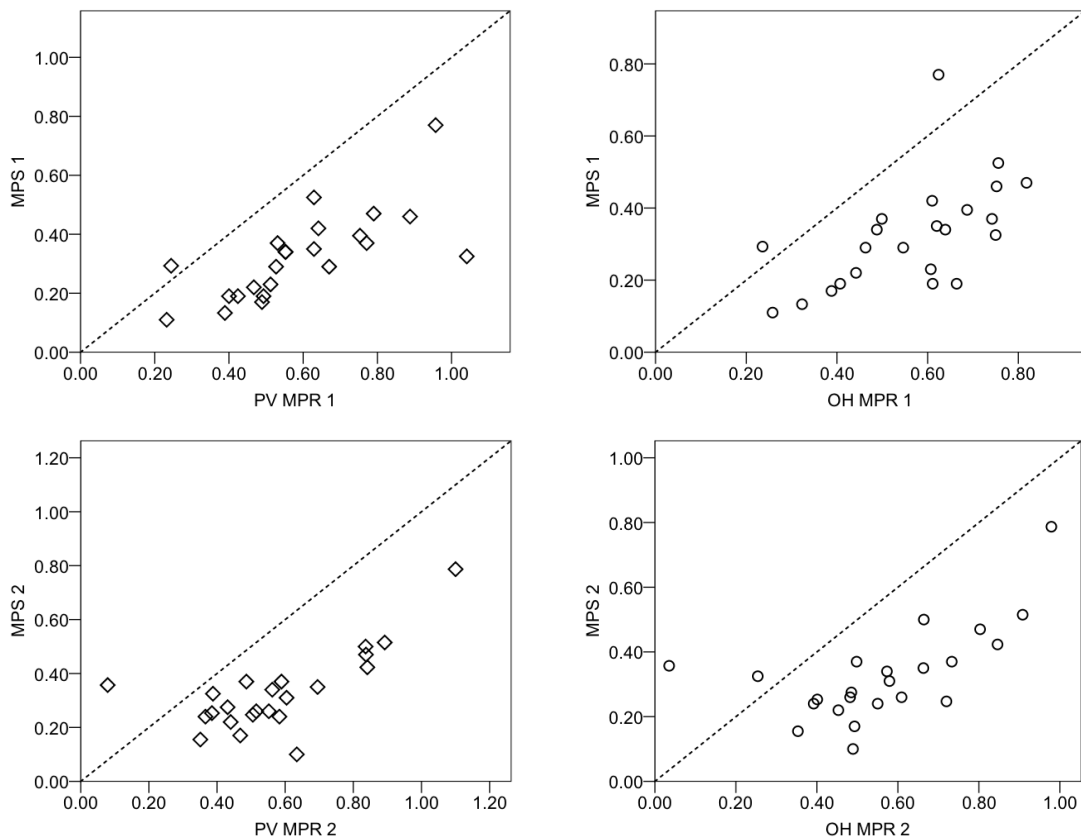
Figure 7.5 The relationship between MPOD measured with the MPS (always operator OH) and MPOD measured with the MPR (operator OH or PV), at visits one and two.

MPS1 vs PV MPR1: r=0.721, p<0.0005. MPS1 vs OH MPR1: r=0.580, p=0.004. MPS2 vs PV MPR2: r=0.703, p<0.0005. MPS2 vs OH MPR2: r=0.614, p=0.002.

The diagonal dotted lines indicate what a perfect match between the two methods would look like.

REPEATABILITY AND REPRODUCIBILITY

As stated in chapters 2 and 3, accurate analysis of test-retest data can be achieved using the CR (Bland and Altman 1986), which gives the 95 percent confidence limits for the amount of difference between two sets of results. It is calculated as 1.96 multiplied by the standard deviation of the differences between two sets of data, and indicates the amount of change that can occur between readings and still be classed as measurement noise. In other words, only increases or decreases in MPOD greater than the CR should be classed as clinically significant. Table 7.2 and figure 7.6 show the inter-session agreement between MPOD values measured with the MPS for all 27 participants. Also shown is the inter-session agreement for C-only MPOD (peripheral estimate provided by the MPS, based on subject age). The CRs were 0.08 and 0.06 for MPOD and C-only MPOD respectively, using the protocol as per chapter 3. This shows that when the same

operator is taking repeated MPS readings over time, changes in MPOD greater than these values can be classed as clinically significant.

	MPS1 – MPS2	C-only MPS1 – MPS2
Mean difference	-0.003	-0.005
SD of mean difference	0.043	0.032
Coefficient of repeatability	0.084	0.063

Table 7.2 Determination of inter-session CR values for MPS-based MPOD (n=27).
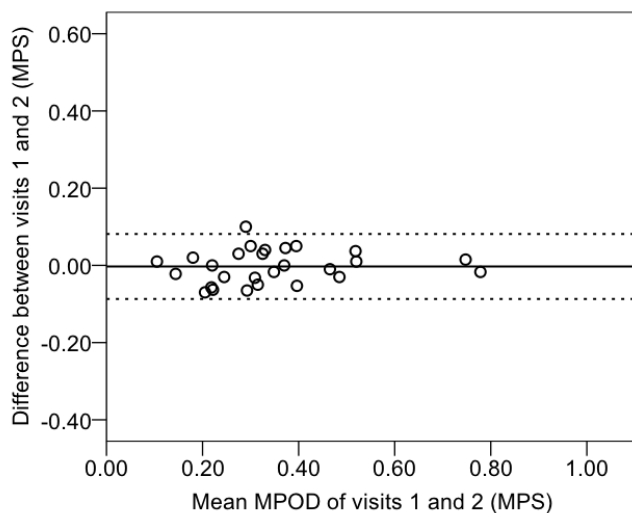
Figure 7.6 Bland-Altman plot representing the difference in MPS-based MPOD readings between visits one and two, compared with the mean of both visits (n=27).
The solid line represents the mean difference, and the dashed lines represent the 95% confidence limits.

Table 7.3 shows the inter-session agreement between MPOD values measured with the MPR for the 23 qualifying participants. Using the highest value of the two for repeatability, the data suggests that when the same operator is taking repeated MPR readings over time, only changes in MPOD of more than 0.4 can be classed as clinically significant (figure 7.7). The same was true for two different operators taking repeated readings over time. Using the highest value of the two for intra-session reproducibility, the data suggests that if two different operators are assessing MPR-based MPOD within the same session, differences of less than or equal to 0.25 are not clinically significant (figure 7.8). For the equivalent L/Z repeatability and reproducibility values, see table A9.2 in appendix 9.

	MPR repeatability		MPR reproducibility			
	PV1-PV2	OH1-OH2	PV1-OH1	PV2-OH2	PV1-OH2	PV2-OH1
Mean difference	0.019	-0.001	0.028	0.008	0.027	0.009
SD of mean difference	0.204	0.180	0.126	0.086	0.196	0.203
Coefficient of repeatability/reproducibility	0.400	0.353	0.247	0.169	0.384	0.397

Table 7.3 Determination of coefficient of repeatability/reproducibility values for MPR-based MPOD (n=23).

PV1 = MPR-derived MPOD at visit one by operator PV. PV2 = MPR-derived MPOD at visit two by operator PV. OH1 = MPR-derived MPOD at visit 1 by operator OH. OH2 = MPR-derived MPOD at visit 2 by operator OH.
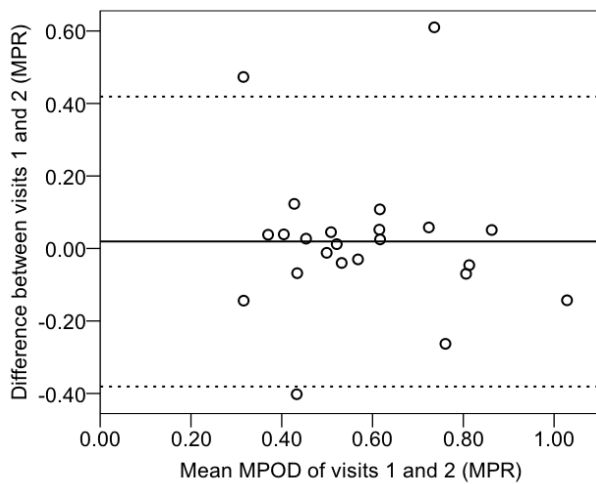
Figure 7.7 Bland-Altman plot representing the difference in MPR-based MPOD readings between visits one and two (operator PV), compared with the mean of both visits (n=23).
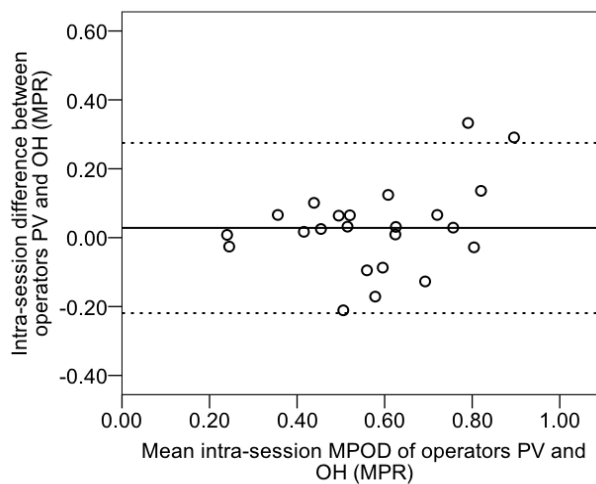
Figure 7.8 Bland-Altman plot representing the difference in MPR-based MPOD readings between operators (at visit one), compared with the mean of both (n=23).

7.4 Discussion

This study is the first independent assessment of the MPR, and as such will be beneficial to other independent research groups using this instrument. It also offers a further comparison with the MPS, whose repeatability using a set protocol was assessed for the first time.

The overall mean MPOD for 27 subjects using the MPS was 0.35±0.16 (0.33±0.15 for n=23), and the overall mean MPOD for 23 subjects using the MPR was 0.57±0.20. These values are in line with previously reported MPS and MPR means (van de Kraats et al. 2006; Makridaki et al. 2009; van der Veen et al. 2009a; van der Veen et al. 2009c; Bartlett et al. 2010c; de Kinkelder et al. 2011). The overall means for L and Z considered separately on the MPR were 0.27±0.13 and 0.30±0.14 respectively, and these means were statistically different (z=-3.398, p=0.001). Van de Kraats et al. (2008) found L and Z means of 0.20 and 0.49, so while a higher level of Z than L is common to both studies, the difference between the two appears to be less in the present study. It has been reported that the ratio of L:Z in the central 0 to 0.25 mm (~1º) of the human retina is 1:2.4 (Bone et al. 1988). This equates well with the results of van de Kraats et al. (2008) but not with the results from the present study, which perhaps indicates that the instrument, or use of the instrument, was not optimized in the current study, or that it was used in a different way from van de Kraats and colleagues.

As per previous MPR/MPS comparative studies (van der Veen et al. 2009a; van der Veen et al. 2009b; de Kinkelder et al. 2011), in the vast majority of cases, MPOD measured with the MPS was lower than MPOD measured with the MPR (see figure 7.5). This is considered to be largely as a result of the 'edge hypothesis' of HFP – the well-adopted belief that HFP-based MPOD is mediated by the edge of the stimulus, so that for a one-degree stimulus, the recorded MPOD corresponds to the MP level at 0.5 degrees from the centre of the fovea, rather than the true peak level at zero degrees, or the total MP in the central one degree (Werner et al. 1987; Hammond et al. 1997c). In contrast, the MPR measures and averages MPOD over the whole one-degree area (van der Veen et al. 2009b), and can therefore be expected to give higher mean MPOD values than the MPS. It has also been suggested that the difference may be attributable, in part, to the peripheral reference of the MPS having residual MP, thus causing an underestimation in MPOD (van der Veen et al. 2009a; van der Veen et al. 2009b). The MPR does not rely on any such peripheral reference area. There is an assumption with HFP that the reference area always has negligible MP, and is therefore just a measure that accounts for any blue light absorption by the crystalline lens. Evidence for the latter has been demonstrated in

several ways (see Wooten et al. 1999; Cuilla et al. 2001b; Makridaki et al. 2009), but the negligible MP part of this assumption has been challenged (Robson et al. 2003; Rodriguez-Carmona et al. 2006; Bhosale et al. 2007). In fact, in two studies where the MPR itself was modified to determine MPOD spatial profiles, a measurable level of MP was still present eight degrees from the fovea (van de Kraats et al. 2008; van der Veen et al. 2009b), i.e., at the reference point used by the MPS and many other HFP devices. Interestingly, however, because the MPS also provides a fixed estimate peripheral measure based on subject age, it allowed for a further comparison with the MPR in the present study, without the bias of any potential peripheral MP. The number of cases with lower MPS than MPR-based MPOD remained the same, but as in chapter 2, the mean C-only MPOD was significantly higher than the mean actual MPOD (paired-samples t-tests of MPS/C-only MPS at visits one and two, $p<0.0005$ in both cases for n=23). They were, though, very well correlated (r=0.97 in both cases for n=23, $p<0.0005$), with the straight-line regression equations indicating that C-only MPOD is always slightly higher than actual MPOD (e.g., $y = 1.09x + 0.01$ for visit one). Consequently, a small but measurable level of MP at the peripheral reference may actually be the cause of this particular discrepancy, although it is clearly not enough to explain the large differences between MPS and MPR MPOD.

Agreement (Pearson's r) between the MPR and MPS varied from 0.58 to 0.72. Previously reported correlations are 0.78 (n=26, van der Veen et al. 2009a), 0.72 (n=19, van der Veen et al. 2009b) and 0.87 (n=23, de Kinkelder et al. 2011), so while the correlations in the present study are good, they are generally not as good as these earlier correlations. This inconsistency is discussed below.

A high level of inter-session repeatability is important if MPOD is to be accurately monitored over time, especially in an intervention scenario, where a patient is increasing their L/Z/MZ intake via dietary modification and/or supplement use. In this study, the protocol previously described for reducing the CR on the MPS to a level at which clinically significant changes in MPOD can be realistically detected, was put to the test. The result was an inter-session CR of 0.084, which is the best repeatability for the MPS so far reported, and as good as, if not better than, any stated HFP reliability of the last 15 years (Bartlett et al. 2010b; Howells et al. 2011). It could be argued that the results were influenced by the fact that about half of the participants were already familiar with the MPS. However, further analysis of the data, using only those with no prior experience on the MPS (n=14) resulted in a CR of 0.072 (and 0.043 for C-only MPOD), thereby countering this issue.

The inter-session CR of the MPR was between 0.35 and 0.40 in this study. By comparison, van de Kraats et al. (2006) took inter-session repeat measurements for ten subjects, and although the CR was not stated in that particular paper, it was cited some time later as 0.18 by de Kinkelder et al. (2011). While it is still questionable whether the latter CR would allow accurate detection of MPOD changes with L/Z/MZ dietary modification/supplementation (see below), the roughly double CR obtained in the present study would almost certainly not. This is disappointing and requires evaluating.

Firstly, it is worthwhile considering whether the differing number of participants in the two studies could be a reason for the CR discrepancy. Looking back over previous papers that have produced reliability indicators for MPOD measuring techniques, there appears to be a trend towards better inter-session repeatability being associated with fewer subject numbers, and vice-versa. This is illustrated in figure 7.9 (r=0.64, p=0.007). (Note that only HFP data was used here, since there is very limited data on inter-session repeatability for any of the other MPOD measurement techniques.) The fact that there were more than double the number of subjects in the present study than in the van de Kraats study could, in part, explain the difference in recorded repeatability.
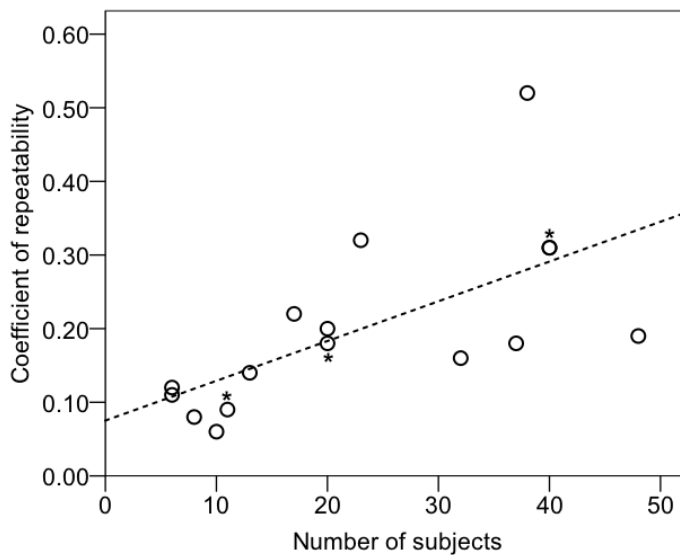
Figure 7.9 A summary of inter-session CR data from HFP studies.
It shows the number of subjects tested and the associated CR (see table A9.3 in appendix 9 for derivation and references). The dotted line is the trend line. Not every study explicitly provided the CR, but it was calculable where a mean difference and standard deviation was given. The correlation, r, is 0.64 (p=0.007).
* = published CR data for the MPS (van der Veen et al. 2009a; Bartlett et al. 2010c; de Kinkelder et al. 2011).

A second consideration is whether differing methodology might have influenced the data in the present investigation. The method followed here was carried out according to the instruction of one of the MPR developers. However, this does differ in several ways from the method described in the original published paper by van de Kraats et al. (2006). The main difference was in the procedure for dark calibration, which in the current study involved setting the lens focusing dial to the subject's known distance refraction, calibrating with the appropriate tube, and then beginning measurements on the subject, as described in section 7.2. The van de Kraats approach was more protracted: the lens focusing dial was set to the subject's spectacle refraction and adjusted, if necessary, until the subject reported that the edges of the light spot were sharply focused; a further lens dial adjustment was made, if required, while looking at the generated reflection spectrums for the highest amplitude outputs (with the subject set up as described in section 7.2); the dark calibration was carried out; and lastly, the actual measurements were taken.

The issue of lens focusing adjustments was addressed with some of the first subjects in the present study. It was found that in general, those subjects reported an approximately ±1.00 DS range (from their spectacle prescription) over which they thought the edge spot was in clear focus. It therefore seems unlikely that this would affect the results. Moreover, van de Kraats et al. (2006) remarked that "dark calibration changed very little over the course of hours, thus in fact it could have been taken less frequently", i.e., not on every subject. Carrying out a lens focus adjustment while observing some initial outputs may have proved useful, though. With this in mind, the reflection outputs and data from the current study were re-examined. Twelve of the 23 subjects showed at some point an inter-session MPOD difference of greater than 0.1. Of these, all but two were still deemed to have good reflection outputs. Some reflection spectrums were visibly different between visits one and two, but they were also equally acceptable according to the prior stated criteria, and thus there was no reason to exclude them. The remaining two subjects were, in hindsight, of less optimal quality. Removing them from the analyses reduced the majority of the CRs considerably, as depicted in table 7.4. In addition, all the MPR/MPS correlations improved (table 7.5), putting the MPR/MPS instrument agreement in line with the previously published articles. Of interest, perhaps, is that from the original 25 participants who had MPR measurements taken, four were eventually removed, and three of these had a BVS spectacle prescription of -6.25 DS or higher (-6.25, -6.50 and -8.75). The lens dial on the MPR has markings from +5 to -6, but it will only turn as far -5.25 for myopic prescriptions (the plus goes up well beyond +5). Subsequently, it seems advisable to limit future study intakes to participants with BVS prescriptions lower than

-5.25 DS. No mention of this was made in the earlier publications.

	MPR repeatability		MPR reproducibility			
	PV1-PV2	OH1-OH2	PV1-OH1	PV2-OH2	PV1-OH2	PV2-OH1
Original coefficient of repeatability/reproducibility	0.400	0.353	0.247	0.169	0.384	0.397
Coefficient of repeatability/reproducibility after removal of two subjects' data	0.244	0.215	0.226	0.175	0.230	0.289

Table 7.4 Coefficient of repeatability/reproducibility values for the MPR, before and after the removal of two subjects' data – see text for details.

Comparison	Original Pearson's r	Pearson's r after removal of two subjects' data
MPS 1 and PV MPR 1	0.72*	0.82*
MPS 1 and OH MPR 1	0.58**	0.60**^
MPS 2 and PV MPR 2	0.70*	0.83*
MPS 2 and OH MPR 2	0.61**	0.75*

Table 7.5 Pearson's r correlations between MPOD measured with the MPS and MPR, before and after the removal of two subjects' data – see text for details.

*p<0.0005 **p<0.005

^ Removal of a clear outlier, visible only on this comparison, improved the correlation further to r=0.71 (p<0.0005).

Another methodology difference was in how many MPOD values to use for the analysis. Where mentioned, the suggestion has been that the mean of five measurements was used to give an overall MPOD value (van de Kraats et al. 2006; van de Veen et al. 2009b). In the help guide that came with the MPR, the advice is to repeat measurements three to five times to allow for later *selection*. Selection was the final chosen method in the present study, although it made very little difference to the calculations.

Overall, it is likely that the poor repeatability values of the MPR in the present study are not attributable solely to measurement noise. Small adjustments of the lens focus, and exclusion of participants with a moderate degree of myopia may well improve repeatability. Other things that may improve it are further operator experience/training on choosing an optimal reflection spectrum. The test may be objective on the part of the subject, but it is not for the examiner. This is evidenced by the outputs that varied in

appearance and MPOD value between visits one and two, but of which neither could be chosen as the better, despite following the advised criteria for a good output. Three 'normal' reflection output examples came with the MPR software; it would be helpful to have more, along with some 'abnormal' examples. Even with this knowledge acquired, there is still a lot for the operator to observe all at once, namely; the beam position in the pupil and, away from that, the reflection output on a computer screen – high reflection in the 550 nm region, low reflection in the 450 nm region, small error bars, plus the help guide recommends looking at the MPOD values as they are generated (not done in the present study to avoid bias). It would seem that even in the experienced hands of the instrument developers, a sizeable amount of within-session variation can still occur. Figure 7.10 shows graphs of MPR-derived L and Z levels for three subjects over the course of a six-month Z supplementation trial and ten-month follow-up (van de Kraats et al. 2008). For the first subject, in particular, intra-session readings appear to vary by up to 0.31.

Figure 7.10 Lutein (L) and zeaxanthin (Z) MPR-based measurements for three subjects (S1-S3) over the course of 18 months.

From days 0 to 182, the subjects took a daily Z supplement (20 mg). Individual measurements at each visit are represented by the triangles. Trend lines before and after Z intake are shown; the bolder trend lines are significantly different from zero ($p<0.05$). (Reprinted from van de Kraats et al. 2008.)

If, with a few changes, the repeatability of Aston University's MPR device is brought in line with that reported by de Kinkelder et al. (CR 0.18, de Kinkelder et al. 2011), will that be sufficient to monitor any MPOD change over time? Connolly et al. (2010) recently produced a review table which showed that MPOD rarely increases by more than 0.07, on average, over the course of a 15 to 24 week intervention period with 5 to 30 mg of L, Z or MZ (via supplement use or dietary modification), so at first glance it would appear not. However, in the aforementioned Z supplementation study by van de Kraats et al. (2008), a statistically significant increase in Z was demonstrated by all three participating subjects

over the course of 16 months (figure 7.10). The data from figure 7.10 were subsequently extrapolated in the current investigation to calculate a mean value of L and Z for each subject at each visit, which in turn allowed for a combined MPOD value (L+Z) at each visit. The results are presented in figure 7.11. An increase is evident for all subjects. However, if the results are considered from a more practical and clinical point of view, in which a patient wishes to know whether their MPOD has increased from one eye test to another, the results would be less conclusive. For example, if one takes the MPOD at approximately -7 days as the initial eye test, i.e., a week before beginning Z supplementation, then the MPOD at the end of the supplementation period (approximately 180 days) as the six-month follow-up eye test, the MPOD increase is 0.06, 0.07 and 0.13 for subjects one to three respectively. None of these would be considered clinically significant, going by the CR of 0.18. Nevertheless, it could be rightfully argued that the MPR is only a research-based instrument, in which more regular visits are to be expected, and therefore MPOD differences would be picked up.
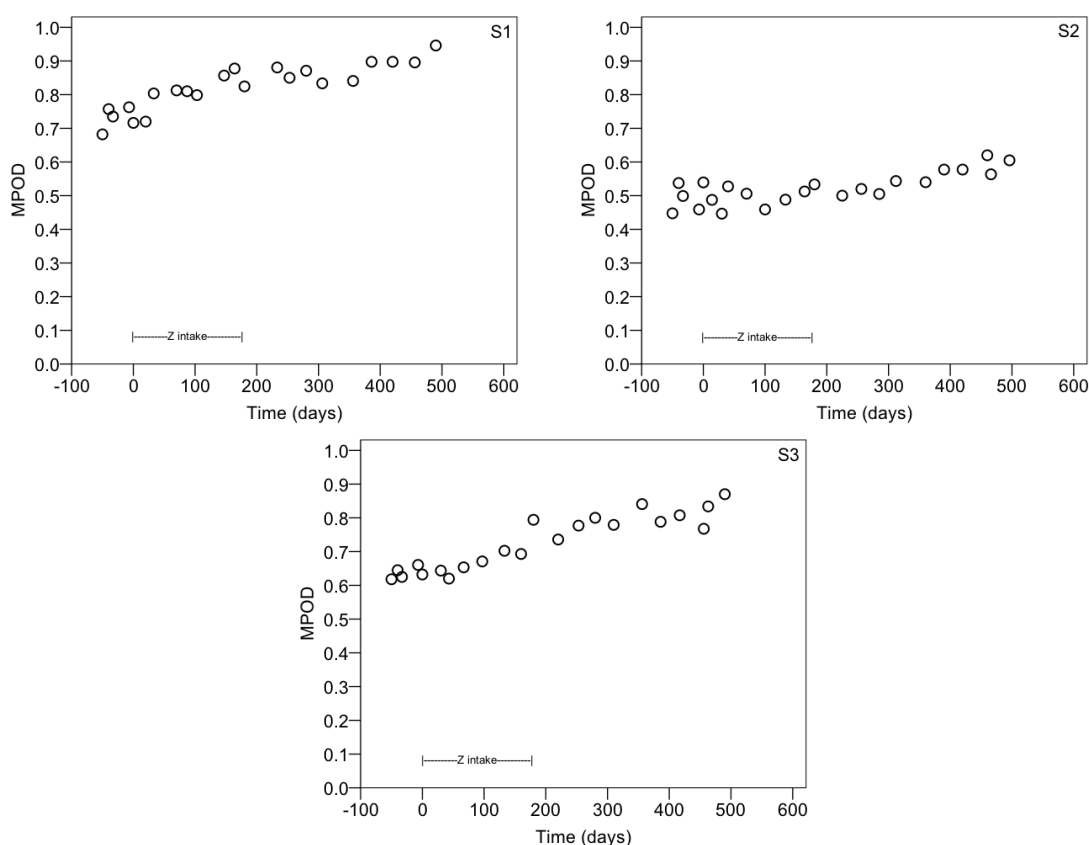
Figure 7.11 Data extrapolated from van de Kraats et al. (2008) (figure 7.10) to show MPOD values for three subjects (S1-S3) over the course of 18 months.

From days 0 to 182, the subjects took a daily Z supplement (20 mg).

In conclusion, the MPR, as used in the current study, is a straightforward and fast task for the subject, requiring them simply to stare at a bright light for about one minute in total. It is not repeatable enough to detect changes in MPOD over time, but it does provide an accurate one-off measure of MPOD for most individuals, as assessed by comparison with the MPS. Modification to the methodology would increase the time required from the subject (by approximately two minutes), but it would potentially improve repeatability. The MPS, as used in the current study, is a more demanding task for the subject, requiring concentration and time (between 10 and 20 minutes in total, depending on the results). However, the repeatability is excellent, and as a result, it is more likely to detect a significant change in MPOD than the MPR.

7.5 Summary

This chapter has introduced a second MPOD measuring instrument, the MPR, whose reliability did not match that of the MPS. On account of its availability, however, the MPR is also used (along with the MPS) in the final study, in which another attempt to increase MPOD is made, this time using dietary modification.

CHAPTER 8: MACULAR PIGMENT RESPONSE TO DIETARY MODIFICATION WITH KALE: AN EXPLORATORY STUDY

In the previous chapter, the inter-session repeatability of the MPS was shown to be very good when used in a set way. The inter-session repeatability of the MPR (an objective MPOD device) was not so good, although with some method modification it's possible that its repeatability could be improved, as was the case with the MPS. In this final investigation, both instruments are used to monitor MPOD in a second intervention study. Unfortunately, the present research had already started before the last research had finished, so the same MPR protocol is employed. Here, a classic dietary modification study takes place using kale, a vegetable frequently advocated to patients by optometrists and ophthalmologists as being a rich source of L, yet also a vegetable that had yet to be included in any MPOD-based supplementation studies.

8.1 Background and rationale

Over the last 14 years there have been at least 31 studies in which MPOD has been monitored in humans following an increase in supplement or dietary intake of L, Z or MZ (Hammond et al. 1997a; Landrum et al. 1997; Berendschot et al. 2000; Johnson et al. 2000; Aleman et al. 2001; Duncan et al. 2002; Bone et al. 2003; Cardinault et al. 2003; Bernstein et al. 2004; Koh et al. 2004; Richer et al. 2004; Franciose et al. 2006; Kopsell et al. 2006; Kvansakul et al. 2006; Rodriguez-Carmona et al. 2006; Wenzel et al. 2006a; Wenzel et al. 2006b; Aleman et al. 2007; Bone et al. 2007b; Schalch et al. 2007; Trieschmann et al. 2007; Wenzel et al. 2007b; Johnson et al. 2008; Stringham and Hammond 2008; van de Kraats et al. 2008; Berson et al. 2010; Bone and Landrum 2010; Connolly et al. 2010; Zeimer et al. 2010; Borel et al. 2011; Nolan et al. 2011). The dietary interventions have been fewer in number than the supplement interventions; they have focused on the foods spinach, corn and eggs, and all have used the HFP technique to measure MP (Hammond et al. 1997a; Johnson et al. 2000; Kopsell et al. 2006; Wenzel et al. 2006b).

Subjects in a dietary MPOD study by Hammond et al. (1997a) consumed 60 g of spinach and/or 150 g of corn per day for up to 15 weeks. This equated to 10.8 mg of L and 0.3 mg of Z per day for the spinach, and 0.4 mg of L and 0.3 mg of Z per day for the corn. In observational MPOD studies, the mean daily intakes of L and Z have varied from 1.1 mg (Ciulla et al. 2001a) up to 3.7 mg (Beatty et al. 2001), so the spinach consumption represented a considerable increase in L/Z compared with a typical diet. In the spinach and corn combined group (n=11), MPOD notably increased for most of the subjects

(n=8), referred to as 'retinal responders', but it was unchanged for the remaining subjects (n=3), referred to as 'retinal nonresponders'. In the corn only group (n=2), there was one retinal responder and one retinal nonresponder (Hammond et al. 1997a). A dietary MPOD study by Johnson et al. (2000) followed an almost identical premise to the previous one by Hammond et al., except that there were no subjects who just ate corn. The group as a whole (n=7) saw a statistically significant rise in MPOD (from 0.40 at baseline to 0.47 at 15 weeks, p<0.05), but individual responses were not provided. Kopsell et al. (2006) randomly assigned 30 volunteers to one of three treatment groups: high L spinach (n=10), low L spinach (n=10), and no spinach (n=10). The high and low L spinach groups each consumed 50 g of spinach, five days a week for 12 weeks. The high L spinach contained 6.05 mg of L per 50 g serving, and the low L spinach contained 4.2 mg of L per 50 g serving. The high L group demonstrated a statistically significant increase in MPOD (from 0.34 at baseline to 0.37 at 12 weeks, p=0.021), but the low L group and no spinach group did not. Individual responses were not provided. Of note, the high L spinach still contained less L than the spinach in Hammond et al.'s study, but this was probably because the L values provided by Kopsell et al. related to fresh mass spinach, whereas the L values provided by Hammond et al. related to cooked spinach, which would result in different L quantities (USDA 2010). Moving away from spinach, Wenzel et al. (2006b) assigned 24 volunteers to one of three treatment groups: supermarket-bought eggs (n=8), organic-farm-bought eggs (n=8) and a placebo sugar pill (n=8). The egg groups ate six eggs per week (no more than two per day) for 12 weeks. Eggs are known to be a highly bioavailable source of L and Z (Handelman et al. 1999; Chung et al. 2004), so although the supermarket and organic eggs equated to only 0.284 mg of L/Z per day and 0.826 mg of L/Z per day, respectively, there were statistically significant increases in MPOD for both groups (p=0.001 and p=0.04). The pill group saw no increase (p=0.51). Interestingly, the lower L/Z egg resulted in a greater augmentation of MPOD (by ~0.09 compared with ~0.05 in the higher L/Z egg), which may have been because the baseline MPOD of this group was significantly lower than the higher L/Z egg group (0.18±0.02 vs 0.37±0.06), and hence there may have been a limitation as to how much more L and Z could be captured by the retina in the latter group (Wenzel et al. 2006b). Like Hammond et al. (1997a), Wenzel and colleagues reported on individual changes in MPOD; there were 11 retinal responders and five retinal nonresponders.

Like spinach, kale is a vegetable that eye professionals frequently recommend to their patients, and according to the USDA national nutrient database, kale contains more L/Z per 100 g than spinach (USDA 2010). Nevertheless, it had yet to be included in any interventional MPOD studies, so the study described here aimed to explore the effect on

MPOD of incorporating a regular intake of kale to the diet. If kale were proven to increase MPOD, this would lend support to the recommendation of kale to members of the public.

8.2 Methods

SUBJECTS

Six reliable and trustworthy subjects were personally recruited (3 males, 3 females); four were staff members from Aston University's optometry department, one was an undergraduate optometry student, and the other was a close family member of the principle investigator (OH). They were all in good general and ocular health, with a VA of 0.12 logMAR (Snellen $6/7.5^{-1}$) or better in the eye being tested. Refractive error (BVS) was between +3.00 DS and -8.00 DS (mean±SD: -2.50±4.50 DS). Exclusion criteria were: age younger than 18 years; VA worse than 0.2 logMAR (Snellen 6/9.5); the presence of any ocular disease; and, as a precaution, any kidney or gallbladder problems, because kale contains oxalates, which can be problematic for individuals with kidney or gallbladder troubles, although kale is considered a 'low-oxalate' vegetable (Heaney and Weaver 1990).

Aston University's Ethics Committee approved the study. All subjects signed an informed consent form, and all procedures adhered to the tenets of the Declaration of Helsinki.

DIETARY MODIFICATION PROTOCOL

The majority of kale was bought from one of two supermarkets. It was UK-grown, uncooked and supplied in 200 g bags, which were passed on weekly to the study participants for 16 consecutive weeks. One 200 g bag per week was chosen because it seemed a decent amount of kale to consume – a lot more than the vast majority of individuals would be expected to eat in a week, but not an unachievable amount to eat either. Going by the USDA database, it was also expected to contain a considerable amount of L/Z, somewhere in the realm of 36 mg per week (USDA 2010), although in hindsight this was an oversimplification (see discussion). The participants were asked to spread the 200 g of kale over three to five portions, cooking the kale each time and incorporating it into a meal (e.g., a stir-fry) or eating it with a meal (e.g., a roast dinner).

At the end of the study period a short questionnaire was sent to each participant to establish more details on how the kale was cooked, what it was eaten with, exactly how much of it was eaten, etc. A copy of the questionnaire is provided in appendix 10.

MPOD MEASUREMENTS

Measurements of MPOD were taken on seven separate occasions using the MPS and the MPR. There were two baseline measurements separated by 7 to 14 days (visits 1 and 2), followed by measurements after 4, 8, 12 and 16 weeks of kale eating (visits 3 to 6), then a final measurement four weeks after finishing the kale eating part of the study (visit 7).

For measurement of MPOD using the MPS, at the first visit subjects received verbal instructions and completed a short practice test, after which the prior established protocol was used (chapter 3). After the initial visit, instructions were given only as necessary for individual subjects. The averages of the central minima and peripheral minima were used to calculate MPOD. Using this technique, all participants at every visit had two to three good quality central and peripheral curves to derive MPOD from. Measurements were taken in the right eye only, except for one subject whose MPOD was measured in both eyes (for reasons explained below).

After the MPS-based measures, the MPR was used to measure MPOD as described in the previous chapter (section 7.2 in chapter 7), i.e., after the initial setup, subjects were asked to remain as still as possible while staring into the centre of a bright spot of light, which was aligned in the upper half of their pupil at a distance of one to two cm from the eye. Five or six spectral reflection outputs were saved according to criteria provided in chapter 7. In the subsequent analysis, only five outputs (spread between three subjects) were deemed too poor for inclusion (out of a total of 185 outputs). One subject participating in the study was also the investigator (OH), so whereas it was possible to self-complete MPOD measurements on the MPS, it was not possible to self-complete MPOD measurements on the MPR. Consequently, it was decided that they would measure MPS-based MPOD in both eyes instead of one, to examine the possibility of asymmetric changes; most studies of this nature tend to measure MPOD in one eye only (e.g., Kopsell et al. 2006; Rodriguez-Carmona et al. 2006; Trieschmann et al. 2007; van de Kraats et al. 2008; Connolly et al. 2010), but in studies in which both eyes have been assessed, an asymmetric change has been reported once (Bone et al. 2007b).

The total time per visit was between 10 and 20 minutes.

MASKING

The investigator avoided looking at previous MPOD results prior to each session, in order to help prevent any inadvertent bias. For the MPR in particular, the spectral reflection

outputs were chosen purely on their appearance (at the time of data collection and in the later selection process) and not on what the MPOD readings were.

SAMPLE SIZE

As an exploratory study, a sample size of six was considered adequate, and it was also in line with several other diet and supplement modification MPOD studies (Berendschot et al. 2000; Johnson et al. 2000; Koh et al. 2004; Wenzel et al. 2006a; Wenzel et al. 2007b; van de Kraats et al. 2008; Connolly et al. 2010).

STATISTICS

Microsoft Excel and IBM SPSS were used for data analysis. Each data set met the assumption of normality, so mean MPOD values over time were compared by one-way repeated measures ANOVA. The differences between data sets were also normally distributed, so paired-samples t-tests were used when comparing mean MPOD values between two visits.

8.3 Results

MEAN MPOD

Table 8.1 shows the mean MPOD over time for the six participants consuming 200 g of kale per week, using the MPS and MPR devices. Also shown is the mean C-only MPS-based MPOD (peripheral estimate provided by the MPS, based on subject age), because for the purposes of monitoring change in MPOD over time, this test is acceptable, and the test-retest repeatability is better than it is for the standard (centre and peripheral derived) MPOD (see chapter 7). The MPR gives the breakdown of MPOD into its L and Z components, so this information is provided in the table as well.

Visit	Time	Mean (±SD) MPOD				
		MPS	C-only MPS	MPR	Lutein	Zeaxanthin
1	Baseline i	0.24 ± 0.11	0.24 ± 0.11	0.51 ± 0.14	0.28 ± 0.07	0.22 ± 0.09
2	Baseline ii	0.23 ± 0.09	0.24 ± 0.09	0.51 ± 0.12	0.33 ± 0.13	0.18 ± 0.09
3	4 wks of kale	0.23 ± 0.09	0.25 ± 0.09	0.50 ± 0.16	0.29 ± 0.06	0.21 ± 0.12
4	8 wks of kale	0.22 ± 0.08	0.24 ± 0.06	0.41 ± 0.10	0.21 ± 0.07	0.20 ± 0.08
5	12 wks of kale	0.23 ± 0.09	0.24 ± 0.08	0.45 ± 0.11	0.28 ± 0.12	0.18 ± 0.10
6	16 wks of kale	0.22 ± 0.07	0.24 ± 0.07	0.46 ± 0.12	0.24 ± 0.05	0.21 ± 0.10
7	4 wks post-kale	0.22 ± 0.08	0.23 ± 0.09	0.54 ± 0.14	0.32 ± 0.10	0.22 ± 0.15

Table 8.1 Mean MPOD of the six participants over the course of the study, determined using the MPS (n=6) and the MPR (n=5).

By observation only, taking into account the inter-session repeatability of each MPOD device as determined in the previous chapter, there did not appear to be any noticeable differences in the mean MPODs. Paired-samples t-tests comparing the two baseline measurements yielded insignificant differences (p=0.820, 0.921, 0.929, 0.576 and 0.250 for MPS MPOD, C-only MPOD, MPR MPOD, L and Z, respectively), so in the subsequent analyses MPOD values were compared with the second baseline measure, i.e., just before kale intake began. Repeated measures analysis confirmed that there was no statistically significant change in MPOD from baseline to the end of the dietary modification period (table 8.2), as measured by the MPS or the MPR, including C-only MPOD, and L and Z. Paired-samples t-tests were used to compare two visits at a time. The results for baseline MPOD (visit 2) versus MPOD after 16 weeks of kale-eating (visit 6) are shown in table 8.2, but the results of the other comparisons with baseline MPOD can be found in appendix 11 (table A11.1). In all cases there were no statistically significant differences. Figure 8.1 illustrates the lack of MPOD change over time.

Comparison	Statistic value	Significance (p) value
MPS MPOD visit 2 to 6	F = 0.883	0.489
MPS MPOD visit 2 vs 6	t = 1.136	0.299
C-only MPS MPOD visit 2 to 6	F = 0.156	0.959
C-only MPS MPOD visit 2 vs 6	t = -0.183	0.861
MPR MPOD visit 2 to 6	F = 1.102	0.389
MPR MPOD visit 2 vs 6	t = 1.787	0.149
Lutein (MPR) visit 2 to 6	F = 1.716*	0.245
Lutein (MPR) visit 2 vs 6	t = 2.062	0.108
Zeaxanthin (MPR) visit 2 to 6	F = 1.019	0.427
Zeaxanthin (MPR) visit 2 vs 6	t = -1.122	0.325

Table 8.2 Statistical comparisons of the mean MPOD from baseline (visit 2) through to the end of the dietary modification period (visit 6), and between visits 2 and 6 specifically. 'F' refers to a one-way repeated measures ANOVA, and 't' refers to a paired-samples t-test (each data set, and the differences between each data set, were normally distributed).

*Here, Mauchly's test indicated that the assumption of sphericity was not met, and as a result, the Greenhouse-Geisser sphericity correction was used.
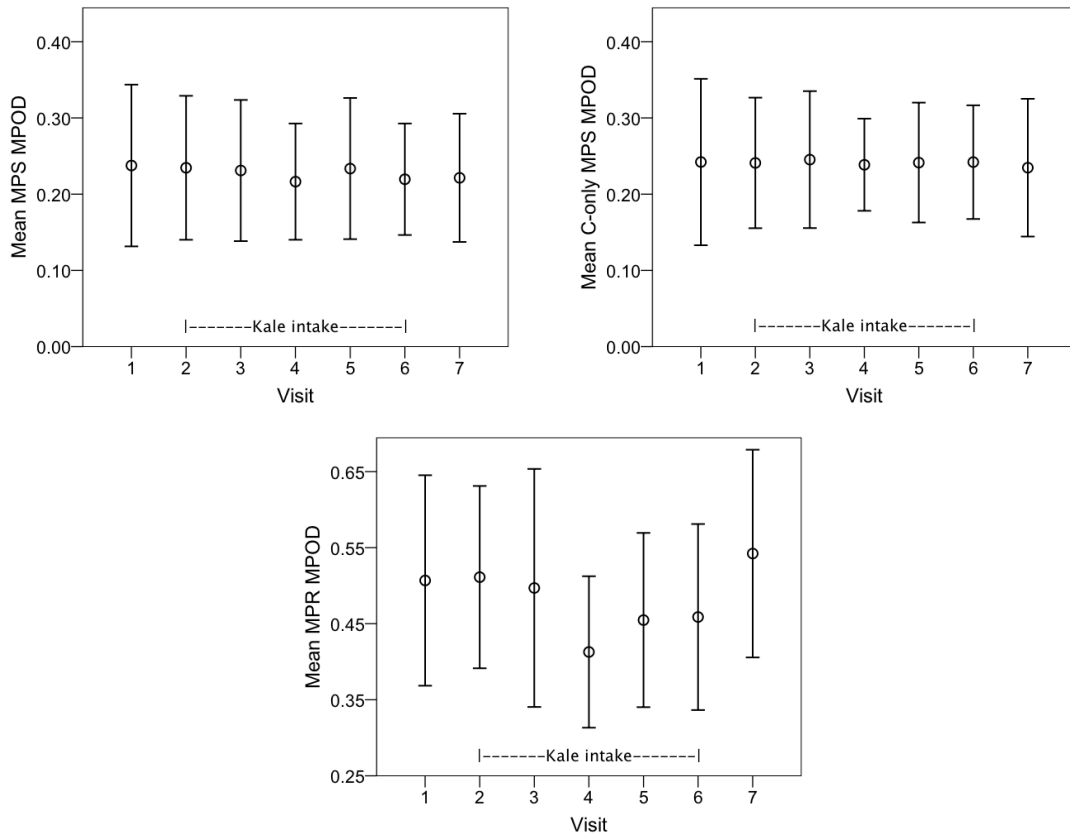
Figure 8.1 Mean MPOD (as measured by the MPS and the MPR) at each visit.

The error bars are ± 1 SD.

Visits: 1 = baseline i; 2 = baseline ii; 3 = after four weeks of kale intake; 4 = after eight weeks of kale intake; 5 = after twelve weeks of kale intake; 6 = after sixteen weeks of kale intake; 7 = four weeks post kale intake.

INDIVIDUAL MPOD – SUBJECT 1

Background: 25-year-old Caucasian male. Had never eaten kale before beginning the study and did not eat any further kale between the final supply and the four-week post-study check. The kale was eaten in addition to his usual vegetables, not as a replacement. It was stir-fried with three to four tablespoons of olive oil and eaten with a meal, e.g., pasta.

MPOD: See table 8.3 and figure 8.2.

MPS proficiency: Very good – virtually all optimal curves throughout the study.

Outcome: No change in MPOD.

| Visit | MPOD | | | | |
	MPS	C-only MPS	MPR	Lutein	Zeaxanthin
1	0.11	0.13	0.33	0.21	0.12
2	0.10	0.17	0.49	0.46	0.03
3	0.12	0.17	0.29	0.26	0.02
4	0.12	0.19	0.31	0.21	0.10
5	0.10	0.16	0.47	0.46	0.01
6	0.11	0.18	0.37	0.28	0.09
7	0.12	0.14	0.48	0.48	0.00

Table 8.3 MPOD over time for subject 1.

Figure 8.2 MPOD over time for subject 1.

Background: 48-year-old Caucasian male. Had never eaten kale before beginning the study and did not eat any further kale between the final supply and the four-week post-study check. The kale was eaten mainly in addition to his usual vegetables, not as a replacement. It was boiled and eaten with bacon as part of a soup (15% of the time); stir-fried with vegetable oil (15% of the time) or steamed (70% of the time), and eaten with a meal, e.g., chicken curry.

MPOD: See table 8.4 and figure 8.3.

MPS proficiency: Excellent – consistently optimal curves produced throughout the study.

Outcome: No change in MPOD.

Visit	MPOD				
	MPS	C-only MPS	MPR	Lutein	Zeaxanthin
1	0.19	0.22	0.63	0.28	0.35
2	0.25	0.23	0.68	0.47	0.21
3	0.23	0.21	0.52	0.26	0.26
4	0.25	0.24	0.41	0.12	0.29
5	0.21	0.23	0.35	0.17	0.18
6	0.22	0.22	0.59	0.27	0.31
7	0.22	0.22	0.72	0.37	0.35

Table 8.4 MPOD over time for subject 2.
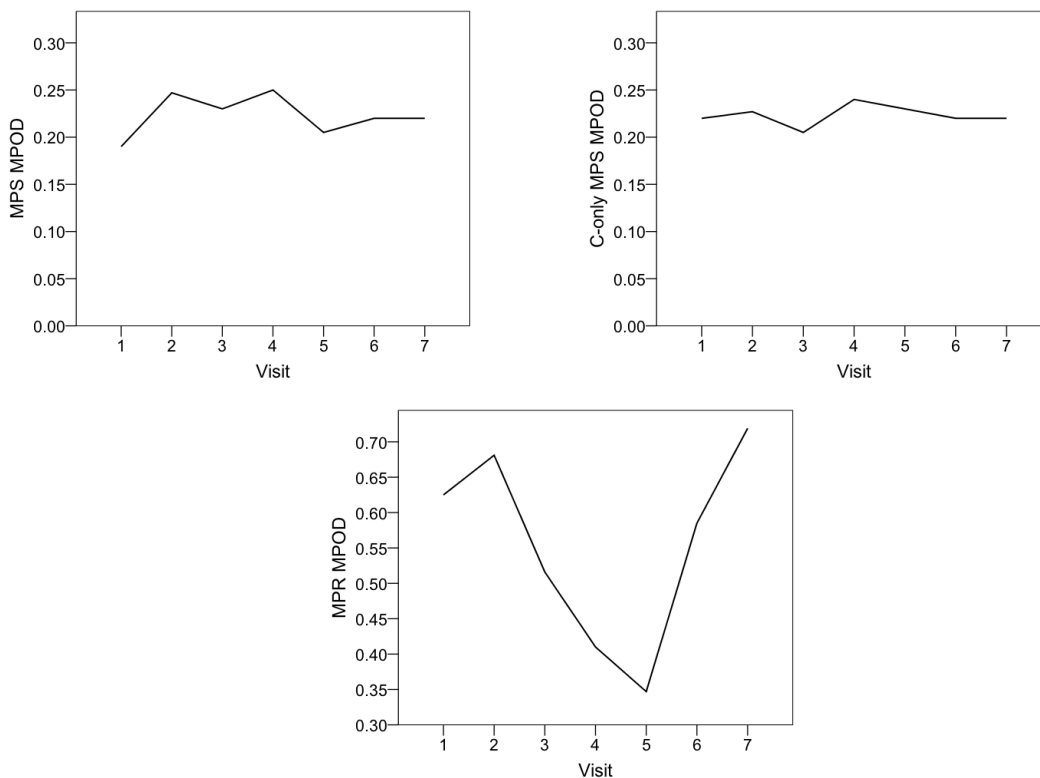
Figure 8.3 MPOD over time for subject 2.

INDIVIDUAL MPOD – SUBJECT 3

Background: 34-year-old Caucasian male. Had occasionally eaten kale before beginning the study. Did not eat any further kale between the final supply and the four-week post-study check. The kale was sometimes eaten in addition to his usual vegetables, and sometimes as a replacement for broccoli. It was stir-fried (most of the time) with vegetable oil, and boiled (about once a week or every other week). Always eaten with a main meal, e.g., beef stew, chicken curry, roast dinner.

MPOD: See table 8.5 and figure 8.4.

MPS proficiency: Very good – virtually all optimal curves throughout the study.

Outcome: No change in MPOD.

| Visit | MPOD | | | | |
	MPS	C-only MPS	MPR	Lutein	Zeaxanthin
1	0.13	0.12	0.39	0.23	0.17
2	0.16	0.12	0.35	0.18	0.17
3	0.12	0.13	0.40	0.23	0.17
4	0.13	0.14	0.32	0.17	0.15
5	0.18	0.14	0.33	0.17	0.16
6	0.14	0.14	0.30	0.16	0.14
7	0.13	0.12	0.36	0.22	0.14

Table 8.5 MPOD over time for subject 3.
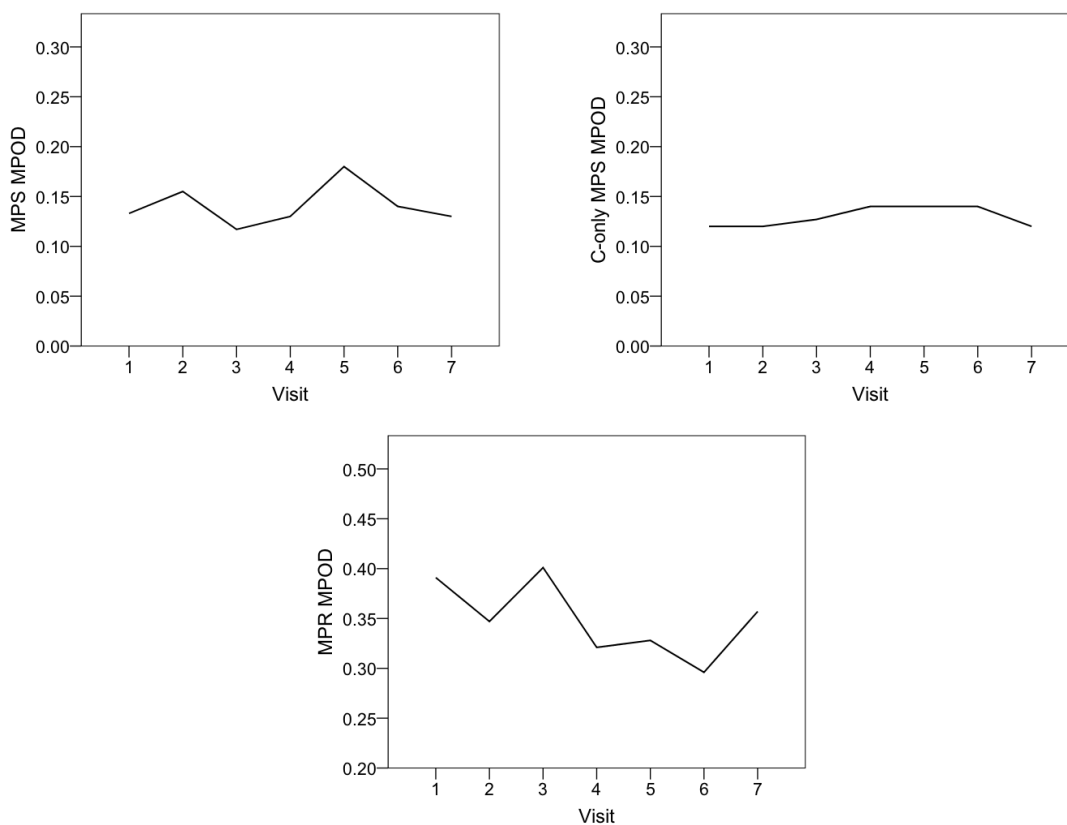
Figure 8.4 MPOD over time for subject 3.

158

INDIVIDUAL MPOD – SUBJECT 4

Background: 27-year-old Asian female. Had never eaten kale before beginning the study but did continue to eat it regularly between the final supply and the four-week post-study check. The kale was eaten in addition to her usual vegetables, not as a replacement. It was stir-fried with one and a half tablespoons of sunflower oil and eaten with a meal, e.g., vegetable curry.

MPOD: See table 8.6 and figure 8.5.

MPS proficiency: Good – required three tests at a few visits but this always resulted in at least two optimal central and peripheral curves.

Outcome: No change in MPOD.

| Visit | MPOD | | | | |
	MPS	C-only MPS	MPR	Lutein	Zeaxanthin
1	0.35	0.42	0.62	0.38	0.24
2	0.31	0.38	0.54	0.28	0.26
3	0.31	0.38	0.68	0.38	0.30
4	0.23	0.31	0.54	0.28	0.26
5	0.34	0.36	0.57	0.33	0.24
6	0.26	0.37	0.50	0.28	0.22
7	0.29	0.38	0.61	0.29	0.32

Table 8.6 MPOD over time for subject 4.
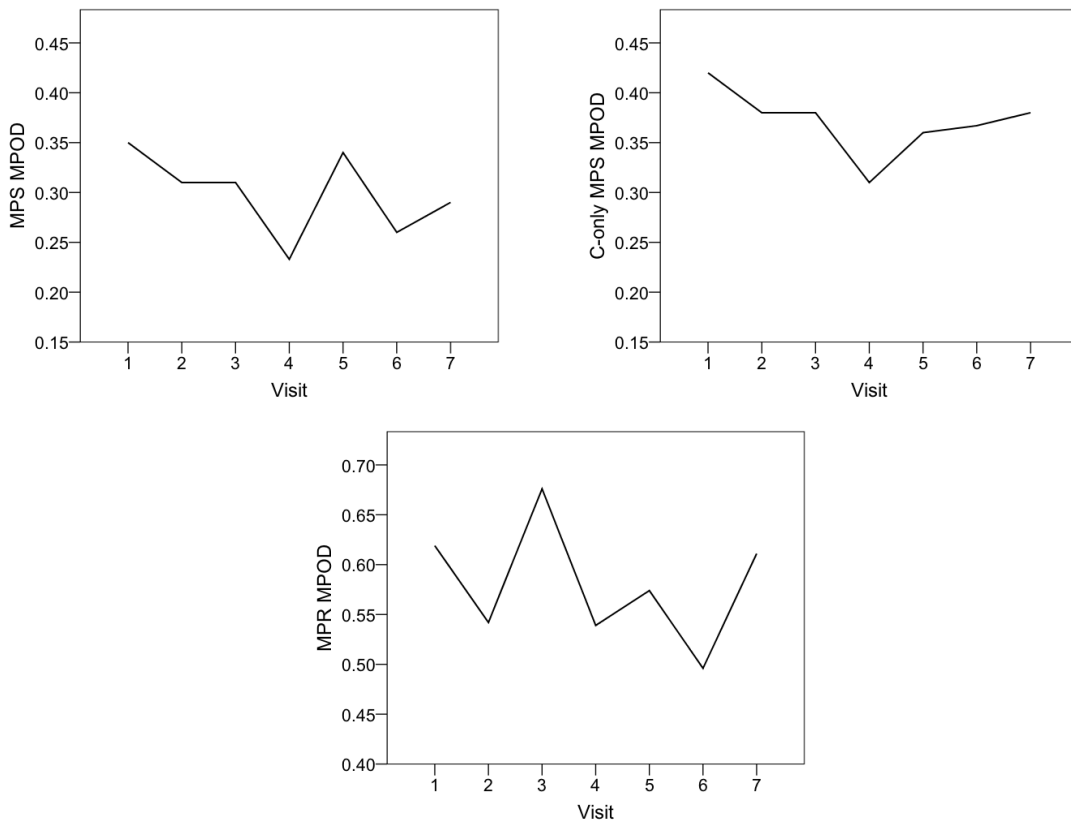
Figure 8.5 MPOD over time for subject 4.

INDIVIDUAL MPOD – SUBJECT 5

Background: 52-year-old Caucasian female. Had occasionally eaten kale before beginning the study and did continue to eat it fairly regularly (in homemade soup) between the final supply and the four-week post-study check. The kale was eaten in addition to her usual vegetables, not as a replacement. It was boiled with a little butter and eaten as part of a soup, stir-fried with olive oil and eaten as part of a stir-fry meal, or boiled and eaten with a meal e.g., meat casserole, pasta, roast dinner.

MPOD: See table 8.7 and figure 8.6.

MPS proficiency: Excellent – consistently optimal curves produced throughout the study.

Outcome: No change in MPOD.

| Visit | MPOD | | | | |
	MPS	C-only MPS	MPR	Lutein	Zeaxanthin
1	0.19	0.19	0.57	0.33	0.24
2	0.17	0.22	0.50	0.26	0.24
3	0.19	0.22	0.61	0.31	0.30
4	0.18	0.23	0.48	0.26	0.22
5	0.17	0.22	0.56	0.27	0.28
6	0.22	0.24	0.55	0.23	0.32
7	0.17	0.22	0.55	0.27	0.28

Table 8.7 MPOD over time for subject 5.

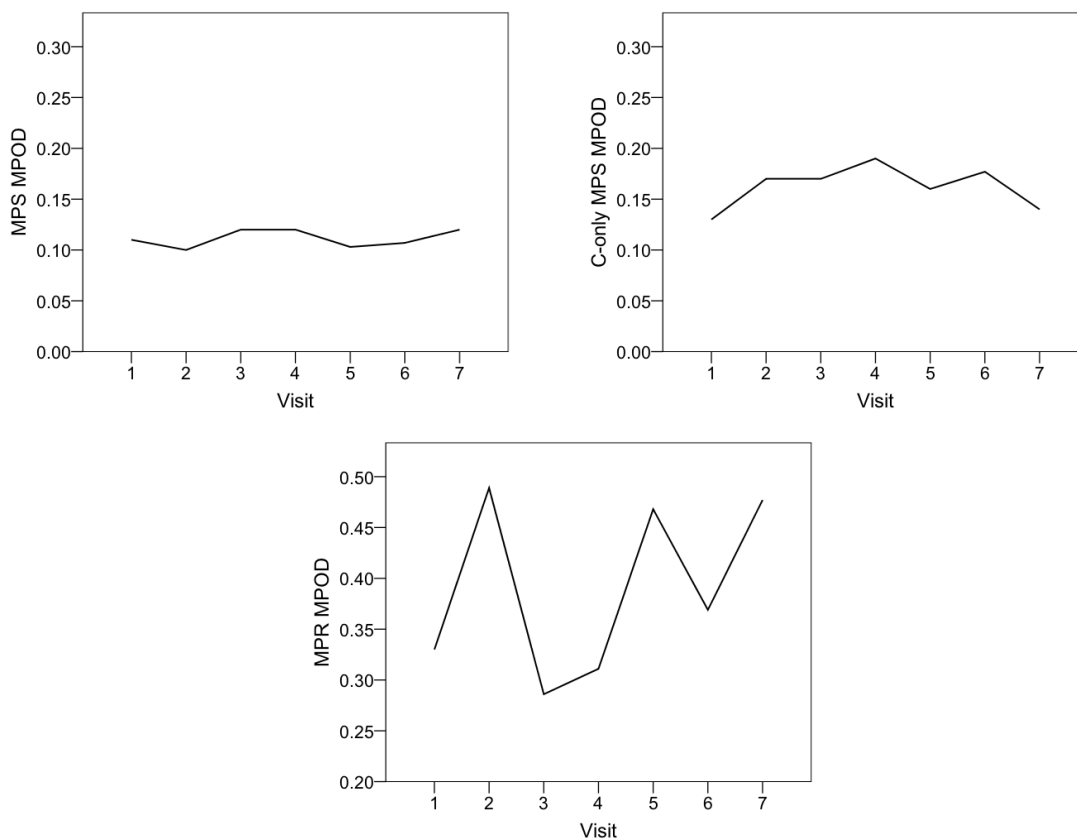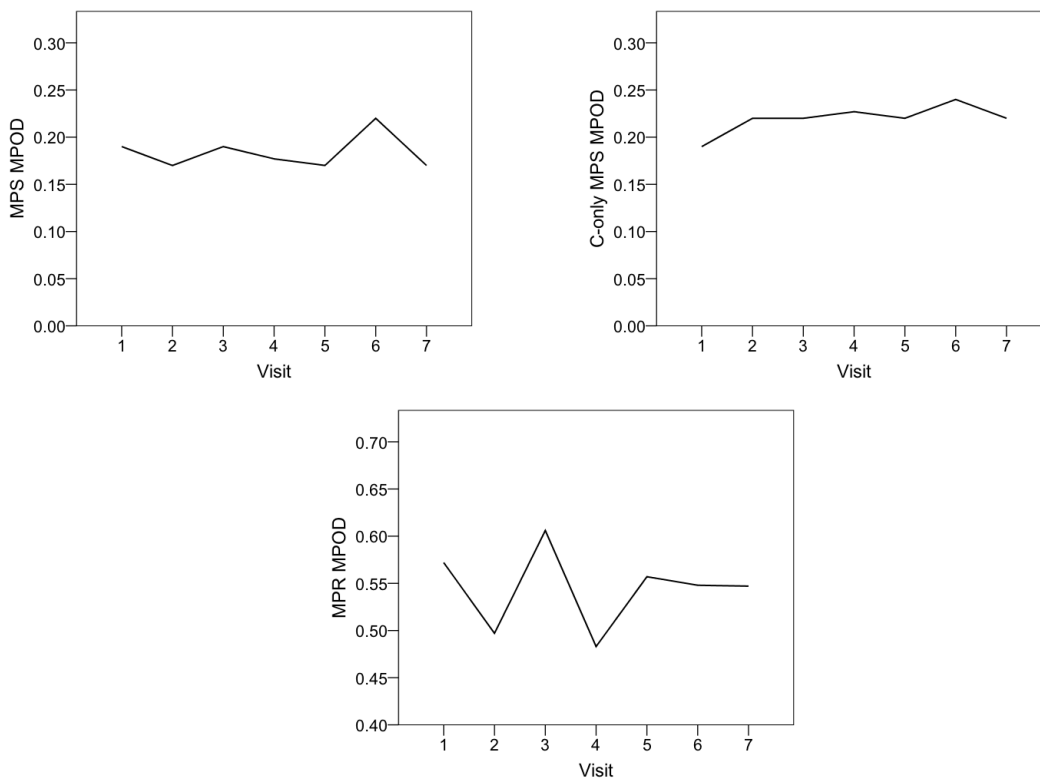**Figure 8.6** MPOD over time for subject 5.

INDIVIDUAL MPOD – SUBJECT 6

Background: 28-year-old Caucasian female. Had sometimes eaten kale in the past, but none at all for two months before beginning the study. Continued to eat kale on and off between the final supply and the four-week post-study check. The kale was sometimes eaten in addition to her usual vegetables, and sometimes as a replacement for broccoli or sweetcorn. It was stir-fried (most of the time) with sprayed rapeseed or sunflower oil, and boiled (about once a week or every other week). Always eaten with a main meal, e.g., pasta, roast dinner, stir-fry.

MPOD: See table 8.8 and figure 8.7.

MPS proficiency: Good – required three tests at a few visits (only in one eye at any one visit) but this always resulted in two optimal central and peripheral curves.

Outcome: No change in MPOD.

| Visit | MPOD | | | |
	MPS RE	C-only MPS RE	MPS LE	C-only MPS LE
1	0.34	0.29	0.35	0.33
2	0.31	0.27	0.35	0.30
3	0.31	0.29	0.34	0.33
4	0.29	0.26	0.32	0.30
5	0.30	0.27	0.34	0.31
6	0.29	0.26	0.30	0.29
7	0.29	0.26	0.33	0.30

Table 8.8 MPOD over time for subject 6.
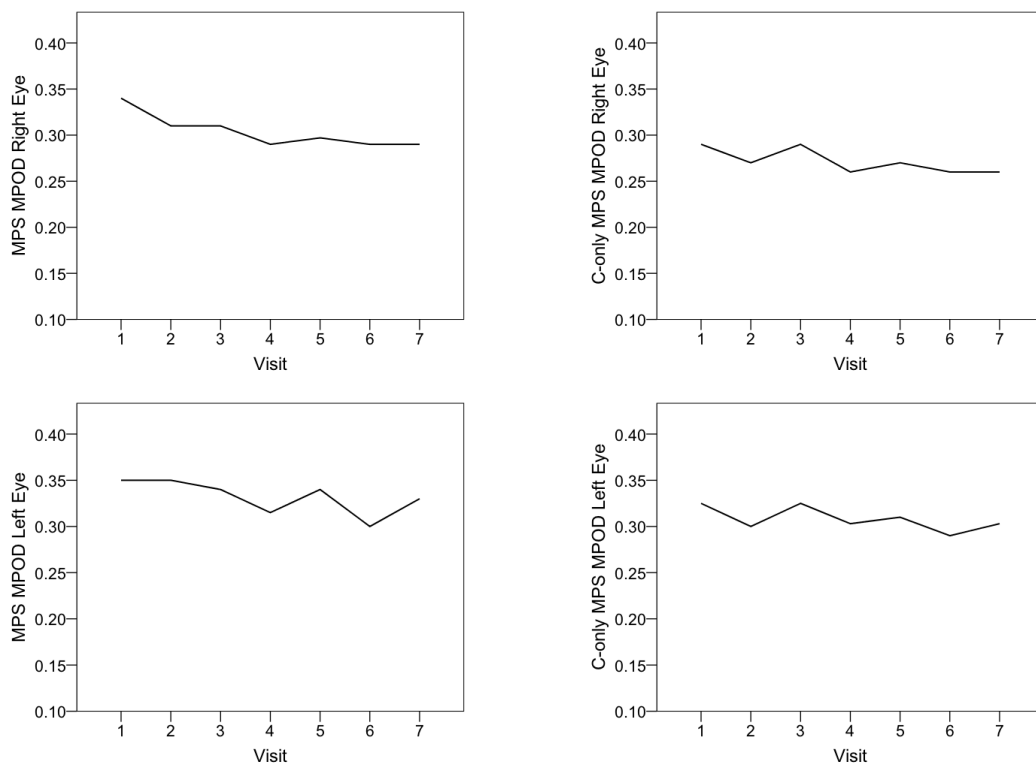
Figure 8.7 MPOD over time for subject 6.

8.4 Discussion

The study described here explored MPOD over time in a group of healthy volunteers, following a substantial, but realistically achievable, increase in the consumption of the L-rich vegetable, kale. As a possible preventer of AMD, efforts to increase MP levels through simple dietary methods are important, and consumption of other L-rich foods had resulted in increases in MPOD in previous studies, thus setting a precedent for this study.

For 16 weeks, six subjects ate 200 g of UK-grown kale per week, distributed over several main meals. There was no apparent change in MPOD for the group as a whole or for any one individual, which raises the question, why not?

Firstly, and a major limitation for this study given the insignificant results, the actual L/Z content of the kale distributed to the participants was unknown. The USDA database (USDA 2010) is a catalogue of nutrient data for foods available in the United States, compiled by comprehensively reviewing the literature for such data (e.g., Mangels et al. 1993; Holden et al. 1999). From this it was assumed that the kale would contain a high level of L/Z (the values are combined in the database); 'kale, cooked, boiled, drained, without salt' is reported to contain 18.2 mg of L/Z per 100g (USDA 2010). As a result, 200 g of cooked kale in the present study was expected to provide around 36 mg of L/Z, i.e., 9 mg per meal if spread over four days per week. However, here lies the first problem with this assumption; it is unclear whether the 100 g of kale (containing 18.2 mg of L/Z after boiling) represents 100 g of originally raw kale that has subsequently been boiled and drained, or 100 g of boiled and drained kale. Boiled and drained kale increases in weight from the raw weight (e.g., 50 g of raw kale becomes 70 g drained, following ten minutes of boiling), so if the USDA data refers to the boiled and drained weight, then 200 g of kale, if boiled, would be expected to contain around 51 mg of L/Z, i.e., 12 to 13 mg per meal if spread over four days per week. Conversely, raw kale decreases in weight when stir-fried or microwaved – 50 g of raw kale becomes 38 g following stir-frying in one tablespoon of oil for eight minutes, and it becomes 20 g following microwaving without water (as per preparation instructions) for two minutes. Judging by the USDA data on spinach, which offers L/Z data on raw and cooked spinach, it would seem that the weights do refer to the weights of the foodstuffs as prepared, e.g., the weight of cooked kale after boiling and draining. Unfortunately, though, the USDA database does not provide L/Z information on raw, stir-fried, microwaved or steamed kale, so it is difficult to estimate how much was in the kale supplied in the present investigation, particularly as all the participants stir-fried at least some, if not all, of the kale. In looking elsewhere for

this information, it became abundantly clear just how much the reported L values in kale vary. For instance, reported L levels for raw kale vary from approximately 2.5 mg per 100 g (Kobori et al. 2011) up to 51.4 mg per 100 g (Updike and Schwartz 2003), with values in between generally falling somewhere between 7 mg and 34 mg per 100 g (e.g., Khachik et al. 1986; Mercadante and Rodriguez-Amaya 1991; Humphries and Khachik 2003). Few studies have measured the Z content of kale in any form, but it seems that this is because the Z levels are zero or close to zero in kale (Humphries and Khachik 2003; Perry et al. 2009). Only one study (from Brazil) measured the L content of stir-fried kale; after ten minutes of stir-frying, there was 3.15 mg of L per 100 g of cooked kale, on average. This would therefore equate to less than 5 mg of L in the entire 200 g bag of kale from the current study (assuming a 25% weight loss on stir-frying). Besides the method of cooking, a recent series of papers by Lefsrud, Kopsell and co-workers has illustrated the range of variables that can affect the quantity of L in pre-harvested kale, such as type, season, air temperature, amount of light irradiance, and maturity (Kopsell et al. 2004; Lefsrud et al. 2005; Lefsrud et al. 2006a; Lefsrud et al. 2006b; Lefsrud et al. 2007). Additionally, the conditions and duration of storage after kale has been harvested can affect its L levels (Kobori et al. 2011). Unfortunately, there do not appear to have been any L/Z papers published on UK kale, although research by Lefsrud et al. demonstrated that the L concentration of kale peaked when cultivated in an air temperature of 30ºC, and decreased linearly at air temperatures of 25, 20 and 15ºC (Lefsrud et al. 2005). Therefore, considering the typical climate in the UK, it is likely that UK-grown kale contains lower L levels than kale from warmer countries. Interestingly, the same study also showed that the L concentration of spinach peaked in an air temperature of 10ºC and decreased linearly as the air temperature increased to 15, 20 and 25ºC. Consequently, it may be that in the UK, spinach is a better source of L than kale. In any case, it is evident that the amount of L provided by this 16-week kale study could well have been a lot lower than anticipated, and thus it could offer one explanation for the lack of MP response. However, it could also be that even if the kale did contain the levels of L originally expected, this might not have been enough to influence the measured MPOD.

Aside from the actual L concentration of the kale, another possibility for the lack of increase in MPOD is that the L wasn't particularly bioavailable to the body. By instructing the subjects to eat the kale with a main meal, it was taken for granted that it would be consumed with a reasonable amount of fat. However, it was perhaps naïve to make this assumption, and instead there should have been a specific instruction to eat the kale with a fat source, since it has been demonstrated that absorption of L is enhanced by consumption with some form of dietary lipid (Roodenburg et al. 2000; Unlu et al. 2005;

Takeda et al. 2009). That said, L does appear to be considerably more bioavailable than another carotenoid, beta-carotene (Castenmiller et al. 1999; van het Hof et al. 1999), and all the post-study questionnaires completed by the volunteers did indicate that the kale was cooked and/or eaten with fat of some variety.

Continuing with the bioavailability theme, carotenoids such as L and beta-carotene have been documented as competing with each other for absorption from the small intestine (Kostic et al. 1995; van den Berg and van Vliet 1998; Wang et al. 2010), so this is another possibility for the underlying cause of the poor macular response to kale intake, although if anything, L appears to compete more successfully for absorption than beta-carotene (van den Berg and van Vliet 1998). Moreover, spinach contains comparable amounts of beta-carotene to kale (USDA 2010), yet in the studies discussed earlier (section 8.1), dietary modification with spinach did increase MPOD (Hammond et al. 1997a; Johnson et al. 2000; Kopsell et al. 2006). However, it should be noted that in these studies the spinach was consumed more regularly than in the present study. In fact, in their questionnaire responses, four out of the six subjects stated that they ate the kale over the course of only two to three meals, so there may have been a limit to how much L could actually be absorbed at the serum or retinal level in one sitting.

So-called 'retinal nonresponders' have been reported in other MPOD studies (e.g., Hammond et al. 1997a; Cardinault et al. 2003; Trieschmann et al. 2007; Johnson et al. 2008; Stringham and Hammond 2008; Connolly et al. 2010; Nolan et al. 2011), so it should not be ruled out that all the subjects in the present investigation fit into that category. In several studies, though, it is often the subjects with the lowest baseline MPOD that demonstrate the greatest rise in MPOD in response to dietary or supplement L/Z modification (Wenzel et al. 2006b; Aleman et al. 2007; Richer et al. 2007; Borel et al. 2011), and in this respect there were at least three subjects who could be classed as having a low starting MPOD. It has also been suggested that by not taking into account the spatial distribution of MP during supplementation, increases in MP at more eccentric locations may be missed, or indeed central MPOD increases may be missed or underestimated if the peripheral reference point accumulates any MP (Rodriguez-Carmona et al. 2006; Bone et al. 2007b; Schalch et al. 2007; Wenzel et al. 2007b; Johnson et al. 2008; Connolly et al. 2010). With respect to the former, those studies demonstrating increases in MP beyond the central measure still tend to show the highest increases at the central points (Berendschot et al. 2000; Wenzel et al. 2007b; Connolly et al. 2010), so it is unlikely that an MPOD increase would have occurred outside of the central fovea in the current study. With respect to the latter, by observing the C-only

MPOD values as well as the actual MPOD values, any underestimation of MPOD caused by increases in peripheral MP was avoided, and there was still no change in MPOD over time.

The duration of dietary modification was similar to most other MP studies, so this should not have been a limitation to achieving an MPOD increase, although in a recent study in which participants took a daily 12 mg L plus 1 mg Z supplement, there was no statistically significant increase in group MPOD until 12 months of supplementation (measurements were taken at baseline, three months and six months), despite statistically significant serum increases in L by three months (Nolan et al. 2011). Measurements of serum L/Z would have been extremely useful in the current investigation, because dietary L/Z intake is more closely correlated with serum L/Z than MPOD (Beatty et al. 2004), hence it would have provided a means of assessing whether there was a significant amount of L in the kale.

A final possibility for the findings would be that the kale was used in place of other vegetables that may also have contained L/Z. As it turns out, though, this was generally not the case for any of the subjects (see section 8.3), so this can probably be ruled out.

In conclusion, there was no MP response to dietary modification with kale in this exploratory study, which was most likely due to a lower than anticipated L content in the kale itself. In addition, it may simply have been that the amount of kale consumed was not enough to affect MPOD. For any similar future study, determination of the L/Z content of the kale, plus serum measures of L/Z, would be highly recommended. Furthermore, it would be wise to insist on boiling the kale rather than stir-frying it, to advise more thoroughly on the types of food to consume the kale with, and finally, to make sure that the kale is shared over more than three meals.

8.5 Summary
The results of the study described here cannot be used to add further support to the recommendation of kale to members of the public. This chapter marks the end of this body of research in which a variety of studies have aimed to add to MP knowledge. In the concluding chapter to follow, the findings of these studies are brought together.

CHAPTER 9: CONCLUSION

Through use of a novel MPOD-measuring device (the MPS), which was first explored and optimized for accurate employment in the research and clinical environment, the investigations described over the course of the previous chapters aimed to contribute new information to the MP literature, with MPOD-based studies on anisometropia, South Asian race, blue-light-filtering CLs, and dietary modification with kale.

In chapters 2 and 3, the aforementioned efforts to maximize the accuracy and consistency of MPOD measurements taken with the MPS were carried out. The eventual protocol that was established (chapter 3) proved useful for obtaining good inter-session repeatability (chapter 7). In fact, the CR (0.08, n=27) was half that of another MPS inter-session repeatability study declaring 'high agreement' (de Kinkelder et al. 2011) (CR=0.18, n=20). It was also far better than that of an objective MPOD device (the MPR), which was assessed alongside the MPS in chapter 7.

In chapter 4 it was established that occasional interocular MPOD differences could not be explained by anisometropic refractive errors, but the idea of refractive error having at least some influence on MPOD was not ruled out altogether. A future study could examine MPOD in two groups of individuals with contrasting refractive errors, such as myopic refractive errors greater than -5.00 DS and hyperopic refractive errors greater than +2.00 DS.

A notable and statistically significant finding was obtained in chapter 5; South Asian individuals were found to have higher MPOD, on average, than Caucasian individuals. Within the Asian group, males had a statistically significant higher mean MPOD than females, and there was a tendency for participants with lighter irides to have lower MPODs; both these outcomes have been reported in MPOD studies with Caucasian subjects, and they have also been reported as possible associations with AMD (i.e., female gender and light iris colour), which is of course the chief motivation behind most MPOD research.

As discussed in chapter 1, there is no definitive proof of a MP-AMD relationship, but there is enough evidence to justify the continued investigation of MP, particularly as it remains one of the few potential associations that is modifiable. However, novel and traditional efforts to augment MPOD were unsuccessful in the research studies described here. In chapter 6, blue-light-filtering CLs had no effect on MPOD, which could have been due to

the ages of the volunteers taking part, and in chapter 8, dietary modification with kale made no difference to MPOD either, which may well have been the result of a lower than anticipated L content in the kale itself. It was observed that reported L levels in kale vary widely, and moreover, that there does not appear to be any published L data for kale grown in the UK. In view of the latter, it would be beneficial to conduct a study in which the L (and Z) in UK-grown kale is quantified, the results of which might have a bearing on the advice given to patients regarding the ocular benefits of kale.

Should a relationship between L/Z/MZ and AMD truly exist, it seems logical to suggest (in view of its location) that the amount of L and Z in the macula (i.e., MPOD) would have a stronger relationship with AMD than L and Z found elsewhere in the body. From the wide range of information available up to this point in time, combined with the results of the studies performed throughout the research period described here, it is the author's opinion that MPOD does have an influence on the development of AMD, but that supplementing or modifying a diet to include high amounts of L and Z may not have as much effect as one would hope. The reasoning behind this belief comes from the knowledge that L/Z intake is not directly related to serum L/Z, which in turn is not directly related to MPOD. There appear to be many uncontrollable inter-individual variations that regulate the digestion, absorption, transport and eventual retinal uptake and maintenance of L and Z, and while some of these factors may eventually turn out to be controllable to some degree, it is likely that the majority will not. The body is a carefully balanced equilibrium, and arguably the best way to keep it that way is to adhere to the classic 'everything in moderation' principle. For example, with a healthy diet and regular exercise (neither to excess), essentially there is there is a natural weight and shape for a body, and this varies from person to person. Analogous to this, it seems that MP also has a natural level, which like body weight and shape, varies from one individual to the next. It is receptive to a high intake of L etc., much like an extreme diet will cause most people to lose weight, and it may also be receptive to other alterations in lifestyle, such as cessation of smoking, but ultimately if a normal, healthy diet and lifestyle is followed, an individual's MP level is what it is. Nevertheless, the results of the second Age-Related Eye Disease Study (AREDS2 2011), which is monitoring the progression of AMD in subjects taking 10 mg of L and 2 mg of Z (as well as other supplements and combinations of supplements), will be of great interest and will hopefully support the continued investigation of MP.

In summary, this research aspired to add to the growing body of MP knowledge, and hopefully this was achieved.

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APPENDICES

Appendix 1: Peer-reviewed publication

Appendix 2: References for table 1.1 in chapter 1

See reference list (starting on page 168) for the references in full.

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Appendix 3: MPS instructions to patient

In a moment I'll ask you to look into the instrument with your right eye, where you should see three black spots on a white background.

Throughout the test you'll be detecting when the middle spot appears to flicker, although you won't always be looking directly at it.

In the first part of the test you'll be looking straight at the central spot, which will change to a blue colour instead of being black. Keep looking at the blue spot all the time. At some point it should appear to start flickering. At the point when you first notice it flickering, press and release the response button. *(Demonstrated to subject.)* The flicker will stop when you've pressed the button. Have a blink or two then wait for the next flicker and press again.

We'll start with a short practice so you know what to expect. Press the button every time you see the middle spot start to flicker. This will happen about five times. *(Subject set up on the MPS and central practice started.)*

Now you're going to do the proper test. It starts like the practice run you just did. The blue spot will then probably change to a paler or darker blue. It may look almost white in colour. Again, keep looking directly at it and push the button when you first notice it beginning to flicker. You'll do about 15-20 presses in total. Remember to blink after each button click and any other time you need to. Don't worry if sometimes the flicker seems to take longer to occur than other times – that's normal. Let me know if you want me to pause or stop the test at any time. *(Central test started.)*

Okay, in the second part of the test you'll see a larger red spot to the left of the centre one *(to the right when testing the left eye)*. You'll need to look straight at this red area. At the same time you should be aware of the blue spot in your side vision. Try hard to keep your fixation on the red, not the blue, but when you first become aware of the blue spot flickering out of the 'corner' of your eye, press the button. This test can be tricky because sometimes it might feel like the blue spot is disappearing, so blinking regularly becomes even more important. You should blink once or twice after each push of the button, and anytime in between if you need to. Another thing you can do if the blue seems to be disappearing is to change your viewpoint on the red spot frequently – as long as you stay within the red area, that's fine.

We'll start with a practice again. Press the button when you first detect any flicker. This'll happen about five times. *(Subject set up on the MPS and peripheral practice started.)*

Now let's do the proper test. It starts just like the practice test, and then the blue spot will probably become paler or darker again. Remember to keep looking within the red area, press when you first notice the blue spot start to flicker, and blink at least after each press. Again, you'll do about 15-20 button presses in total, and sometimes the flicker will seem to take longer to appear than other times. *(Peripheral test started.)*

You're now going to repeat the process for your left eye. *(Central and peripheral tests completed for left eye.)*

If the subject's central or peripheral curve is poor, offer this further advice:

If you think the blue spot might be flickering but you're not really sure, just wait a little bit longer and then it should become easier to tell.

O.Howells
11/02/09

Appendix 4: Additional analysis for chapter 2

See chapter 2 for further explanation, if required.

	Comparison	Difference (significance, p)
SD between measures 1, 2 and 3	CP RE and C-only RE	t = 3.223 (p = 0.004)
	CP LE and C-only LE	t = 4.094 (p<0.0005)
	CP RE and CP LE	t = 2.013 (p = 0.055)
	C-only RE and C-only LE	t = 2.910 (p = 0.008)
CV between measures 1, 2 and 3	CP RE and C-only RE	t = 3.669 (p = 0.001)
	CP LE and C-only LE	t = 4.449 (p<0.0005)
	CP RE and CP LE	t = 1.916 (p = 0.067)
	C-only RE and C-only LE	t = 3.223 (p = 0.004)

Table A4.1 Comparisons of the mean individual standard deviations of the three repeated MPOD measurements, and comparisons of the mean individual coefficients of variation (n=25).

't' refers to a paired-samples t-test (the differences between each data set were normally distributed).

Comparison	Difference (significance, p)	Correlation (significance, p)
CP R1 and L1	t = 1.075 (p = 0.293)	r = 0.699 (p<0.0005)
CP R2 and L2	z = -1.864 (p = 0.062)	rho = 0.655 (p<0.0005)
CP R3 and L3	t = -0.079 (p = 0.938)	r = 0.846 (p<0.0005)
C-only R1 and L1	z = -0.931 (p = 0.352)	r = 0.865 (p<0.0005)
C-only R2 and L2	z = -0.521 (p = 0.602)	r = 0.921 (p<0.0005)
C-only R3 and L3	z = -0.242 (p = 0.809)	rho = 0.918 (p<0.0005)

Table A4.2 Interocular mean MPOD comparisons, plus correlations (n=25).

't' refers to a paired-samples t-test, which was used where the differences between each data set were normally distributed. Otherwise, the Wilcoxon signed rank test ('z') was used. 'r' refers to Pearson's r, which was used where both data sets were normally distributed. Otherwise, Spearman's rho was used.

Appendix 5: Lifestyle questionnaire

LIFESTYLE QUESTIONNAIRE

1. **Age:** _____

2. **Gender:** ☐ Male   ☐ Female

3. **How would you describe your ethnic group?** The categories below were those used in the 2001 census and are recommended by the Commission for Racial Equality.

A   White
   ☐ British
   ☐ Irish
   ☐ Other White background (please state) _____

B   Mixed
   ☐ White and Black Caribbean
   ☐ White and Black African
   ☐ White and Asian
   ☐ Other Mixed background (please state) _____

C   Asian or Asian British
   ☐ Indian
   ☐ Pakistani
   ☐ Bangladeshi
   ☐ Other Asian background (please state) _____

D   Black or Black British
   ☐ Caribbean
   ☐ African
   ☐ Other Black background (please state) _____

E   Chinese or other ethnic group
   ☐ Chinese
   ☐ Any Other (please state) _____

4. **Height:** _____

5. **Weight:** _____

6. **Iris colour:**   ☐ Black/dark brown   ☐ Brown   ☐ Light brown   ☐ Hazel
                      ☐ Green   ☐ Grey   ☐ Blue
                      ☐ Other (please state, e.g. light brown/hazel) _____

7. **Do you smoke?** ☐ Yes   ☐ No

   If yes, please go to question 8. If no, please go to question 9.

8. **a) Approximately how many cigarettes do you smoke in a week?** _____

   b) What brand(s) of cigarette do you smoke most regularly?

   _____

   c) How many years have you smoked for? _____

9. **Have you ever been a regular smoker in the past?** ☐ Yes   ☐ No

   If yes, please go to question 10. If no, please go to question 11.

10. **a) Approximately how many cigarettes did you smoke in a week?** _____

    b) What brand(s) of cigarette did you smoke most regularly?

    _____

    c) How many years did you smoke for? _____

    d) How long has it been since you stopped smoking? _____

11. **Do you drink alcohol?** ☐ Yes   ☐ No

    If yes, approximately how many units do you consume in a week? _____

    1 alcopop bottle = 1.4 units.

    1 bottle of average strength beer/lager/cider = 1.7 units.

    1 can of average strength beer/lager/cider = 2.2 units.

    1 pint (568 ml) of average strength beer/lager/cider = 2.8 units.

    1 strong cocktail = 4 units.

    25 ml spirit/shot = 1 unit (gin, rum, sambuca, tequila, vodka, whisky).

    35 ml spirit/shot = 1.3 units.

    1 bottle of average strength wine (12% vol) = 9 units.

    1 small glass (125 ml) of wine = 1.5 units.

    1 standard glass (175 ml) of wine = 2.1 units.

    1 large glass (250 ml) of wine = 3 units.

    For more information, go to www.units.nhs.uk/howMany.html

12. **a) Which of the following best describes your dietary background?**

    ☐ Meat eater   ☐ Vegetarian   ☐ Partly vegetarian   ☐ Vegan

    Please specify as appropriate, e.g. 'meat eater but no beef' or 'vegetarian but eat eggs' or 'partly vegetarian (eat fish)'. _____

    b) Has this been your dietary background for at least one year?

    ☐ Yes   ☐ No   If no, how long? _____

13. On average, how many servings of vegetable do you eat *per week*? _____

14. On average, how many servings of fruit do you eat *per week*? _____

15. On average, how many eggs (including yolks) do you eat *per week*? _____

16. On average, how many servings of oily fish do you eat *per week*? _____
    For a list of what counts as oily fish, please go to
    http://www.eatwell.gov.uk/healthydiet/nutritionessentials/fishandshellfish/

17. Do you exercise regularly? ☐ Yes   ☐ No
    (The government recommends 30 minutes of moderate exercise five days a week.)

18. Approximately how many daylight hours *per week* do you spend outdoors (i.e. outside of buildings/vehicles and therefore exposed to light) in:
    Autumn/Winter months? _____   Spring/Summer months? _____

19. Are you exposed to strong sunlight regularly (e.g. more than 1 sunny holiday per year)? ☐ Yes   ☐ No

20. Do you use sunbeds or tanning booths regularly? ☐ Yes   ☐ No

21. Is your skin particularly sensitive to sunlight? ☐ Yes   ☐ No

22. In bright conditions, how often do you wear sunglasses?
    ☐ Always   ☐ Most of the time   ☐ Sometimes
    ☐ Occasionally   ☐ Very rarely   ☐ Never

23. Do you have any kind of medical condition? ☐ Yes   ☐ No
    If yes, what? _____

24. Do you take any regular medication? ☐ Yes   ☐ No
    If yes, what? _____

25. Do you take any regular vitamins or supplements? ☐ Yes   ☐ No
    If yes, what? (Please include as much detail as possible, e.g. Superboots Multivitamin A-Z, 1 tablet three times per week, and Seven Oceans 650mg fish oils, 1 capsule daily.)

    _____

    _____

26. **Do you wear glasses or contact lenses?** ☐ Yes   ☐ No

    If yes, please go to question 27. If no, please go to question 28.

27. **a) Are your glasses/contact lenses worn:**

    ☐ Full-time?

    ☐ Part-time (e.g. just for distance activities)?

    ☐ Occasionally?

    b) In public, do you mainly wear:

    ☐ Glasses?   ☐ Contact lenses?   ☐ Both equally?   ☐ Neither?

    c) For contact lens wearers, if you know the brand and/or the power of your lenses, please give the details here:

    _____

    _____

    d) If you know approximately or exactly what your glasses prescription is, please write it here:

    _____

    _____

28. **Do you have any kind of eye condition, besides refractive error?**

    ☐ Yes   ☐ No

    If yes, what? _____

29. **Are you aware of any history of age-related macular degeneration in your family?** ☐ Yes   ☐ No

    If yes, who? _____

    If you have any further comments regarding any of the questions above, please write them here:

    _____

    _____

    _____

    Thank you for taking the time to complete this questionnaire. If you need any help or advice with any of the questions, please contact Olivia Howells by telephone: 0121 204 4135, or email: o.howells@aston.ac.uk.

FOOD DIARY

Instructions on how to fill in your food diary

Please fill out this food diary for three days. Every time you eat or drink something, write it down in the diary provided under the correct day.

Try and describe the food as accurately as possible. For example:

One large bowl of cornflakes with skimmed milk and a level teaspoon of sugar
Two slices of medium thickness wholemeal toast, thickly spread with butter

Try to give rough estimates of the food and drink consumed. For example:

One small cup of tea or one large cup of coffee
Large, medium or small banana
Two chocolate biscuits
Three tablespoons of baked beans

Try to be as accurate as possible (it would be great if you could include weights!).

Remember to include all foods and drinks consumed at home and at other places such as restaurants and friends' houses etc.

Try to fill in the diary as you eat, instead of leaving it until the end of the day. This ensures that you won't forget what you have eaten.

Day 1 (weekday)	
Main meal 1 (e.g. breakfast):	Main meal 3 (e.g. dinner):
Main meal 2 (e.g. lunch):	Snacks and drinks:

Day 2 (weekday)	
Main meal 1 (e.g. breakfast):	Main meal 3 (e.g. dinner):
Main meal 2 (e.g. lunch):	Snacks and drinks:

Day 3 (weekend day)	
Main meal 1 (e.g. breakfast):	Main meal 3 (e.g. dinner):
Main meal 2 (e.g. lunch):	Snacks and drinks:

THANK YOU FOR YOUR TIME

Appendix 7: Additional analysis for chapter 5

See chapter 5 for further explanation, if required.

Variable	Comparison	Statistic value	Significance (p) value
MPOD	Male/Female	t = 2.285	0.027
	Indian/Pakistani	t = 0.249	0.804
	Meat-eater/Vegetarian	t = -0.294	0.770
Body mass index	Male/Female	t = 4.007	0.001
	Indian/Pakistani	t = -1.631	0.120
	Meat-eater/Vegetarian	t = 0.572	0.570
Vegetable + Fruit + Egg intake per week	Male/Female	t = 0.607	0.547
	Indian/Pakistani	t = 0.623	0.536
	Meat-eater/Vegetarian	U = 137.5	0.156
Energy (kilocalories)	Male/Female	t = 4.196	<0.0005
Carbohydrate (grams)	Male/Female	U = 141.0	0.004
Total fat (grams)	Male/Female	U = 94.0	<0.0005
Lutein + Zeaxanthin (milligrams)	Male/Female	U = 214.0	0.173
	Indian/Pakistani	U = 205.0	0.650
	Meat-eater/Vegetarian	U = 104.0	0.023
	VFE ≤15/VFE >15	U = 258.5	0.366

Table A7.1 Statistical comparisons of mean values for the 50 Asian subjects with completed questionnaires and food diaries (see table 5.3 for details).

VFE ≤15 = less than or equal to 15 vegetable+fruit+egg servings per week.

't' refers to an independent-samples t-test, which was used when each data set was normally distributed. Otherwise, the Mann-Whitney U test ('U') was used. For all t-tests, the assumption of homogeneity of variance was checked; where this assumption was not met, the adjusted t and p values are reported.

Nutrient	Mean (±SD) from food diaries	Nutrient	Mean (±SD) from food diaries
Alcohol g	2.00 ± 5.53	Pantothenic acid mg	4.34 ± 1.93
Biotin mcg	22.4 ± 9.4	Phosphorus mg	1165 ± 422
Calcium mg	797.6 ± 302.1	Polyunsaturated fat g	15.3 ± 7.3
Carbohydrate g	248.4 ± 80.3	Potassium mg	2718 ± 881
Carotene mcg	2760 ± 2744	Protein g	75.8 ± 32.9
Chloride mg	3801 ± 1332	Retinol mcg	375.1 ± 271.3
Cholesterol mg	201.1 ± 129.2	Riboflavin mg	1.43 ± 0.67
Copper mg	1.22 ± 0.75	Saturated fat g	26.5 ± 10.5
Energy kcal	1950 ± 615	Selenium mcg	40.1 ± 19.6
Fat (total) g	78.2 ± 29.6	Sodium g	2.66 ± 1.03
Fibre g	17.1 ± 7.1	Starch g	141.9 ± 47.8
Folate mcg	228.7 ± 99.9	Sugars g	96.9 ± 42.4
Iodine mcg	118.4 ± 60.8	Thiamin mg	2.38 ± 3.26
Iron mg	11.8 ± 4.9	Vitamin B6 mg	1.94 ± 0.89
Lutein and Zeaxanthin mg	1.31 ± 2.46	Vitamin B12 mcg	3.87 ± 2.31
Magnesium mg	240.4 ± 86.2	Vitamin C mg	109.9 ± 76.5
Manganese mg	2.92 ± 1.28	Vitamin D mcg	1.56 ± 1.26
Monounsaturated fat g	24.5 ± 10.3	Vitamin E mg	9.03 ± 3.93
Niacin equivalent mg	20.0 ± 10.3	Vitamin K mcg	21.9 ± 81.1
Nitrogen g	12.4 ± 5.3	Zinc mg	7.45 ± 3.09

Table A7.2 Mean nutrient values per day, derived from analysis of the 50 food diaries.

Appendix 8: Additional analysis for chapter 6

See chapter 6 for further explanation, if required.

	Comparison	Yellow CL eye		Normal CL eye	
		Statistic (t) value	Significance (p) value	Statistic (t) value	Significance (p) value
MPOD (Centre and Peripheral)	Measure 1 vs 2	0.262	0.802	-1.214	0.270
	Measure 1 vs 3	1.805	0.121	-1.478	0.190
	Measure 1 vs 4	0.214	0.838	-1.483	0.188
	Measure 1 vs 5	-0.617	0.560	-1.592	0.162
	Measure 1 vs 6	-0.064	0.951	0.811	0.448
	Measure 2 vs 3	0.800	0.454	-1.309	0.238
	Measure 2 vs 4	-0.030	0.977	-0.198	0.850
	Measure 2 vs 5	-0.664	0.532	0.431	0.681
	Measure 2 vs 6	-0.252	0.810	1.485	0.188
	Measure 3 vs 4	-1.494	0.186	0.659	0.534
	Measure 3 vs 5	-1.508	0.182	0.914	0.396
	Measure 3 vs 6	-1.333	0.231	1.641	0.152
	Measure 4 vs 5	-1.095	0.315	0.451	0.668
	Measure 4 vs 6	-0.283	0.787	1.410	0.208
	Measure 5 vs 6	0.508	0.630	2.499	0.047
Centre-only MPOD	Measure 1 vs 2	-0.982	0.364	0.440	0.675
	Measure 1 vs 3	0.383	0.715	-0.402	0.702
	Measure 1 vs 4	-0.378	0.718	-0.127	0.903
	Measure 1 vs 5	-0.634	0.549	1.641	0.152
	Measure 1 vs 6	-0.673	0.526	1.887	0.108
	Measure 2 vs 3	1.228	0.266	-1.511	0.182
	Measure 2 vs 4	0.426	0.685	-0.609	0.565
	Measure 2 vs 5	0.084	0.936	1.350	0.226
	Measure 2 vs 6	0.357	0.734	1.262	0.254
	Measure 3 vs 4	-0.633	0.550	0.510	0.628
	Measure 3 vs 5	-0.766	0.473	1.609	0.159
	Measure 3 vs 6	-0.942	0.382	1.435	0.201
	Measure 4 vs 5	-0.346	0.741	1.196	0.277
	Measure 4 vs 6	-0.256	0.807	1.130	0.302
	Measure 5 vs 6	0.251	0.810	0.577	0.585

Table A8.1 Mean MPOD comparisons (n=7) from baseline (measure 1) to one month post CL wear (measure 6), assessed by paired-samples t-tests (the differences between each data set were normally distributed).

Appendix 9: Additional analysis for chapter 7

See chapter 7 for further explanation, if required.

	Comparison	Statistic value	Significance (p) value
Within-operator	MPS 1 and MPS 2	t = -0.463	0.648
	PV MPR 1 and PV MPR 2	z = -0.198	0.843
	PV Lutein 1 and PV Lutein 2	z = -0.122	0.903
	PV Zeaxanthin 1 and PV Zeaxanthin 2	z = -0.821	0.411
	OH MPR 1 and OH MPR 2	z = -1.065	0.287
	OH Lutein 1 and OH Lutein 2	t = -0.603	0.553
	OH Zeaxanthin 1 and OH Zeaxanthin 2	z = -0.03	0.976
	MPS 1 and C-only MPS 1	t = -4.278	<0.0005
	MPS 2 and C-only MPS 2	t = -4.398	<0.0005
Between-operator	PV MPR 1 and OH MPR 1	t = 1.078	0.293
	PV MPR 2 and OH MPR 2	t = 0.427	0.673
	PV Lutein 1 and OH Lutein 1	t = 1.279	0.214
	PV Lutein 2 and OH Lutein 2	z = -0.593	0.553
	PV Zeaxanthin 1 and OH Zeaxanthin 1	z = -0.274	0.784
	PV Zeaxanthin 2 and OH Zeaxanthin 2	t = 0.078	0.938
All	MPR: PV1/PV2/OH1/OH2	F = 0.373	0.774
	Lutein: PV1/PV2/OH1/OH2	χ^2 = 2.528	0.470
	Zeaxanthin: PV1/PV2/OH1/OH2	F = 0.372	0.774
Between-instrument	MPS 1 and PV MPR 1	t = -8.927	<0.0005
	MPS 1 and OH MPR 1	t = -7.941	<0.0005
	MPS 2 and PV MPR 2	t = -7.399	<0.0005
	MPS 2 and OH MPR 2	t = -6.637	<0.0005

Table A9.1 Mean MPOD comparisons (n=23).

't' refers to a paired-samples t-test, which was used where the differences between each data set were normally distributed. Otherwise, the Wilcoxon signed rank test ('z') was used. 'F' refers to a one-way repeated measures ANOVA, which was used where each data set was normally distributed. Otherwise, Friedman's ANOVA ('χ^2') was used. (For the one-way repeated measures ANOVAs, the assumption of sphericity was not met according to Mauchly's test, so with the sample size in mind [n=23], multivariate test statistics were used for interpretation.)

	L/Z repeatability		L/Z reproducibility			
	PV1-PV2	OH1-OH2	PV1-OH1	PV2-OH2	PV1-OH2	PV2-OH1
Lutein original	0.413	0.278	0.264	0.194	0.403	0.345
Lutein after removal of two subjects' data	0.244	0.222	0.201	0.201	0.261	0.297
Zeaxanthin original	0.148	0.164	0.094	0.083	0.154	0.147
Zeaxanthin after removal of two subjects' data	0.078	0.105	0.069	0.087	0.077	0.088

Table A9.2 Coefficients of repeatability/reproducibility for L and Z on the MPR (n=23).

Number of subjects	Coefficient of repeatability	Reference
6	0.11	Koh et al. 2004
6	0.12	Tang et al. 2004
8	0.08	Hammond and Caruso-Avery 2000
10	0.06	Hammond and Fuld 1992
11	0.09	Van der Veen et al. 2009a
13	0.14	Hammond et al. 1997a
17	0.22	Liew et al. 2005
20	0.2	Hammond et al. 1995
20	0.18	de Kinkelder et al. 2011
23	0.32	de Kinkelder et al. 2011
32	0.16	Hammond et al. 1997c
37	0.18	Hammond et al. 1998
38	0.52	Bartlett et al. 2010a
40	0.31	Bartlett et al. 2010c
40	0.31	Gallaher et al. 2007
48	0.19	Snodderly et al. 2004

Table A9.3 A summary of inter-session CR data from HFP studies (derivation for figure 7.9, plus references).

KALE QUESTIONNAIRE

1. Had you eaten kale before beginning this study, and if so, how often?

2. In general, over how many meals did you spread the bag of kale during a week?

3. Did you eat the kale as a replacement for one or more of your usual vegetables, or did you eat it in addition to these? If yes, what vegetable(s) did you eat less of?

4. How did you cook your kale, e.g., was it boiled, stir-fried, steamed, microwaved? Please record all methods used, along with an average frequency of each.

5. When cooking the kale, was any kind of fat added to it, and if so, what kind?

6. Please indicate the most common dishes with which you ate the kale, e.g., in a stir-fry, with pasta/meat casserole/risotto, as part of a roast dinner, in soup, etc.

7. Did you eat the kale stalks in each bag? (Please highlight as appropriate)
   ☐ All/nearly all   ☐ A lot   ☐ Some   ☐ A few   ☐ Virtually none

8. Do you think you will continue to eat kale? If yes, how often do you think you will eat it (on average), and does this equate to more, less, or the same as you have been eating over the course of the study?

Thank you for your time. If you have any further comments, please feel free to write them below.

O.Howells
15/05/11

Appendix 11: Additional analysis for chapter 8

See chapter 8 for further explanation, if required.

	Comparison	Statistic (t) value	Significance (p) value
MPS MPOD	Visit 2 vs 3	0.459	0.662
	Visit 2 vs 4	1.478	0.190
	Visit 2 vs 5	0.109	0.917
	Visit 2 vs 6	1.136	0.299
	Visit 2 vs 7	2.039	0.088
Centre-only MPS MPOD	Visit 2 vs 3	-0.735	0.490
	Visit 2 vs 4	0.203	0.846
	Visit 2 vs 5	-0.087	0.933
	Visit 2 vs 6	-0.183	0.861
	Visit 2 vs 7	1.457	0.195
MPR MPOD	Visit 2 vs 3	0.201	0.851
	Visit 2 vs 4	1.834	0.141
	Visit 2 vs 5	0.793	0.472
	Visit 2 vs 6	1.787	0.149
	Visit 2 vs 7	-2.154	0.098
Lutein (MPR)	Visit 2 vs 3	0.637	0.559
	Visit 2 vs 4	1.624	0.180
	Visit 2 vs 5	0.785	0.476
	Visit 2 vs 6	2.062	0.108
	Visit 2 vs 7	0.227	0.831
Zeaxanthin (MPR)	Visit 2 vs 3	-2.156	0.097
	Visit 2 vs 4	-1.084	0.339
	Visit 2 vs 5	0.431	0.689
	Visit 2 vs 6	-1.122	0.325
	Visit 2 vs 7	-1.204	0.295

Table A11.1 Mean MPOD comparisons (n=5/6) from baseline (visit 2) through to 16 weeks of kale intake (visits 3 to 6), and four weeks post kale intake (visit 7), assessed by paired-samples t-tests (the differences between each data set were normally distributed).