CHAPTER 3 - MATERIALS AND METHODS

The project was designed in three phases linked to the desired objectives, namely:

Phase 1 - EMC production

Phase 2 - Chemical analyses

Phase 3 - Sensory analyses

The experimental work was carried out at a company called FlavourCraft in New Germany, KwaZulu Natal, South Africa. FlavourCraft produces flavours and foods including top notes, soups, maize snack dustings and soya minces. Research and development of their products is carried out in their laboratories. The company is locally owned and has been in business since 1993. FlavourCraft commissioned this study due to the high import cost of EMC, which they use as an ingredient in their products.

3.1 PHASE 1 - EMC PRODUCTION

The key parameters in the production of an EMC are the type of enzyme, the substrate and the production method.

3.1.1 Enzymes and other additives

3.1.1.1 *Enzyme Selection*

As discussed, EMC is made with a lipase, protease or a combination of the two. A few companies such as Novo Nordisk, Christian Hansen and BioCatalysts produce EMC enzymes but FlavourCraft has a relationship with BioCatalysts so their enzymes were used. FlavourCraft specified that the enzymes should have kosher status so that the final EMC could obtain kosher status if so required, to make the product acceptable to the largest market possible. BioCatalysts only produce one kosher lipase (Lipomod 187) and one kosher protease (Promod 215) both of which are typically used for Cheddar EMC (CEMC) production (BioCatalysts, 1996). Their specific characteristics are shown in Table 6.

Since enzymes have varying activities, the BioCatalysts suggested dosages listed in Table 6 were used for the experimental work. Cheddar cheese curd is approximately pH 5.5 (Rosenthal, 1991), so

Table 6 Parameters for a lipase and protease which typically are used to produce EMCs (BioCatalysts, 1996)

Enzyme name	Lipomod 187	Promod 215
Enzyme type	Lipase	Protease
Typical activity	Esterase 11000 units/g (Tributyrin substrate)	Endo-proteinase (caseinase) 120 units/g
pH optimum	5.0 - 7.0	7.0
Temperature range for maximum activity	40 - 50°C	45 - 55°C
Maximum temperature for long incubation (≥16 h)	45°C	45°C
Enzyme source	Fungal	*Aspergillus* sp.
Suggested dosage	0.1% (w/w) curd solids	0.1% (w/w) curd solids

the pH was not adjusted since it is within the optimum pH range for the lipase and near the optimum pH for the protease. An incubation temperature of 45°C was used since it is the maximum suitable for long incubations.

3.1.1.2 *Other additives*

Many additives other than enzymes have been used in the production of EMC and as can be seen from the Literature Review the most often mentioned additive is GSH. Researchers have reported that the addition of GSH greatly enhances flavour if used at a level of 0.01% (w/w) curd (Kristoffersen, 1967; Sutherland, 1991) so GSH was added at that level.

The other additive commonly mentioned in commercial preparations of EMC is an anti-microbial agent such as potassium sorbate, which is needed because of the high digestion temperatures which also would promote microbial growth (Cliffe & Law, 1990). South African food law (Act 965 of 1977, page 907) limits addition of potassium sorbate to 1 g/kg (0.1% w/w) so this rate was used to increase the safety of the product for the sensory evaluation in Phase 3.

3.1.2 Substrate selection

As discussed, the starting substrate for EMC can be milk, fresh cheese curd or young cheese. BioCatalysts (1996) recommended the use of fresh curd as the substrate for EMC production and so did Kristoffersen *et al*. (1967), the inventors of EMC.

The curd was sourced from a small-scale cheese farm that produces curd using non-animal rennets so that the curd could be certified as kosher if so required. The farm was run by Francis and Alan Webster and is in Weenen in the KwaZulu Natal Midlands in South Africa. The farm details are as follows:

Name: Foundation Jersey Farm

Telephone: (03635) 41714

Postal Address: P.O. Box 219, Weenen, 3325, South Africa.

3.1.2.1 *Curd production*

The EMC was made using unpasteurised, full cream Jersey milk curd sourced from a small-scale cheese farm in KwaZulu Natal, South Africa. The milk was added to a 250 l tank during milking and the starter (type O - R704 ex. Christian Hansen, Hørsholm, Denmark) added to the tank shortly after the first milk entered. Once the starter had been in the tank for 1 h with constant stirring the rennet (Chymogen 900 ex. Christian Hansen, Hørsholm, Denmark) was added at the recommended dose. After one hour the curd was cut and stirred while the temperature was increased to 37°C. Once the titration reached 0.15% titratable acidity, the whey was drained and the curds stacked. The curd was left until the runoff whey reached 0.7% titratable acidity. The curd was milled into approximately 1 cm^3 cubes and approximately 2% salt by weight was mixed into it by hand. The curd was left overnight at room temperature to complete the lactic acid production as recommended by BioCatalysts (1996) before storage in a freezer (Plate 1).

3.1.3 Production method

The EMC production method of West & Pawlett (1996) was used. This method is based on the original method of Kristoffersen *et al.* (1967).

3.1.3.1 *Incubation method*

Heat-sealed plastic bags were used for the EMC incubation (Takafuji, 1993; Horwood *et al.*, 1994) since this would reduce problems with contamination during sampling and would also simplify incubation and heat treatment. Each batch of curd slurry was divided up with approximately 45 g in four bags for the 0, 4, 8 and 12 h samples and the remainder into a fifth bag for the 16 h sample and sensory testing. The division of the batch into separate pouches allowed sampling at 4 h intervals

without contaminating the rest of the batch. The 45 g of curd slurry was calculated as being sufficient for analytical testing. The main disadvantage was that the batch could not be stirred continuously but the flexibility of the bags allowed them to be agitated at frequent intervals.

Initially, sterile stomacher bags were used for incubation but after incubation and subsequent heat treatment it was discovered that the bags were permeable as evidenced by the fact that they smelled very strongly of butyric acid and had a greasy feel. A second attempt with laminated foil bags, used for packaging products such as potato crisps, was successful. They are "vapour-tight" at ambient temperature, whereas stomacher bags are not. However, they were not sterile and since they cannot withstand temperatures greater than 100°C they cannot be autoclaved. To reduce contamination, the bags were heat-treated empty at 80°C for 2 h.

3.1.3.2 *Enzyme incubation*

The curd was turned into a smooth paste by mixing 150 g sterilised 5% saline solution with 400 g curd in a liquidiser to get a total moisture content of 45%, which was within the recommended moisture content limits of 40-55% (BioCatalysts, 1996). The total batch size of 550 g was the capacity limit of the liquidiser and provided sufficient material for subsequent chemical and sensory testing. The liquidising was done at 45°C since at lower temperatures the curd was too firm to liquidise. The slurry was heat-treated in a sealed bag for 10 min at 80°C and allowed to cool to below 50°C (to reduce enzyme denaturation) before mixing the enzymes and other additives into the curd slurry in a liquidiser (Plate 2). It was crucial that the slurry was liquidised immediately after the heat treatment to ensure a slurry with a small particle size since the high temperatures caused the macerated curd to amalgamate into a solid mass. The solid mass of curd was also easier to liquidise at elevated temperatures. The additives were 0.01% GSH (w/w), 0.1% potassium sorbate (w/w) and 0.1% enzymes (w/w), all added by weight of dry curd solids. Nitrogen was bubbled into the curd slurry for 5 min while liquidising since Horwood *et al.* (1994) observed that dissolved and headspace oxygen increased off-flavours during EMC incubation.

The samples were incubated at 45°C for 16 h with sampling every 4 h including zero time (Plate 3). Zero time was taken as when the slurry was introduced into the incubator. The finished product and 4 h samples were heat-treated at 80°C for 30 min as soon as they were taken from the incubator. This

heat treatment was done to denature the added enzymes and therefore halt the proteolysis and/or lipolysis.

3.1.3.3 *Microbiological safety*

During the initial experimentation the EMC (after 16 h incubation and subsequent heat treatment) was tested for common pathogens and a pathogen indicator, since the EMC would need to be of a good microbiological standard for human consumption. Swift microbiological laboratory, Durban, South Africa (part of CSIR Foodtek) carried out the testing. A total plate count was done as well as testing for the following pathogens: Coliforms, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, and salmonella and *Escherichia coli*, which is an indicator of faecal contamination. All the tests were carried out using conventional plate count methods, except for salmonella where the Malthus method was used.

3.2 PHASE 2 - CHEMICAL ANALYSES

3.2.1 Measurement of FAN to assess degree of proteolysis

A useful way to measure the progress of flavour formation in Cheddar cheese is to measure the breakdown products of the proteins and lipids, since they and their reaction products are the main contributors to flavour formation. Protein breakdown is normally measured using FAN and the lipid breakdown is measured using FFA (Fox & Wallace, 1997).

The FAN was measured using a combination of methods by Jarrett *et al.* (1982) and the European Brewery Convention (1975). There was an error in Jarrett *et al.* (1982) where the concentration of sulphuric acid should be 3.95 M and not 16.8 M (Aston, Durward & Dulley, 1983a).

The EMC was blended into a sodium citrate solution (emulsifier) and then the solids were removed by centrifuging. The supernatant was then acidified with sulphuric acid and phosphotungstic acid (PTA) which caused the soluble proteins to precipitate leaving only the single amino acids and peptides smaller than 600 Da in solution. The dibasic amino acids and proline are also precipitated out of solution. The solution was filtered and the remaining PTA and sulphuric acid was neutralised with sodium hydroxide. An aliquot was reacted with trinitrobenzene sulphonic acid (TNBS) in a phosphate

buffer medium at a constant temperature. Since TNBS is a chromophoric agent it reacted with the amino acids, and any peptides remaining in solution, to form compounds that absorb light at 340 nm (Plate 4). Quartz cuvettes were used instead of glass since glass cuvettes start to absorb light near 325 nm.

The method was calibrated using a standard glycine-sodium citrate solution together with a pre-precipitated sulphuric acid-PTA mixture as specified by Jarrett *et al.* (1982). When sulphuric acid and PTA are mixed at the levels used in the method then PTA precipitates out of solution creating a solution saturated in PTA, which is filtered before use in calibrating the method using the spectrophotometer. This step is crucial since PTA also absorbs at 340 nm therefore the level of PTA must be the same in both the standards and samples so that only the different concentrations of the TNBS-amino acid complex are detected. The glycine was also dissolved in sodium citrate solution so that the levels of sodium citrate were the same in both the sample and standard.

3.2.1.1 *Spectrophotometer calibration*

The spectrophotometer was calibrated each time it was used for FAN analysis. The straight lines that were fitted to the calibration data (glycine concentration versus absorbance) had a mean r^2 of 0.990 across all the calibrations. The good curve fit confirmed that the spectrophotometer was precise.

3.2.2 Measurement of FFA to assess degree of lipolysis

The general analytical method for FFA such as that described by the American Association of Cereal Chemists (1983) (originally based on a method from the American Oil Chemists' Society) was used. The sample of EMC was titrated in hot neutral ethanol to an endpoint detected by phenolphthalein (Plate 5). The sodium hydroxide used in the method was standardised using potassium hydrogen phthalate as detailed by Skoog *et al.* (1988). All the free fatty acids are titrated by the base and the results are reported as if only a single FFA was in the sample. The American Association of Cereal Chemists method reports the FFA as '% FFA as oleic acid' but in cheese butyric acid is commonly used as the basis for FFA (e.g. Fox *et al.*, 1996). Therefore, the molecular weight of butyric acid was used in the formula and the results are reported as % FFA expressed as butyric acid.

3.2.3 Statistics

The means of the values for the three replicates were plotted against their incubation time. On the same plot the error bars were shown, which represent the standard error of the mean.

3.3 PHASE 3 - SENSORY ANALYSIS

The quantitative flavour profiling (QFP) technique (Stampanoni, 1993) was used for taste panel tasting. A variation of the technique described by Heymann (1995b) was used which allows the panellists to generate the descriptors but the panel leader may modify the list by combining, adding or deleting terms. This adjustment of terms was done to reduce the descriptor list to approximately 10 descriptors, which is the maximum that a panellist can evaluate at a single sitting (pers. comm. - Mrs Henrietta L. de Kock, Department of Food Science, University of Pretoria).

3.3.1 Base for taste testing

The EMC was taste-tested in an umami base/savoury soup based on a method supplied by Kilcawley (EMC PhD student, Dairy Products Research Centre, Fermoy, Ireland, 1998 - pers. comm.). One major change was the addition of food colouring to offset any visible colour differences between the different EMCs caused by different degrees of breakdown of the lipids and proteins since the fat is yellow and the protein is white. The savoury soup recipe is given in Table 7.

The soup powder (10 g) was blended with 200 ml water and then the EMC was homogenised into the soup at a 1.75% (w/v) level. The soup was heated to approximately 40°C before tasting. The level of 1.75% (w/v) was based on a simple pre-trial where the EMC was diluted using a serial dilution and tasted until an acceptable level was found. The combination of EMC and the savoury soup was called EMC soup.

3.3.2 Taste Testing

3.3.2.1 *Panellists*

The panellists chosen for taste testing were staff at FlavourCraft. Most of the panellists had been on sensory training courses and are involved in research and development work on products mostly of a

Table 7 Recipe of 'umami' soup for sensory analysis of EMC

Ingredient	Quantity	Supplier
Creamer	25 g	Bordens econo creamer
Quicksperse	15 g	Universal Starch
Salt	15 g	Cerebos
Mono-sodium glutamate	1.5 g	Ajinomoto
Yeast extract	1.5 g	Gistex
Citric acid	0.25 g	Savannah Fine Chemicals
Sunset yellow supra	0.03615 g	Pointings

savoury nature, such as cheese flavoured maize snack dustings, soya minces, instant sauces and soups. The details of the panellists appear in Table 8.

Table 8 Sex and age details of taste panellists

Initials	Sex	Age
BW	Female	22
BS	Male	26
DO	Female	21
LT	Female	22
MC	Female	27
NS	Female	21

3.3.2.2 *Developing descriptors*

During an initial sensory session, the panellists each provided a list of terms whereby they described the flavours of the different EMC soups including both a control (processed curd and soup) and blank (soup only). After comparing this to a lexicon developed by Heisserer & Chambers (1993) for cheese taste testing, it was decided to modify their lexicon slightly rather than developing a new lexicon, since this would allow this study to be compared with other works which may use a similar lexicon.

Through training and elimination the 30 terms originally identified were reduced to those listed in Table 9 as being the best terms to describe the EMC. Some generic terms such as 'cheesy' and 'salty' had to be excluded even though all panellists were able to taste them since the list of

Table 9 Final EMC sensory descriptors with their respective standards and dilutions (adapted from Heisserer & Chambers, 1993)

Flavour Category	Flavour Term	Reference standard	Reference standard dilution
Dairy	Buttery	†	†
Dairy	Cooked Milk	†	†
Dairy	Dairy Fat	†	†
Dairy	Dairy Sweet	†	†
Fatty-acid/animal	Butyric Acid	†	†
Fatty-acid/animal	Goaty	Hexanoic acid	1% (w/w) in propylene glycol
Fatty-acid/animal	Sweaty	Isovaleric acid	5% (w/w) in Vema 45‡
Fatty-acid/animal	Waxy	Undecanoic acid	10% (w/w) in propylene glycol
Other Aromatics	Fruity	Trans-2-hexanal	10% (w/w) in propylene glycol
Other Aromatics	Pineapple	Ethyl Hexanoate*	1% (w/w) in propylene glycol
Fungal	Mouldy	2-ethylfenchol*	0.1% (w/w) in propylene glycol
Fungal	Mushroom	1-octen-3-ol*	0.1% (w/w) in propylene glycol
Mouthfeelings	Sharp	Propionic acid	1% (w/w) in propylene glycol
Fundamental tastes	Bitter	†	†
Fundamental tastes	Sweet	†	†

*Reference standard altered from that of Heisserer & Chambers (1993)

†Due to panellists' experience, no standards were required

‡Vema 45 is a fat manufactured by Hudson and Knight that melts at 45°C

descriptors was too long for panellists to evaluate at a single sitting. Standards were only used for those terms where panellists had difficulty identifying the descriptor. The standards for a few descriptors were changed due to lack of availability and these were done on the advice of a flavourist at FlavourCraft. All the terms and standards appear in Table 9 and where the standard has been changed the chemical is marked with an asterisk. The pure chemicals are too overpowering to be smelled in pure form so they were diluted before their use as a reference standard.

3.3.2.3 *Sensory sheet*

The copy of the final marking sheet used in the sensory evaluation appears in Figure 3. The use of non-numerical scales, recommended by Stone & Sidel (1985) (according to Stampanoni, 1993), was used. Random 5 digit numbers were used for each sample which were changed after each batch of taste testing and also the samples were taste tested in random order at each sitting. The layout for sensory evaluation can be seen in Plate 6.

Name:
Sample No.:

Dairy

Buttery	Low	———	High
Cooked Milk	Low	———	High
Dairy Fat	Low	———	High
Diary Sweet	Low	———	High

Fatty-acid/animal

Butyric Acid	Low	———	High
Goaty	Low	———	High
Sweaty	Low	———	High
Waxy	Low	———	High

Other Aromatics

Fruity	Low	———	High
Pineapple	Low	———	High

Fungal

Mouldy	Low	———	High
Mushroom	Low	———	High

Mouthfeelings

Sharp	Low	———	High

Fundamental tastes

Bitter	Low	———	High
Sweet	Low	———	High

Figure 3 The panellist sensory marking sheet

3.3.3 Calculations and statistics

A large quantity of data points was collected. The sensory profiles were measured in millimetres from the marking sheets (Figure 3) and converted to a dimensionless number by dividing by the total length of the line, which was 186 mm. Each data point had the following format:

Samples:	Control and EMCs made with protease only, lipase only, lipase and protease, 1:1 blend of lipase only and protease only and soup.
Panellist:	BW, BS, DO, LT, MC or NS
Run:	Run 1, Run 2 or Run 3.
Replicate:	Replicate 1, Replicate 2 or Replicate 3
Descriptor:	Buttery, Cooked Milk, Dairy Fat, Dairy Sweet, Butyric Acid, Goaty, Sweaty, Waxy, Fruity, Pineapple, Mouldy, Mushroom, Sharp, Bitter or Sweet.

Total data points = No. Samples x No. Tasters x No. Runs x No. Replicates x No. Descriptors

= 6 x 6 x 3 x 3 x 15

= 4860 points

3.3.3.1 *Panellist consistency*

The panellist's consistency was tested i.e. whether their three tasting replicates were similar. The standard error was calculated for each panellist for each sample and descriptor. So if for example BW taste tested three replicates of Run 1 of the lipase only sample for the Buttery descriptor and got scores of 0.156, 0.081 and 0.124 then the standard error would be 0.022. If all these standard errors were averaged for each panellist then they would give an indication of panellist consistency.

3.3.3.2 *Flavour wheel of flavour descriptor*

The dimensionless means of the descriptors were sorted according to the standard deviation of the means for each descriptor to aid the visualisation of the data. The data were then plotted clockwise on a flavour wheel so that the descriptor with the least score variation appears at the 12 o' clock position with increasing score variability through the revolution.

3.3.3.3 *Plot of descriptor error bars*

The mean dimensionless scores for each descriptor and sample were plotted together with standard error bars.

3.3.3.4 *Principal Components Analysis*

Principal components analysis (PCA) was carried out on the data using XLSTAT v4.3 which is an add-in for Excel 97 for Windows. The software used the standard method of calculating the correlation coefficients and from these the independent factors. From this the two eigenvectors that account for the maximum variance were plotted against each other. Two plots were created, one each for the samples and the descriptors.

3.4 COLOUR PLATES OF EXPERIMENTAL WORK

Plate 1 Frozen curd

Plate 2 Curd slurry in liquidiser together with enzymes and other additives

Plate 3 Batches of EMC in incubator

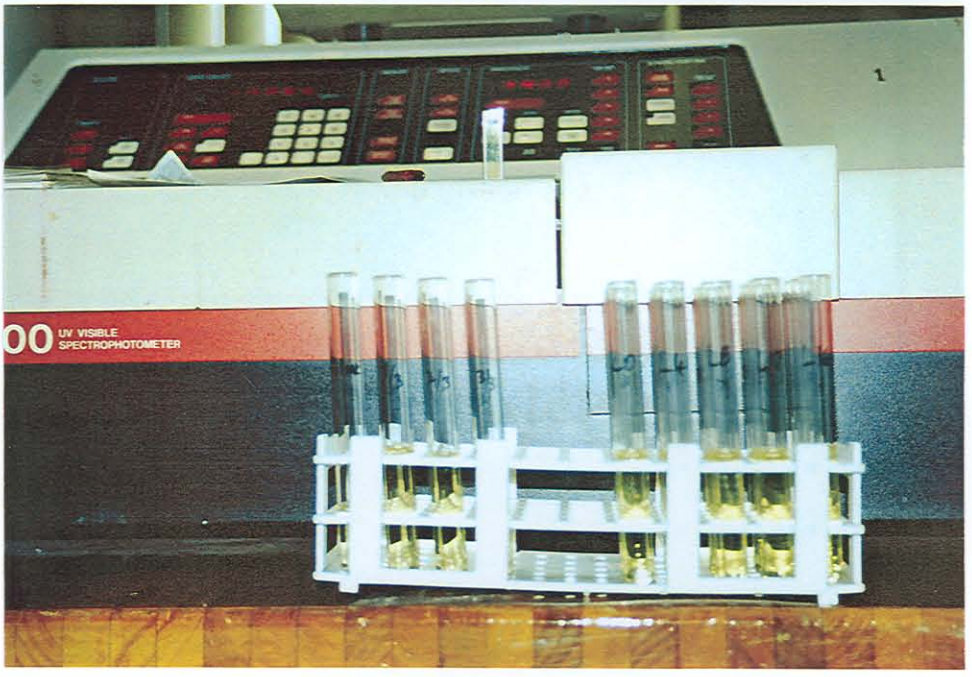

Plate 4 TNBS-PTA-FAN solutions and spectrophotometer

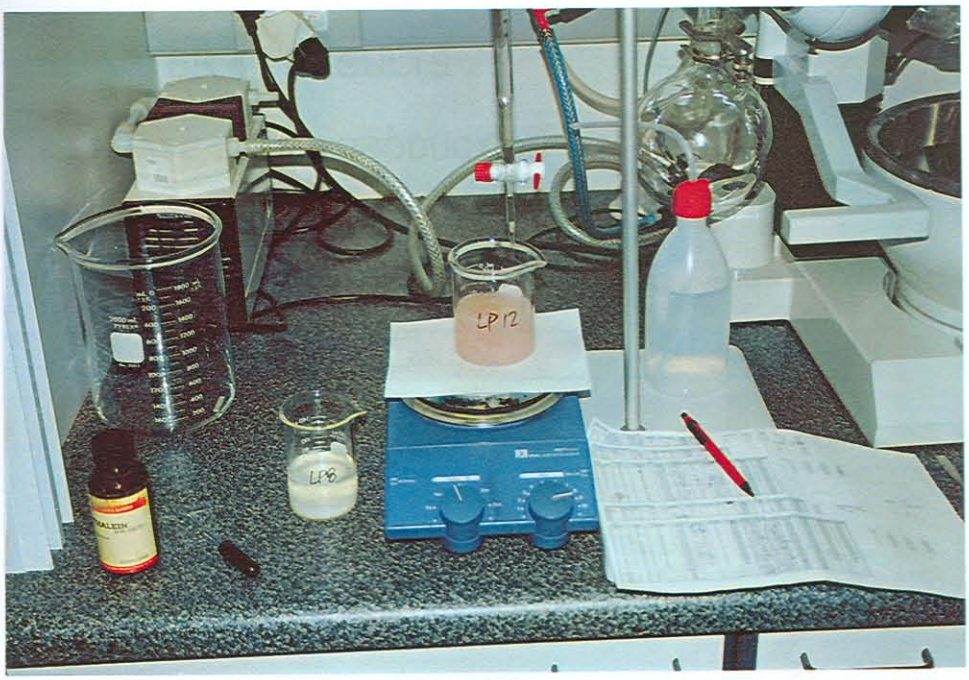

Plate 5 FFA titrations

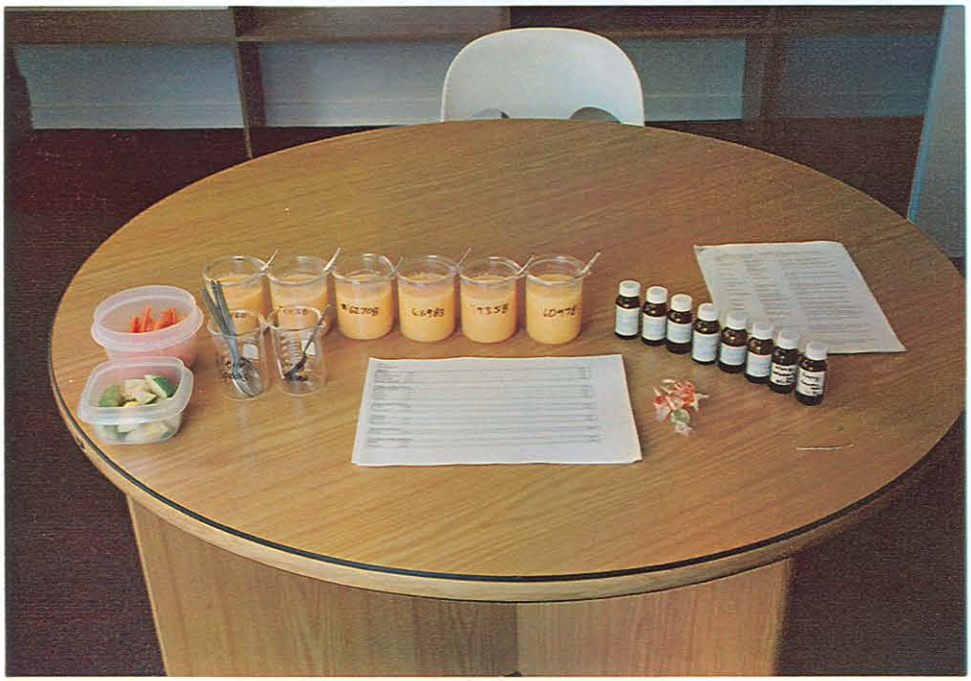

Plate 6 Taste testing samples, carrot, apple and sensory reference standards