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The quantification of citral in lemongrass and lemon oils by near-infrared spectroscopy

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Abstract

Previous work has demonstrated the capability of near-infrared (NIR) spectroscopy to determine the cineole content (not less than 70% w/w) of eucalyptus oil with an accuracy comparable with that of the British Pharmacopoeia (BP) assay method. The aim of the present study was to determine if the same method was capable of quantifying other chemical constituents at similar levels in essential oils and also to ascertain if NIR spectroscopy can accurately quantify compounds present at much lower levels in essential oils. Lemongrass oil contains citral at concentrations of approximately 65–85% w/w, and lemon oil contains citral at a concentration of approximately 2–5% w/w. A total of 26 samples of pure lemongrass oil and 35 samples of pure lemon oil (both including samples that were ''spiked'' with citral to increase the calibration range) were scanned on the FOSS NIRSystems 6500 Rapid Content Sampler using a reflectance vessel as sample presentation method. The reference method for both types of oil was the BP monograph titration assay for the citral content of lemon oil and calibrations were constructed using these reference data. For the lemongrass oils, the mean accuracy was found to be 1.00% or less and the mean bias was 0.09% or less. For the lemon oils, the mean accuracy was found to be 4.28% or less and the mean bias was −0.71% or less. The NIR method developed was rapid, simple and non-destructive and may prove beneficial for the accurate determination of the citral content of lemongrass oils and for the approximate citral content of lemon oils.

Introduction

Near-infrared (NIR) spectroscopy has been used previously to develop a robust yet sensitive calibration for the determination of cineole (eucalyptol) content in eucalyptus oils, which is present at levels in excess of 70.0% w/w (Wilson et al 2001). The aim of the present study was to determine if NIR spectroscopy was capable of quantifying chemical constituents other than cineole in essential oils at similar concentrations. A further aim of this study was to ascertain if NIR spectroscopy could accurately quantify compounds present at much lower levels in essential oils using a simple means of data analysis. There are only a few reports concerning quantification of low-concentration essential oil analytes in the literature. The quantification of secondary metabolites in tea drugs and spice plants has been carried out (Schulz et al 1999a), as has the quantification of constituents present in the leaves and oil of peppermint (Schulz et al 1999b). Citrus oils have been studied both qualitatively and quantitatively (Steuer et al 2001; Schulz et al 2002), including the total aldehyde content of a number of citrus oils. Although the standard errors of calibration appear low, the small amount of constituent present also has to be taken into account for these results to be meaningful. In all these cases, more complicated techniques such as partial least squares and/or principal component analysis was carried out. The purpose of the work was to show that the use of more simple techniques such as multiple linear regression (MLR) for the construction of NIR calibrations can also be useful. Moreover, this serves to dispel the ''black box'' image often viewed by those not thoroughly acquainted with NIR spectroscopy.

Lemongrass oils and lemon oils were studied, both of which contain the carbonyl compound citral. Lemongrass oil contains citral at levels of approx. 65–85% w/w

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(Lawless 1999) and most lemon oil contains citral at a concentration of approximately 2–5% w/w (Tyler et al 1988). By using these two oils it can be determined if a single calibration could be constructed to accurately quantify citral at both high and low concentrations.

Lemongrass (*Cymbopogon citratus*) oil is obtained by steam distillation from finely chopped fresh and partially dried leaves. There are two main types of lemongrass oil, West Indian and East Indian, each giving different characteristics to the oil. It is thought to have antiseptic, deodorant and insecticidal properties and is also used in the cosmetic industry (Lawless 1999).

Lemon oil is produced around the Mediterranean, California and North America, Australia and parts of Africa. It is known to have antiseptic properties and is claimed to have medicinal benefits in the treatment of high blood pressure, dyspepsia and arthritis, although its main use is in the cosmetic and flavouring industries (Evans 1996). The quality of lemon oil in terms of citral content varies greatly depending on country or area of origin, time of harvesting and other factors. It is also known that the citral content of lemon oil varies greatly depending on the method used for extraction (Guenther 1952). Methods of production of lemon oil such as steam distillation will cause hydrolysis and oxidation of citral and thus considerably lower the quality of the oil (Samuelson 1999). The main constituent of lemon oil is limonene, a monoterpene hydrocarbon (approx. 90% w/w). Although some oils have a citral content as high as 13%, a high quality oil has an optimum range of 2–4% w/w citral. Citral content is an important factor in determining the purity of lemon oil and commercial purchasers of lemon oil will carry out a determination of citral content, along with a physical and chemical examination of the oil, as it is common to adulterate lemon oil with citral obtained from cheaper sources such as lemongrass oil (Tyler et al 1988).

Citral (3,7-dimethyl-2,6-octadienal) from natural sources is a mixture of two geometric isomers, geranial and neral, otherwise known as citral a and b, respectively (The Merck Index 1996). It is the citral a form which largely predominates in nature and is responsible for the odour and flavour of lemon and lemongrass oils (Guenther 1957).

The British Pharmacopoeia (2001) monograph (reproduced in the European Pharmacopoeia 1997) for lemon oil states that the oil must be obtained by suitable mechanical means, without the aid of heat, from the fresh peel of *Citrus limon* (L.) Burman fil. and contain not less than 2.2% w/w and not more than 4.5% w/w of carbonyl compounds calculated as citral. The assay described in this monograph for the determination of citral content was used as the reference method in this study and NIR calibrations constructed for the determination of citral in lemongrass and lemon oils.

The British Pharmacopoeia (BP) assay is a titration method in which hydrochloric acid is liberated upon the reaction of hydroxylamine hydrochloride with the aldehyde (citral) in the oil and then titrated against a standardized solution of ethanolic potassium hydroxide.

It must be noted that other aldehydes (such as citronellal) are known to be present in lemongrass oil and to a lesser extent in lemon oil. Thus, the BP assay for citral in lemon oil (and the reference method for both types of oil used in this study) is more correctly an assay for total aldehyde content rather than for citral content alone.

Development of an NIR calibration for the accurate determination of the citral content in lemongrass and lemon oils would result in a rapid, simple and non-destructive procedure that could be used to replace conventional methods.

Materials and Methods

Materials

Citral (a mixture of geranial and neral) was obtained from Avocado Research Chemicals Ltd, Heysham, Lancs, UK and was stated to contain more than 95% of the pure substance. Samples (n = 25) of pure lemon oil and pure lemongrass oil (n = 14) were obtained from retail outlets such as pharmacies and health food stores. To expand the range of concentration of initial citral content in the samples obtained, samples of lemon oil (n = 10) and lemongrass oil (n = 11) were "spiked" with citral (a few drops were added to the sample). Details for the preparation of all the reagents required for the assay are in the British Pharmacopoeia (2001; Appendix 1A: General Reagents).

BP method

Approximately 9.000 g of the lemon oil was accurately weighed and mixed with 20 mL absolute ethanol. Then, 10.0 mL of hydroxylamine hydrochloride solution and 0.4 mL bromophenol blue solution were added. The mixture was titrated slowly with 0.5 M alcoholic potassium hydroxide until the colour changed from yellow to olive-green. The titrated mixture was then allowed to stand for 5 min and titrated again, if necessary, until it changed colour from yellow to olive-green.

Each millilitre of 0.5 M alcoholic potassium hydroxide was equivalent to 76.1 mg of carbonyl compounds calculated as citral (molecular weight of citral is 152.2).

The lemongrass oils were assayed for citral content in the same way, but the mass of oil used and the volume of reagents used in the assay were modified accordingly, as the citral content was much greater.

Instrumentation and equipment

A FOSS NIRSystems 6500 spectrophotometer with a Rapid Content Sampler module was used. The data acquisition software was NSAS version 3.52 (FOSS NIRSystems, Silver Spring, USA). A reflectance vessel (FOSS NIRSystems) was employed 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 using FOSS NIRSystems Vision software version 2.11.

Near-infrared measurements

Spectral data of all oil samples were obtained using the reflectance vessel and stainless steel disc 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 allowing a thin layer of an oil sample to be sandwiched between the disc and the bottom of the reflectance vessel. It also had three small grooves arranged symmetrically around the rim, allowing easy removal of any air bubbles present. 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.

The lemon and lemongrass oils were scanned over the wavelength range of 1100–2500 nm. Three spectra were obtained for each sample (each spectrum was the average of 32 scans), the vessel being rotated about the centre. The 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.

In addition, a test for the repeatability of the NIR method (six separate NIR spectra obtained throughout a single day) and intermediate precision (a mean NIR spectrum obtained on six consecutive days) for one lemon oil sample and one lemongrass oil sample was carried out. Each reading was obtained using the NIR method described previously, each spectrum being the average of three separate readings.

Results and Discussion

BP method

For the lemongrass oil samples, the range of citral contents was 69.89–76.95% w/w. The addition of the spiked lemongrass oil samples increased the upper limit to 94.6% w/w. In addition to this, a "pure" sample of citral was added to the calibration (a twelfth spiked sample), which was found to contain 99% w/w citral by the BP method.

Although there is a BP monograph for lemon oil, no samples of lemon oils of BP standard were obtained. It was found that the lemon oil samples yielded values ranging from 2.24 to 3.70% w/w. The ten spiked lemon oil samples increased the citral content range to 15.75% w/w.

NIR method development and calibration

Figure 1 shows the mean spectrum obtained for citral and a single sample of each of lemon and lemongrass oil. The reference value (% w/w citral content) for each oil sample was assigned to its corresponding mean NIR spectrum. Samples from the lower, middle and end of the citral content distribution were assigned to either the calibration or validation set. Of the lemongrass oils samples, 18 were assigned to the calibration set and eight samples were assigned to the validation set. For the lemon oil samples, 25 were assigned to the calibration set and 10 to the validation

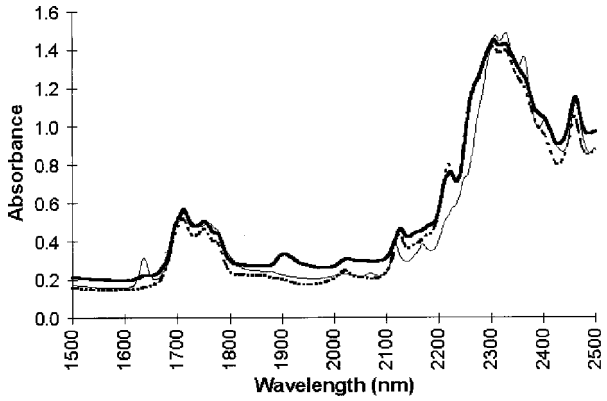

Figure 1 Mean near-infrared spectra of lemon oil (thin line), lemongrass oil (thick line) and citral (broken line) over the wavelength range 1500–2500 nm.

set. Those sample spectra in the calibration set were used to construct the NIR calibration equation and those sample spectra in the validation set were applied to the developed calibration equation to give predicted citral contents.

Sample spectra may be mathematically pre-treated to give improved NIR calibration equations. The most suitable mathematical pre-treatment of the spectra was found to be standard normal variate (SNV) corrected, 2nd derivative spectra (segment size 10, gap size of 0 data points) (Barnes et al 1989). Calculation of the 2nd derivative increased peak resolution but maintained peaks at the same wavelength. SNV is a baseline correction method used to normalize spectra. The spectrum was mean centred and then divided by its standard deviation. This method of pre-treatment was applied to individual mean spectra and their constituent data points. It was used in combination with derivatization of spectra in order to minimize baseline effects and enhance the data (Halsey 1998).

A MLR method across the full wavelength range of 1100–2500 nm was applied to the pre-treated spectra for the development of the calibrations. This MLR program selected the wavelength that had the highest correlation with the reference values assigned to each spectrum.

The quality of a developed NIR calibration equation can be assessed by several statistical factors. The correlation (R^2, the multiple coefficient of determination) of the NIR spectra with the reference data (BP method) allows for a direct estimation of the citral content. A correlation of 1 indicates that there is no difference between the NIR predicted value and the reference value for each sample. The standard error is an absolute value calculated from equation 1 and indicates the average difference between the NIR predicted and reference values for samples in the set. The lower the standard error, the better the calibration. The standard error can be calculated for both the calibration and validation sets, known as the standard error of calibration (SEC) and prediction (SEP) respectively. One or more wavelengths may be used to construct a NIR calibration equation. Addition of extra wavelengths may improve the accuracy of the calibration equation but may result in "over-fitting", whereby the calibration is too

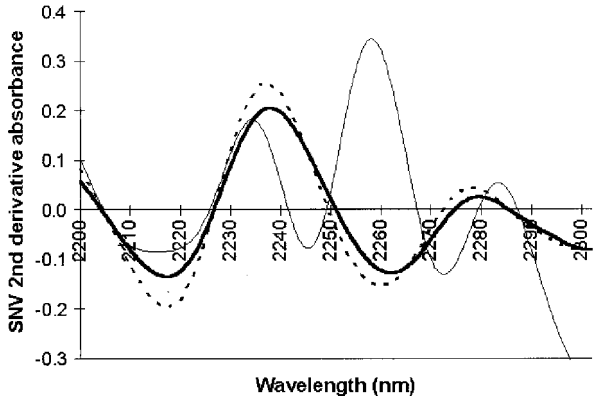

Figure 2 Standard normal variate (SNV) corrected 2nd derivative absorbance spectra of lemon oil (thin line), lemongrass oil (thick line) and citral (broken line) over the wavelength range 2200–2300 nm.

specific to the calibration set samples and the prediction of citral content of the samples in the validation set is less accurate. In this case the SEC would be lowered, but the SEP would be increased. The F value (equation 2) is also a useful tool for detection of possible over-fitting of the calibration to the reference set. In this case, addition of another wavelength may result in a lowered F value. In addition the F value indicates the effectiveness of the wavelength(s) chosen for the calibration. A number greater than 100 is an indication of a good choice of wavelength and the bigger the F value the better. In addition to the standard error, the percentage mean bias and percentage mean accuracy (equations 3 and 4) were also calculated for each calibration, both of which are errors relative to the citral content in sample sets. The accuracy of the calibration was taken as how close the predicted NIR values were to the reference values.

$$\text{Standard error (\% w/w)} = \sqrt{\frac{\equiv(\text{NIR}_{\text{value}} - \text{Reference}_{\text{value}})^2}{n - K - 1}} \quad (1)$$

where n is the number of samples, K is the number of wavelengths or factors. The NIR predicted and reference citral contents are for either the calibration set (SEC) or the validation set (SEP).

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

where n is the number of samples, K is the number of wavelengths and R^2 is the (multiple) correlation coefficient.

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

$$\text{Mean accuracy (\%)} = \frac{\sum_{i=1}^{n} \left\{ \frac{|(\text{NIR}_{\text{value}} - \text{Reference}_{\text{value}}|}{\text{Reference}_{\text{value}}} \right\}}{n} \times 100 \quad (4)$$

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

The lemongrass oil sample set was considered first, as it had a high citral content and a good calibration in terms of accuracy and precision was expected. A wavelength of 2212 nm (selected by the Vision software) was found to have absorbance readings that had the highest correlation with the reference data. This region of the spectrum in its SNV corrected 2nd derivative form is shown in Figure 2. From a visual inspection of this region of the spectrum it can be seen that 2212 nm is an acceptable wavelength to be incorporated into the equation, as it shows the increase in absorbance with an increase in citral content (the peak is negative because the absorbance peaks are reversed in the 2nd derivative). The selection of this particular wavelength is supported by the fact that the aldehydic C-H group (contained in citral) exhibits two characteristic combination bands near 2210 nm and 2250 nm (Whetsel 1968).

A second wavelength (selected by Vision software as providing the highest correlation to the reference data in combination with the first wavelength) was added to the equation, but after consideration of the SEP and the F value, it was not considered beneficial to the calibration. The results for this calibration are summarized in Table 1. The percentage error is a relative error calculated as for the percentage mean accuracy in equation 4, but for the single sample alone. The relative percentage error was less than 2.61 for all samples in the calibration and validation sets. A plot of the NIR predicted versus reference values for the calibration and validation sets is shown in Figure 3. The equation of the line was $y = 1.00x + 1.01$ for the calibration

Table 1 Summary of results for lemongrass oils and lemon oils for the determination of citral (total aldehyde) content.

	Lemongrass oils		Lemon oils	
	Calibration set	Validation set	Calibration set	Validation set
Correlation coefficient (R^2)	0.988	0.997	0.996	0.994
Wavelength(s) selected (nm)	2212		2212, 2258	
F value	1260		2872	
Standard error of calibration/prediction (% w/w)	1.16	0.48	0.20	0.12
Mean bias (%)	0.02	0.09	0.20	−0.71
Mean accuracy (%)	1.00	0.52	4.28	2.86

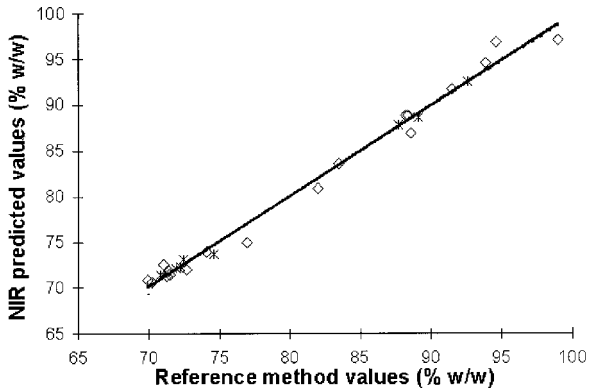

Figure 3 Plot of near-infrared (NIR) predicted values against total aldehyde (citral) content of lemongrass oils determined by the reference method, for the calibration set (◇, thick line, n = 18) and validation set (+, thin line, n = 8).

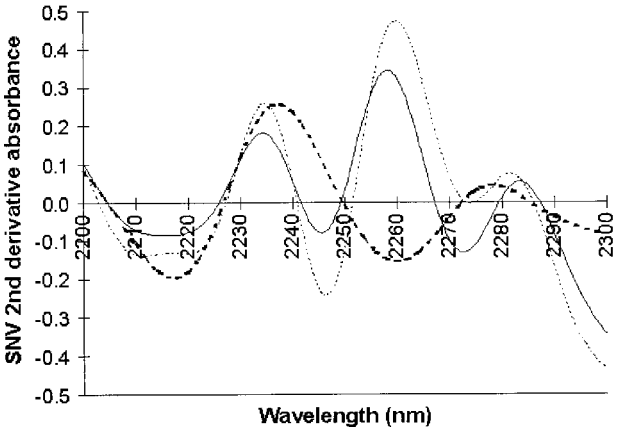

Figure 4 Standard normal variate (SNV) corrected 2nd derivative absorbance spectra of limonene (dotted line), lemon oil (continuous line) and citral (broken line) over the wavelength range 2200–2300 nm.

set samples and y = 0.98x + 1.59 for the validation set, which is in close agreement with y = x. The mean accuracy was 1.00% for the calibration set and 0.52% for the validation set. The errors for the validation set in a calibration produced from a much larger group of samples, such as would be constructed for a commercial purpose, would be expected to be roughly the same or larger than for the calibration set. The relatively small number of samples employed in the validation set for this calibration may account for the improved accuracy relative to the calibration set. The use of a partial least squares method in the construction of the calibration equation means that the bias is as close to zero as possible, but it is useful to compare the bias of the calibration set with the validation set. The mean bias was 0.02% for the calibration set and 0.09% for the validation set.

The lemon oil sample set was then considered. Use of the same MLR program allowing the Vision software to select the wavelength with the highest correlation to the reference data resulted in the selection of the absorbance data for the calibration at 2256 nm, with a mean accuracy of 9.65% and 9.01% for the calibration set and validation set, respectively (data not shown). Although it is known that there is a characteristic aldehyde band in this region of the NIR spectrum, inspection of the SNV corrected 2nd derivative spectra at this point (Figure 4) shows that the lemon oil has a positive peak at this value, whereas citral has a negative peak. This is owing to the fact that the aldehyde absorption band at this point is overlapped with a positive 2nd derivative peak due to a component other than citral. This is likely to be limonene, which is present at levels of roughly 90% m/m in lemon oils. Addition of a second wavelength of 2230 nm (selected by Vision software) improved the calibration to give a mean accuracy of 6.48% and 5.57% for the calibration and validation set, respectively (data not shown). It was not clear on inspection of the spectra at 2230 nm why this particular wavelength provides the best correlation in combination with the absorbance at 2256 nm and so a second approach to the construction of the calibration was therefore considered. If the lemon oil calibration was linear over a much greater range, extending to that of the citral content for the lemongrass oils, the wavelengths selected for both sample sets would be expected to be the same. For this reason, the wavelength selected for the lemongrass oils calibration (2212 nm) was used in the construction of the lemon oil calibration. A second wavelength (selected by the Vision software) was added to the calibration after consideration of the F values and the standard errors. This wavelength was found to be 2258 nm, similar to the 2256 nm wavelength selected originally. The use of the 2258 nm wavelength enabled the presence of limonene to be taken into account in the construction of the calibration equation, thus resulting in an improved calibration with greater quantification accuracy. The SNV 2nd derivative NIR spectra for limonene, lemon oil and citral over the wavelength range 2200–2300 nm are shown in Figure 4. The mean bias and accuracy for the lemon oil for the calibration set were 0.20% and 4.28%, respectively, and for the validation set were −0.71% and 2.86%, respectively. The summary of this calibration for the lemon oil samples is shown in Table 1. The relative error ranged from 0.36% to 10% or less for all but two of the calibration set samples, the remaining two being 13.8% and 15.8%. Re-titration of the oils, visual inspection of the NIR spectra and a brief look at principal components data for the set of lemon oils gave no sign that these two samples were "outliers" that could legitimately be removed from the calibration. Only one of the 10 samples in the validation set (8.56% relative error) was predicted with an accuracy poorer than 4.17. Figure 5 shows the NIR calibration results for the lemon oils in terms of NIR predicted versus reference values for the calibration and validation sets. The equation of the line was y = 1.00x + 0.02 and y = 1.03x − 0.13 for the calibration and validation sets, respectively, both of which are in close agreement with the ideal plot with the line equation y = x.

Comparison with the calibration for the lemongrass oils, also given in Table 1, shows that by taking the relative value of the citral content of the oils into account (given by

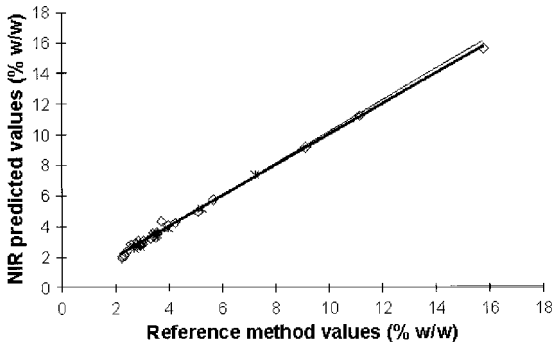

Figure 5 Plot of near-infrared (NIR) predicted values against total aldehyde (citral content) of lemon oils determined by the reference method, for the calibration set (◇, thick line, n = 25) and validation set (+, thin line, n = 10).

the percentage mean accuracy), the prediction error for the lemon oil equation (4.28) is considerably poorer than that developed for the lemongrass oils (1.00). The results for the lemon oil samples may be considered to be unacceptably high. The accuracy of prediction of this method may also be improved by increasing the number of lemon oils in the sample set to take into account other physical and chemical differences (apart from citral content) between the oils, resulting in a more robust calibration.

The addition of a second wavelength in the form of a denominator to that selected initially by the Vision software (i.e. a ratio of the two wavelengths) was considered as an alternative to summation of the two wavelengths for the construction of the MLR calibration equations. Calibrations constructed by partial least squares (using the full wavelength range and the selected portions of the spectrum) were also investigated. None of the calibrations created by these alternative methods for both sets of oils performed better in terms of percentage mean accuracy (data not shown).

Precision of the BP and NIR methods

A single sample of lemon oil and lemongrass oil was assayed for citral content six times using the BP method. Six spectra for a single sample of the oils 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. As for all NIR readings obtained in this study, each spectrum was the average of three spectral readings taken. These were used to obtain NIR predicted citral contents using the two calibration equations constructed previously. The results are summarized in Table 2 for both sets of oils, where the standard deviation, s (equation 5), and the coefficient of variation, CV (equation 7), are given, together with the mean (±95% confidence limit) BP reference method and NIR predicted values.

$$s = \sqrt{\frac{S_{xx}}{n-1}} \tag{5}$$

where S_{xx} is the sum of squares (calculated from equation 6).

$$S_{xx} = \equiv x^2 - \frac{(\equiv x)^2}{n} \tag{6}$$

where x represent the reference or NIR predicted values.

$$CV = \frac{s}{(\bar{x})} \times 100\% \tag{7}$$

where $\bar{x}$ is the mean of the reference or NIR actual values and s is the standard deviation (equation 5).

Precision of the NIR method was good, both on a short-term and intermediate time scale. The confidence interval for determination of both short-term and intermediate repeatability for both calibrations 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.

Conclusion

In conclusion, we propose that the use of NIR spectroscopy with a reflectance vessel as the sample presentation method allows the prediction of citral content in a series of lemongrass oil samples with a mean accuracy (relative difference) of 1% or less. The use of the BP assay as a reference

Table 2 Summary of results for determination of short-term precision (repeatability) and intermediate precision of the near-infrared (NIR) method for a single sample of lemongrass and lemon oils.

			NIR method			
	Reference method		Repeatability		Intermediate precision	
	Lemongrass oils	Lemon oils	Lemongrass oils	Lemon oils	Lemongrass oils	Lemon oils
Mean (% w/w)	75.55	4.29	75.77	4.25	75.11	4.32
Standard deviation (% w/w)	0.27	0.03	0.50	0.10	0.17	0.16
Coefficient of variation (%)	0.36	0.75	0.23	2.44	0.66	3.66
Confidence interval (% w/w)	75.26–75.84	4.26–4.33	74.92–75.30	4.14–4.36	75.24–76.30	4.15–4.48

For the short-term repeatability an average (of three) NIR spectrum was obtained six times throughout a single day and for the intermediate precision an average (of three) NIR spectrum was obtained on six consecutive days.

method in this study limits the accuracy of the NIR method, but they are comparable in both accuracy and precision. It may therefore prove to be a suitable method for the determination of citral content in such oils. 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.

The NIR method is less accurate in comparison with the BP titration method for the assay of lemon oil, although the two methods are comparable in precision. Being a simple and rapid technique, the NIR method could be used to give the approximate citral content. The percentage mean accuracy is decreased (and therefore improved) with an increase in concentration of the constituent of interest. It is suggested that the limit of quantification (percentage mean accuracy) for complex essential oils using this NIR method is approximately 10% w/w or above, but further work would have to be carried out to support this conclusion.

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