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A near-infrared method for the assay of cineole in eucalyptus oil as an alternative to the official BP method

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Abstract

Eucalyptus oil of British Pharmacopoeia (BP) and European Pharmacopoeia standard must contain not less than 70.0 % w/w 1,8-cineole (eucalyptol). The official assay is a freezing-point method which involves the addition of *o*-cresol to the eucalyptus oil, whereupon the *o*-cresol and the 1,8-cineole form a solid complex. The assay has several disadvantages and we aim to show that near-infrared (NIR) spectroscopy is an attractive alternative to this method, in that it is simple to use, requires no sample preparation and is potentially as accurate as the traditional method.

Thirty different eucalyptus oil samples were scanned on the FOSS NIRSystems 6500 Rapid Content Sampler using a reflectance vessel as sample presentation method. The cineole content of each sample was determined by the BP method and these reference data were used to construct two calibration equations for cineole content in the oils using Vision software. The mean accuracy for the NIR method differed by 1.01 % or less, and the mean bias by $\pm$0.33 % or less, compared with the BP method. Calculation of the 95 % confidence intervals for the slope and intercept of plots of NIR predicted values against BP method reference values showed that there was no evidence of fixed or relative systematic errors. Tests for short-term and intermediate repeatability were conducted. The standard deviation was 0.83 % w/w or less and the coefficient of variation was 1.11 % or less. The confidence intervals for both short-term and intermediate repeatability overlapped with that for the BP method, suggesting that there was no evidence for a difference in values obtained by the BP and NIR methods. The range of cineole contents used in the calibrations was extended by incorporating five samples of eucalyptus oil spiked with cineole, and five samples of two essential oils known to have a lower cineole content than eucalyptus oil, to give a range of 52.5 to 99.0 % w/w. The mean accuracy decreased to an error of 1.26 % or less and the bias to $\pm$0.50 % or less. Again, confidence intervals suggested there was no evidence for fixed or systematic errors in the NIR calibrations.

We propose that NIR spectroscopy could be used as an alternative method for the determination of cineole content in eucalyptus oils.

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Introduction

The supplement to the European Pharmacopoeia (1997, 1999) states that eucalyptus oil to the required standard is obtained by steam distillation and rectification from the fresh leaves and terminal branchlets of various species of eucalyptus oil rich in 1,8-cineole. It contains not less than 70.0 % w/w 1,8-cineole (eucalyptol). Eucalyptus oil is a colourless or pale yellow liquid with an aromatic and camphoraceous

odour and a pungent and camphoraceous taste, followed by a sensation of cold. These requirements, together with the European Pharmacopoeia (1997) assay for cineole, are reproduced in the British Pharmacopoeia (1999a, b). Eucalyptus oils purchased commercially from pharmacies are supplied as eucalyptus oil BP and are subsequently referred to as such in this paper. Accordingly, the cineole content assay is referred to as the BP method.

Eucalyptus trees belong to the family Myrtaceae and are native to Australia. In addition they are grown for commercial purposes in those areas of the world with a subtropical or Mediterranean climate. The stated content of volatile oil in the *Eucalyptus* genus varies, but is approximately 0.5 to 3.5% w/w, with the main constituent of the oil being cineole in quantities of approximately 55 to 95% w/w (Bisset & Wichtl 1994; Newall et al 1996). Although there are many species of *Eucalyptus*, only a small number are suitable for medicinal use. The chief requirement is a high cineole content and the absence of appreciable quantities of aldehydes and phellandrene, tests for which are described in monographs for eucalyptus oil (British Pharmacopoeia 1999a). Cineole is classified as a monoterpene, the terpenoids being widely distributed in nature and found in abundance in higher plants.

The BP method for the determination of cineole content in eucalyptus oil was adopted in 1934 from a freezing-point method (Tusting Cocking 1920). Melted *o*-cresol is added to the eucalyptus oil and the *o*-cresol forms an additional compound with the cineole present in the oil (referred to as cresineol). The freezing point is noted and the cineole content of the oil is obtained by reference to a table showing the freezing points of complexes of known cineole content. The accuracy of the procedure is reported to be approximately ± 3% w/w (Tusting Cocking 1920). However, there are several disadvantages associated with this method. The experimental procedure can be time consuming and has no internal standards. In addition, because *o*-cresol is hygroscopic, the presence of water may lower the apparent cineole content by as much as 5% w/w. The method is also suitable for the determination of cineole content of oils of cajuput, camphor, wild marjoram and other cineole-containing oils providing that it is present in amounts of 50% w/w or greater. Other methods, such as gas chromatography, are suitable for the determination of cineole in certain essential oils (Watson 1994); this method can quantify such substances to a high degree of accuracy, but is time consuming and cumbersome. Although there is a general monograph for the use of near-infrared (NIR) spectroscopy in both the British Pharmacopoeia (Appendix IIA, 1999) and the European Pharmacopoeia (method 2.2.40, 1997), as yet there is no monograph for the use of NIR spectroscopy for the quantitative determination of a constituent in a pharmaceutical material. We propose that NIR spectroscopy could be a suitable alternative to the BP method, in that it is simple to use, requires no sample preparation, is non-destructive, rapid and is comparable in accuracy to the traditional method.

Materials and Methods

Materials

Thirty eucalyptus oils of different brands and batch numbers were obtained from pharmacies. Twenty-one of these oils were of BP standard and nine were pure essential oils intended for use in aromatherapy. *o*-Cresol was obtained from Lancaster (Morecambe, Lancs, UK) and was stated to be greater than 98% w/w pure. Cineole of 99% w/w purity was obtained from Avocado Research Chemicals Ltd (Heysham, Lancs, UK).

BP method

The apparatus consisted of a test tube approximately 25 mm in diameter and 150 mm long, placed inside a test tube approximately 40 mm in diameter and 160 mm long. The inner tube was closed by a stopper which carried a thermometer approximately 175 mm long and graduated in intervals of 0.2°C, fixed so that the bulb was approximately 15 mm above the bottom of the tube. The stopper had a hole for the stem of a stirrer made from a glass rod or other suitable material and formed at one end into a loop of approximately 18 mm overall diameter at right angles to the rod. The inner tube with its jacket was supported centrally in a 1-L beaker containing a suitable supercooling liquid (either water or a saturated solution of sodium chloride) to within 20 mm of the top. A thermometer was supported in the cooling bath.

A sample of the oil (3.00 g), recently dried with anhydrous sodium sulfate, was weighed into a dry test tube and 2.10 g melted *o*-cresol was added (these quantities correspond with the molecular masses of cineole and cresol, respectively). The weighed masses were within ± 0.02 g of the stated amounts. On cooling the mixture, at the onset of crystallization there was a small rise in temperature. The highest temperature reached

during solidification (t_1°C) was noted. The mixture was re-melted on a water bath at a temperature that did not exceed t_1 by more than 5°C, and the tube placed in the apparatus, with the supercooling liquid in the beaker maintained at 5°C below t_1. At the onset of crystallization, or when the temperature had fallen to 3°C below t_1, the mixture was stirred continuously. The highest temperature at which the mixture crystallized (t_2°C) was noted. The procedure was repeated until the two highest values obtained for t_2 did not differ by more than 0.2°C. If supercooling occurred, crystallization was induced by adding a small crystal of the complex consisting of 3.00 g cineole and 2.10 g melted *o*-cresol. The cineole content of the eucalyptus oil sample was determined by referral to a table containing values of t_2 and their respective apparent % w/w cineole (British Pharmacopoeia 1999b), if necessary by interpolation.

Instrumentation and equipment

A FOSS NIRSystems (Silver Spring, MD) 6500 spectrophotometer with Rapid Content Sampler module was used. The data acquisition software was NSAS version 3.52 (FOSS NIRSystems, Silver Spring, MD). A reflectance vessel (FOSS NIRSystems) was used for presentation of the sample, in conjunction with a stainless steel cylindrical disc (manufactured by The School of Pharmacy, London, UK). Spectral data analysis was performed on FOSS NIRSystems Vision software version 2.11.

NIR measurements

The eucalyptus oils were scanned over the wavelength range 1100 to 2500 nm. The cineole content of all thirty samples was determined using the BP method described previously. Spectral data of all samples were obtained using the reflectance vessel as a means of sample presentation. The circular stainless steel disc was 3.7 mm in diameter and 9.0 mm thick, with a small ridge (1.0 mm in depth) around the rim of the disc. It also had three small grooves arranged symmetrically around the rim, allowing a thin layer of an oil sample to be sandwiched between the disc and the bottom of the reflectance vessel. Sufficient oil to cover the bottom of the vessel was added and the disc lowered into the glass vessel. The bottom of the vessel was optically clear and the stainless steel disc allowed transflectance measurements of the sample to be taken, the path length being 2 × 1 mm. Five spectra were obtained for each sample, and the vessel was rotated about the centre between spectra. Each spectrum was the mean of 32 scans. The vessel was cleaned and refilled with the sample and a further five spectra obtained. These ten spectra were then averaged on the NSAS software to obtain a single mean spectrum for each sample and the data transferred to Vision version 2.11 software. A Multiple Linear Regression (MLR) method in forward search mode (no pre-selected wavelengths) across the full wavelength range of 1100 to 2500 nm was used for construction of two calibration equations. There were several available mathematical pre-treatments of spectra, each of which could either be used alone or in combination. A number of mathematical pre-treatments of the spectral data set were investigated using the MLR method to select the most appropriate ones for use in the calibrations.

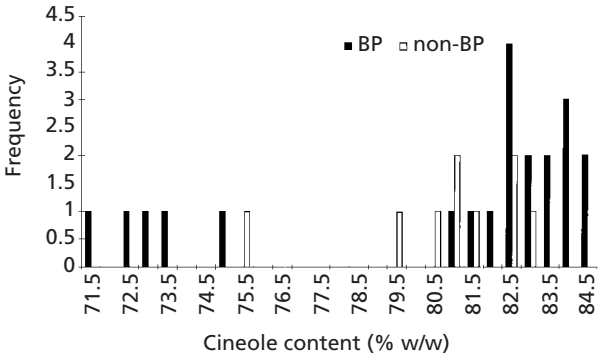

Figure 1 Frequency distribution of cineole content (% w/w) in eucalyptus oil samples (BP samples, n = 21; non-BP samples, n = 9).

Results and Discussion

BP method

Figure 1 shows the frequency distribution of cineole content of the 30 eucalyptus oil samples, as determined by the BP method. These cineole content values were then used as the reference values to develop the NIR method.

The traditional BP and EP freezing-point method for the determination of cineole in the commercial eucalyptus oil samples yielded values ranging from 71.5 to 85% w/w, with the values clustered into two distinct groups. This may be due to species difference, difference in geographical origin or time of harvesting. It was also noted that the non-BP samples used in this investigation were above 70% w/w. The range and distribution of

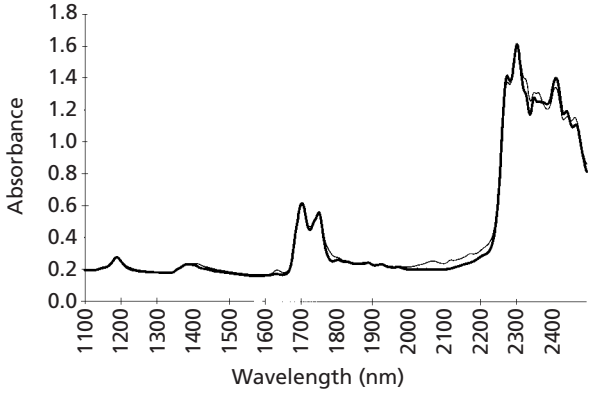

Figure 2 Mean NIR spectra for cineole (thick line) and a sample of eucalyptus oil BP (thin line).

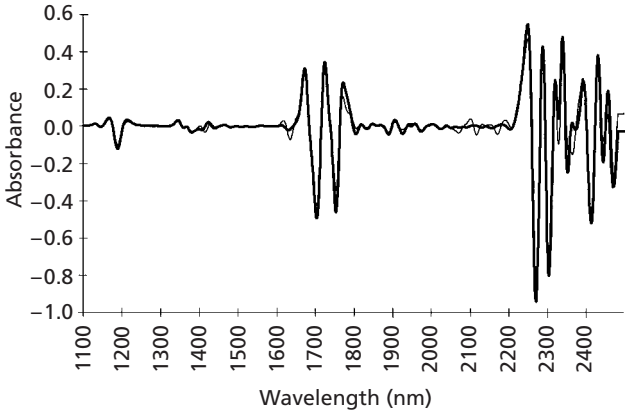

Figure 3 Standard Normal Variate (SNV)-corrected second derivative mean spectra of cineole (thick line) and a sample of eucalyptus oil BP (thin line).

cineole content used in the calibrations is therefore limited by the availability of commercial samples of eucalyptus oils.

NIR method development and calibration

Figure 2 shows the mean spectrum obtained for cineole and a single sample of eucalyptus oil. These two spectra are very similar because of the high cineole content of eucalyptus oil.

A manual sample selection method was applied. This involved manually assigning samples to the calibration set to obtain a representative set of cineole contents for both the calibration and internal validation sets. Samples from the lower, middle and end of the cineole content distribution (Figure 1) were assigned, with 20 samples in the calibration set and 10 samples in the internal validation set. This procedure was repeated, assigning different representative samples between the calibration and validation set to obtain a second set of calibration and validation samples. These two sets of calibration and validation samples were each used to obtain a NIR calibration equation. Two calibration equations were constructed to show that providing samples were chosen equally from the lower, middle and upper range of cineole concentrations, the samples within these could be randomly chosen.

All absorbance spectra were mathematically pre-treated with a Standard Normal Variate (SNV) correction (Barnes et al 1989), followed by a second derivative pre-treatment (segment size 10, gap size of 0 data points). Calculation of the second derivative increases peak resolution but maintains peaks at the same wavelength. SNV is a baseline correction method commonly used to normalize spectra when the effective path length varies among samples in a data set. The spectrum is mean-centred and then divided by its standard deviation. This method of pre-treatment is applied to individual spectra and their constituent data points. It is commonly used in combination with derivatization of spectra to minimize baseline effects and enhance the data (Halsey 1998). Figure 3 gives the SNV-corrected second derivative spectra for cineole and the same sample of eucalyptus oil as in Figure 2.

The MLR program selects the wavelength which has the highest correlation with the reference values assigned to each spectrum. It is then possible to incorporate additional wavelengths into the calibration equation where considered necessary to improve the accuracy of the calibration. The F value was considered (equation 1), which is a useful tool for indication of possible ''over fitting'' of the calibration to the reference set and for determining how many wavelengths should be used in the calibration equation. It also indicated the effectiveness of the wavelength chosen. A number greater than 100 is an indication of a good wavelength, and the larger the number the better. In addition, the correlation, % mean bias (equation 2), and % mean accuracy (equation 3), were calculated (Table 1). For the purposes of this investigation, the accuracy of a calibration was taken as how close the NIR values were to the BP reference values.

$$F = \frac{R^2(n-K-1)}{K(1-R^2)} \quad (1)$$

where n is the number of samples, K is the number of

Table 1 Summary of results for two calibration equations developed for the determination of cineole content in eucalyptus oils.

	Calibration 1		Calibration 2	
	Calibration set	Validation set	Calibration set	Validation set
Correlation coefficient (R^2)	0.955	0.899	0.936	0.960
Wavelength selected (nm)	1758		1756	
F value	382		262	
Mean bias (%)	0.01	−0.33	0.01	0.27
Mean accuracy (%)	0.85	1.01	0.88	0.86
95% Confidence interval (slope)	0.84 to 1.06	0.66 to 1.20	0.81 to 1.07	0.81 to 1.13
95% Confidence interval (intercept, % w/w)	−4.82 to 12.2	−16.3 to 27.5	−5.05 to 15.3	−9.91 to 15.5

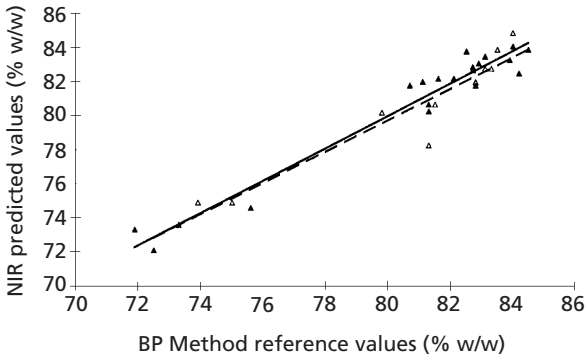

Figure 4 Plot of NIR predicted values against determined cineole content (% w/w) by the BP method, of eucalyptus oils in the calibration (solid line, n = 20) and validation (broken line, n = 10) sets of Calibration 1 for 20 eucalyptus oil samples ($R^2 = 0.956$ and 0.898 respectively).

wavelengths and R^2 is the (multiple) correlation coefficient.

$$\text{Mean bias (\%)} = \frac{\sum_{i=1}^{n}\left(\frac{(\text{NIR value} - \text{Reference value})}{\text{Reference value}}\right)}{n} \times 100 \quad (2)$$

where n was the number of samples in the calibration or validation set.

$$\text{Mean accuracy (\%)} = \frac{\sum_{i=1}^{n}\left(\frac{|(\text{NIR value} - \text{Reference value})|}{\text{Reference value}}\right)}{n} \times 100 \quad (3)$$

Correlation of the NIR spectra with the reference data (BP method) shows that these two methods are comparable for the estimation of the cineole content, with a mean accuracy difference of 1.01% or less for both calibration equations. The NIR method showed little evidence of bias (±0.33% or less). Thus, providing samples are chosen equally from the lower, middle and upper range of cineole concentrations, the samples within these can be randomly chosen.

A plot of predicted values vs reference values should ideally have an intercept of 0 and the slope as 1, if there is no fixed systematic error or relative systematic error in the calibration equation. Linear regression was applied and the 95% confidence intervals for the intercept, a (equation 4, where $\bar{x}$ is the mean of the NIR actual values), and the slope, b (equation 5) were calculated, where t is the Student's *t*-test with n−2 degrees of freedom (n = number of samples). RSD is the residual standard deviation (equation 6) and RSS is the residual sum of squares, where Y is the NIR predicted % w/w cineole content (equation 7), and y is the reference % w/w cineole content. S_{xx} is the sum of squares (equation 8), where x represents the NIR predicted values. Table 1 shows a summary of the results.

$$a \pm t \times RSD \times \sqrt{\frac{1}{n} + \frac{\bar{x}^2}{S_{xx}}} \quad (4)$$

$$b \pm t \times \frac{RSD}{\sqrt{S_{xx}}} \quad (5)$$

$$RSD = \sqrt{\frac{RSS}{n-2}} \quad (6)$$

$$RSS = \sum_{i=1}^{n} (Y_i - y_i)^2 \quad (7)$$

$$S_{xx} = \sum x^2 - \frac{(\sum x)^2}{n} \quad (8)$$

Table 2 Summary of results for determination or short-term precision (repeatability) and intermediate precision.

	BP assay	NIR method			
		Repeatability		Intermediate precision	
		Calibration 1	Calibration 2	Calibration 1	Calibration 2
Mean (% w/w)	75.3	75.4	74.8	74.5	74.8
Standard deviation (% w/w)	0.13	0.49	0.50	0.83	0.80
Coefficient of variation (%)	0.17	0.65	0.67	1.11	1.07
95% Confidence interval	75.2–75.4	74.8–76.0	74.3–75.3	73.6–75.4	74.0–75.6

The absorbance at only a single wavelength was used for construction of both calibration equations after consideration of the F values. All calculated confidence intervals included 1 for the slope and 0 for the intercept, suggesting that there was no evidence for a relative systematic error in either calibration equation.

The calibration equations take the form of that shown in equation 9:

$$C = K_0 + K_1 A_\lambda \tag{9}$$

where K_0 is the intercept, K_1 is the slope for a plot of absorbance (A) at wavelength of λ nm against reference values (concentration, C) for a single wavelength calibration.

The formulae for Calibrations 1 and 2 are given in equations 10 and 11, respectively.

$$C = 8.427 - 333.7A_{1758} \tag{10}$$

$$C = 4.053 - 252.8A_{1756} \tag{11}$$

Plots of the NIR predicted values using Calibration 1 over the range 71.5 to 85% w/w (normal range) for the calibration and validation sets are shown in Figure 4.

Precision of the BP and NIR methods

A sample of eucalyptus oil was assayed for cineole content using the BP method six times. Six spectra for a single sample of eucalyptus oil were obtained on a single day for determination of repeatability (short-term precision) and six spectra were obtained on six consecutive days for determination of intermediate precision. These were used to obtain NIR predicted cineole contents using the two calibration equations constructed previously. The results are summarized in Table 2, where the standard deviation, s (equation 12) and the coefficient of variation, CV (equation 13) are given, together with the mean ($\pm$95% confidence limit) BP reference method and NIR predicted values.

$$s = \sqrt{\frac{S_{xx}}{n-1}} \tag{12}$$

$$CV = \frac{s}{(\bar{x})} \times 100\% \tag{13}$$

Precision of the NIR method was good, both on a short-term and intermediate time scale. The confidence interval for the Calibrations 1 and 2 for determination of both short-term and intermediate repeatability overlapped with that for the BP method, suggesting that there was no evidence for a difference in values obtained by the BP and NIR methods. The reason for the high precision of the BP method is due to the measurement being repeated until the two highest values obtained differ by no more than 0.2°C.

Linearity and range

To extend the range of the NIR assay and to establish linearity at cineole concentrations in eucalyptus oil greater than those occurring naturally (> 85% w/w in the 30 samples examined), cineole (99% pure) was added to five oils. The BP reference method was carried out on these samples to determine the concentration of cineole present in the samples (89.8, 93.7, 95.6, 96.6 and 99.0% w/w). Calibrations were obtained with the original set of thirty eucalyptus oil samples, together with these samples. The same samples were retained in the calibration and validation sets, with the additional samples added to the calibration set.

As it was not practicable to obtain eucalyptus oil samples with cineole contents of less than 70% w/w, three samples of pure niaouli oil and two samples of

Table 3 Summary of results for the determination of linearity for the extension of the range of cineole content values (% w/w).

	Calibration 1		**Calibration 2**	
	Calibration set	Validation set	Calibration set	Validation set
Wavelength (nm)				
71.5–85.0	1758		1756	
71.5–99.0	1774		2462	
52.5–99.0	1772		1772	
F value				
71.5–85.0	382		262	
71.5–99.0	1147		1086	
52.5–99.0	2145		2138	
% Mean bias				
71.5–85.0	0.01	−0.33	0.01	0.27
71.5–99.0	0.01	−0.28	0.02	−0.22
52.5–99.0	0.49	−0.50	0.03	0.22
% Mean accuracy				
71.5–85.0	0.85	1.01	0.88	0.86
71.5–99.0	0.90	0.77	0.89	0.99
52.5–99.0	1.26	0.96	1.26	0.93
Slope				
71.5–85.0	0.84–1.06	0.66–1.20	0.81–1.07	0.81–1.13
71.5–99.0	0.92–1.04	0.80–1.20	0.92–1.04	0.65–1.01
52.5–99.0	0.95–1.03	0.80–1.20	0.95–1.03	0.77–1.13
Intercept				
71.5–85.0	−4.82–12.2	−16.3–27.3	−5.05–15.3	−9.91–15.5
71.5–99.0	−3.41–6.73	−16.9–15.9	−3.49–6.97	−1.16–27.2
52.5–99.0	−2.49–4.63	−16.8–16.2	−2.59–4.57	−9.78–18.6

Table 4 Equation of the best-fit line for the plots of predicted NIR values (y) against BP method reference values (x) for the three ranges of cineole content (% w/w).

	Line equation		R^2	
	Calibration set	Validation set	Calibration set	Validation set
Calibration 1				
71.5–85.0 % w/w	$y = 0.954x + 3.674$	$y = 0.928x + 5.503$	0.956	0.898
71.5–99.0 % w/w	$y = 0.980x + 1.657$	$y = 1.004x - 0.515$	0.981	0.946
52.5–99.0 % w/w	$y = 0.987x + 1.070$	$y = 0.999x - 0.330$	0.987	0.953
Calibration 2				
71.5–85.0 % w/w	$y = 0.937x + 5.137$	$y = 0.968x + 2.799$	0.936	0.964
71.5–99.0 % w/w	$y = 0.979x + 1.738$	$y = 0.834x + 13.04$	0.979	0.976
52.5–99.0 % w/w	$y = 0.988x + 0.990$	$y = 0.947x + 4.421$	0.987	0.960

cajuput oil, (both known to have a lower cineole content than eucalyptus oils) were assayed using the BP method and added to the calibration set together with the five samples to which cineole was added. The cineole content in the niaouli and cajuput oils was between 50 and 60 % w/w. The results are shown in Table 3. The wavelength shown for each sets of samples is that for which the highest correlation coefficient was obtained using the MLR forward search mode, although a single wavelength was used for each set of samples after study of the F values. To determine if the calibration range could be extended using samples to which cineole was added, with or without the addition of oils of lower cineole content, the 95 % confidence intervals for the slope and

intercept of the predicted vs reference values plot were included.

Addition of eucalyptus oils with cineole added and oils with a lower cineole content to the eucalyptus oil samples allowed extension of the range of cineole content values. Confidence intervals for the slope of the calibration and validation sets for both calibration equations include 1 and the confidence intervals for the intercept include 0. Thus there was no evidence for a relative systematic error in either calibration equation.

In addition the F values increased, which suggested that these new calibrations incorporating the extended ranges (71.5 to 99.0% w/w and 52.5 to 99.0% w/w) fitted the reference data better.

The value for the % mean accuracy (equation 3) in the calibration set for each equation for the extended range including samples with cineole added and oils of lower cineole content was greater (and therefore less accurate) than for the normal range of eucalyptus oils alone and with the addition of just the samples with cineole added. However, this is to be expected, as the matrix (chemical constituents) of the lower cineole content niaouli and cajuput oils will be different from that of eucalyptus oil. It may be postulated that inclusion in the data set of eucalyptus oils of lower cineole content would provide a more accurate calibration equation than that obtained with the use of two different pure essential oils. It would have been possible to construct a calibration equation for the determination of cineole content in eucalyptus oil using a single sample of eucalyptus oil with a known cineole content and adding different amounts of pure cineole to obtain an appropriate range of cineole contents. However, because the matrix of eucalyptus oil will vary between samples, although a more accurate calibration is likely to have been achieved, it would be sensitive to small changes in the matrix of other samples and would therefore lead to poor determination of cineole content in new samples. The equation of the lines of best-fit for the plots of NIR predicted values against the BP method reference values for both calibration equations, together with the R^2 values, are given in Table 4.

Although there are no limits of accuracy stated in the BP for this assay, experimental work detailed in the original literature stated a $\pm 3\%$ w/w error (Tusting Cocking 1920). The results obtained for accuracy, linearity, precision and repeatability of the NIR method compare favourably with that of the BP method. The NIR method also has advantages over the BP method, in that once the calibration equation has been developed and validated, it is simpler to carry out, no sample preparation is required and it is more rapid. In addition, no other chemicals are required for the NIR method and the amount of sample used is considerably less.

Conclusion

We propose that the use of NIR spectroscopy with a reflectance vessel as the sample presentation method allows the prediction of cineole content in a series of eucalyptus oil samples to within accuracies that compare highly favourably with the official BP method, and that NIR spectroscopy could be used as an alternative method for the determination of cineole content in eucalyptus oil.

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