

Application of alpha-tocotrienol for detection of palm mid-fraction in dark chocolate formulation

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Abstract Following model studies, the detection of palm mid-fraction (PMF) added to cocoa butter (CB) in chocolate formulations was investigated. Different levels of PMF (0–25%, CB basis) were added to CB in chocolate. High performance liquid chromatography was then used to detect the presence of PMF in chocolate using α -tocotrienol as an indicator. The results, in line with the model studying indicated that increasing the amount of PMF added to CB resulted in a significant ($P < 0.05$) increase in the concentration of α -tocotrienol in chocolate; a linear plot ($R^2 = 0.9837$) was obtained with standard error of 1.986. A validation test was conducted to verify the equation obtained from the regression analysis. The high R^2 -value obtained indicated a good accuracy, reflecting a close relationship between experimental and theoretically predicted values. The applied indicator performed well beyond the level of the statutory limit of 5% PMF addition on a chocolate basis that verified the previously studied model.

Keywords Tocopherol · Tocotrienol · Cocoa butter · Palm mid-fraction · Dark chocolate

Introduction

Cocoa butter (CB) is the continuous phase in chocolate, in which other constituents are dispersed and is responsible for the physical behavior of chocolate. Brittleness at room temperature, quick and complete melting at body temperature is especially unique to CB [1]. A shortage in CB supplies, poor quality of individual harvests, economic advantages and some technological benefits have prompted the development of specialty fats such as CB equivalent (CBE) fully compatible with the physical and sensory properties of CB [2, 3]. The substitution of CB with vegetable fats other than CB to an extent of up to 5% of the total weight in the end product is authorized by the European Commission, under Directive 73/241/EEC [4]. The European Commission legislation also permits the use of mixtures containing only illipe, palm mid-fraction (PMF), sal, shea, kokum and palm kernel fats. These fats are bleached, deodorized, fractionated and formulated into mixtures that are designed to be compatible with CB. The product label has to cover a correct, neutral and objective indication of the presence of such substances, and must not mislead the consumer. On the other hand, the Directive does not preclude the labeling of chocolate products to indicate that CBEs have not been added (so called ‘negative labeling’). Unfortunately, the Directive does not cover aspects regarding methods of analysis for law enforcement. Due to the similarity of CBEs to genuine CB, they are difficult to detect. The Institute of Reference Materials and Measurements (IRMM) of the Joint Research Center (JRC), for the purpose of detection has recently elaborated and validated a suitable method by gas liquid chromatography of triacylglycerols [5]. However, the monitoring of wrongful labeling and the protection against fraud requires not only detection methods for the presence of added CBEs, but also

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methods that can achieve a reliable quantification of these fats [6]. CB and PMF are compatible fat blends due to their similarities in both TAGs (triacylglycerols) and fatty acids [7]. PMF, which has similarities in chemical and physical properties with CB, is suitable as one of the main components of the CBE (in combination with other authorized vegetable fats) to replace the presence of CB in chocolate formulation [8]. Due to the fact that CBEs are very similar to genuine CB, the detection and quantification of these vegetable fats in chocolate is both challenging and analytical.

A method of analysis to enforce compliance is necessary as the European Commission (EC) Directive requires appropriate monitoring [9]. Due to some technological requirements, only certain vegetable fats or mixtures can be used in the production of chocolate [10–12]. Various components of CB have been suggested as indicators for the detection of added vegetable fats other than CB in chocolate. According to Lipp et al. [3] although both tocopherols and tocotrienols form only minor components and have no quantitative roles, they could be traced as indicators for the presence of other vegetable fats in chocolate. Furthermore, Moazami et al. [13] have demonstrated that α -tocotrienol can be used as an indicator to detect and quantify the PMF in a model system. We report in this paper the application of this method to a real system (chocolate) and demonstrate that it provides reliable results albeit over a smaller working range.

Materials and methods

Materials

For detection and quantifying purposes, the CB from the Malaysian Cocoa Board (MCB, Bandar Baru Bangi, Malaysia) and the PMF from the Intercontinental Specialty Fats (ISF, Dengkil, Malaysia) were used. The samples of CB and PMF for validation studies were obtained from ISF and Kempas Edible Oil Sdn. Bhd, respectively. Analytical and high performance liquid chromatography (HPLC) grade solvents and chemicals, and individual standard vitamin E isomers of α -, β -, γ - and δ -tocopherols and tocotrienols were obtained from Merck (Darmstadt, Germany). Lecithin was obtained from the MCB.

Methods

Chocolate preparation

The formulation used for dark chocolate is as follows: cocoa powder (20%), fat content containing the mixture of CB with different percentage of PMF (33%), sugar (47%)

and lecithin (0.4%). The preparation of the mixtures in known proportions from 0 to 25% (w/w) added-PMF was chosen to verify the chocolate model studied by Moazami et al. (2008). Therefore, the mixtures were prepared from the melt at levels of 0, 5, 10, 15, 20, 21, 22, 23, 24 and 25% of PMF (w/w). Cocoa powder, sugar and half of the fat (CB and/or PMF) were mixed before being refined twice to reduce the particle size to 20–30 μm into a paste. Refining carried out using a three-roller refiner (Pascal Engineering Model No. 1802/3466, UK). The refined mass was then conched in a runner mill (Pascal Engineering Model No. 23197, UK) at 63–65 (C for 8 h. The remaining CB, PMF and lecithin were added to the mixture 2 h before the final conching process. Manual tempering was carried out on a clean marble slab, then, the chocolate mixture was poured into mould which was firmly hand-vibrated to remove air bubbles. This tempered dark chocolate was used for the detection of the added PMF after the extraction of the tocopherol and tocotrienol components.

Extraction of the tocopherols and tocotrienols

Vitamin E present in the chocolate samples was extracted using the method described by John et al. [14]. About 5 g of sample were weighed into a round-bottomed flask fitted with a side arm. Antioxidant (ascorbic acid, 100 mg) and ethanol (99.9%) were added and the mixture was swirled to ensure that all samples were wetted. Aqueous KOH (60%w/v) solution was added and mixed with ethanol. A slow stream of nitrogen was introduced via the side arm and the content was boiled under reflux for 5 min at the temperature of 80–85 (C. When saponification was completed, the resultant solution was cooled as quickly as possible by placing the flask in cold water and transferred into a separating funnel using 10 mL distilled water to rinse out the flask. The unsaponifiable matter was extracted by shaking the funnel for 1 min after the addition of 15 mL of petroleum ether (1:1). The organic layer was collected and the lower aqueous layer was discarded. The organic layer was washed carefully with distilled water. Saturated sodium chloride was introduced to remove the excess water. The solvent was then removed from the extract by evaporating it at 40 (C in the presence of nitrogen flow. The residue was dissolved in a 5-mL n-hexane and the mixture was analyzed for quantification of tocopherol and tocotrienol. All determinations were carried out in six replications.

Determination of the tocopherols and tocotrienols

Both tocopherols and tocotrienols were analyzed by the HPLC Model 1090 (Hewlett-Packard, Palo Alto, CA, USA), equipped with an auto sampler (25 μL syringe), a fluorescence detector Model FL 2000 (Spectra-Physics

Analytical, Fremont, CA, USA) and a data acquisition system (HP 3365 Series II Chemstation). A normal-phase Supelcosil LC Diol Column (25 cm × 46 mm, 5 μ particle size; Supelco Inc., Bellefonte, PA, USA) was used and operated at room temperature, with Hexane/propane-2-ol (99:1) as the mobile phase for 30 min at a flow rate of 1 mL/min. The solvent mixture was prepared fresh each day. The fluorescence detector excitation wavelength was 296 nm with an emission wavelength of 330 nm. All tocopherol and tocotrienol determinations were carried out in triplicate.

Recovery, precision, limit of detection and quantification

The recovery test was carried out by spiking α -tocotrienol standard at different levels of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.75 mg/g to 5 g of CB sample. The extraction of the α -tocotrienol was carried out by saponification of samples in five replicates as described above for the extraction of tocopherols and tocotrienols. Precision is expressed as the percentage coefficient of variation (%C.V.) or relative standard deviation (SD) of the replicate measurements. The precision of the analytical method was obtained in 1-day analysis of 0.3 mg/g α -tocotrienol standard in five replications. Limit of detection (LOD) and limit of quantification (LOQ) were defined when the ratio of signal-to-noise (S/N) was 3 and 10 times, respectively, above the blank signal.

Statistical analysis

The Complete Randomized Design of experimental research was employed to indicate the existence of PMF at the level of the statutory limit of 5% foreign fat addition to the CB. The study indicated the combination of six replications for each treatment. The MINITAB software 13.2 (MINITAB, State College, PA, USA) was used for all the statistical analysis. The significant differences between values are at $P < 0.05$ levels. The goodness of fit of the model was evaluated by the coefficient of determination (R^2).

Results and discussion

Determination of the tocopherols and tocotrienols in the dark chocolate

Satisfactory recoveries were obtained for α -tocotrienol used, ranging between 89 and 98%. The SDs were quite low for all adsorbents, ranging between 0.32 and 0.83. According to Parker [15] the acceptable range for recovery rate was between 70 and 120%. The results showed that the recoveries for all spiked levels used in this study were within an acceptable range. The LOD and LOQ were 0.025

and 0.100 mg/g, respectively. The acceptable range for precision is up to 20% [16, 17]. The SD and mean difference (MD) for detection of α -tocotrienol using HPLC was 0.09120 and 0.00497, respectively. The calculated C.V. was 5.45%. Vitamin E isomers (tocopherols and tocotrienols) were proposed for the detection of PMF in a chocolate model system and detailed analysis of the isomers in CB and PMF were investigated by Moazami et al. [13]. Therefore, α -tocotrienol was used as an indicator to determine the PMF in chocolate (real system) to verify the accuracy of the model. The separation of a mixture of tocopherol and tocotrienol standards is shown in Fig. 1. Since the α -tocotrienol was the only vitamin E isomer, which presents in PMF but absent in CB (Figs. 2, 3, respectively), increasing the PMF amount has resulted in an increase in the α -tocotrienol concentration. In the case where the PMF was added, the results showed an excellent correlation between the percentage of the added PMF and the increase of the α -tocotrienol amount (Table 1). When the PMF amount was increased from 1.4 to 2.8% based on the final product (chocolate), the α -tocotrienol concentration had also significantly increased from 0.16 to 0.26 ppm ($P < 0.05$). The α -tocotrienol concentration also increased significantly ($P < 0.05$) from 0.26 to 0.53 ppm where the amount of added PMF was increased from 2.8 to 4.2%. There was a significant difference ($P < 0.05$) in the concentrations of the α -tocotrienol when the amount of the PMF was increased from 4.2 to 5.6%. Increasing PMF amount to 5.9, 6.2 and 6.4% had resulted in slight increase of α -tocotrienol concentrations from 0.61 to 0.61 and 0.62 mg/g respectively, which were not significantly different ($P > 0.05$). Generally, adding PMF from 0 to 5.6% (on final product basis) resulted in a significant difference ($P < 0.05$) between the obtained concentrations of the α -tocotrienol. However, a significant difference was observed when the amount of PMF was increased from point 5.6 to 7%. The results showed that the amount of the added PMF to CB in chocolate is predictable using the applied indicator when PMF is added from 0 to 7% (on chocolate basis). However, the increase in α -tocotrienol concentration was not significant when the PMF level increased from 5.6 to 5.9, 6.2 and 6.4%. So the mentioned PMF levels were not differentiated in this chocolate system. Therefore, the detection of the PMF could be achieved below and over the 5% statutory limit of PMF based on the final product (chocolate). The probability and R^2 -values of the regression model for the correlation between α -tocotrienol and PMF levels are shown in Fig. 4.

Verification of the regression model

The validation was conducted to verify the model obtained from the regression analysis in order to detect and quantify

Fig. 1 Separation of tocopherol and tocotrienol standards by HPLC-FLD

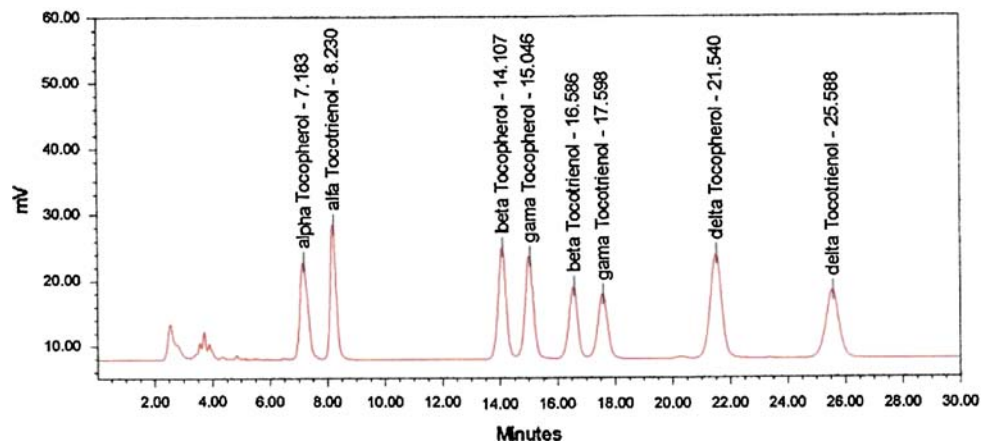


Fig. 2 Separation of tocopherols and tocotrienols in PMF

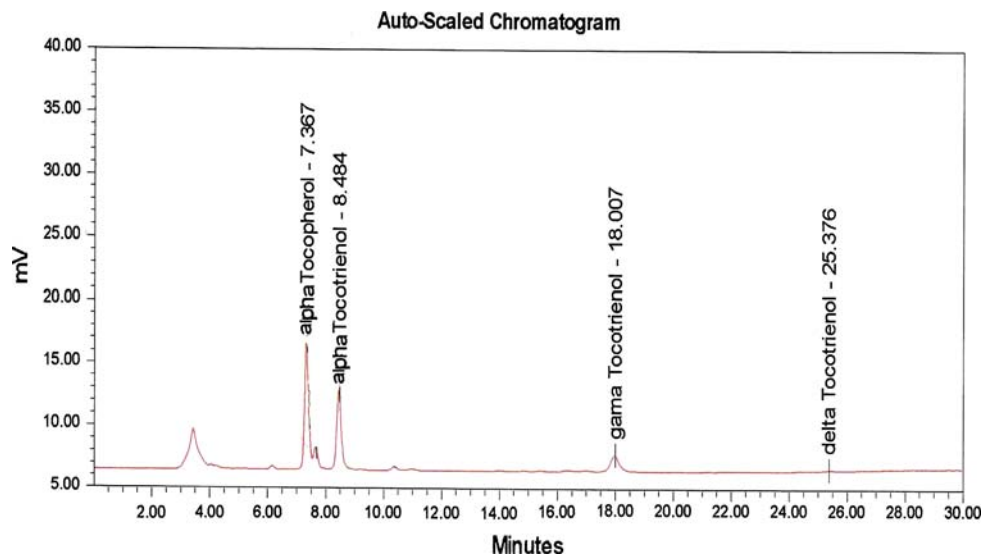
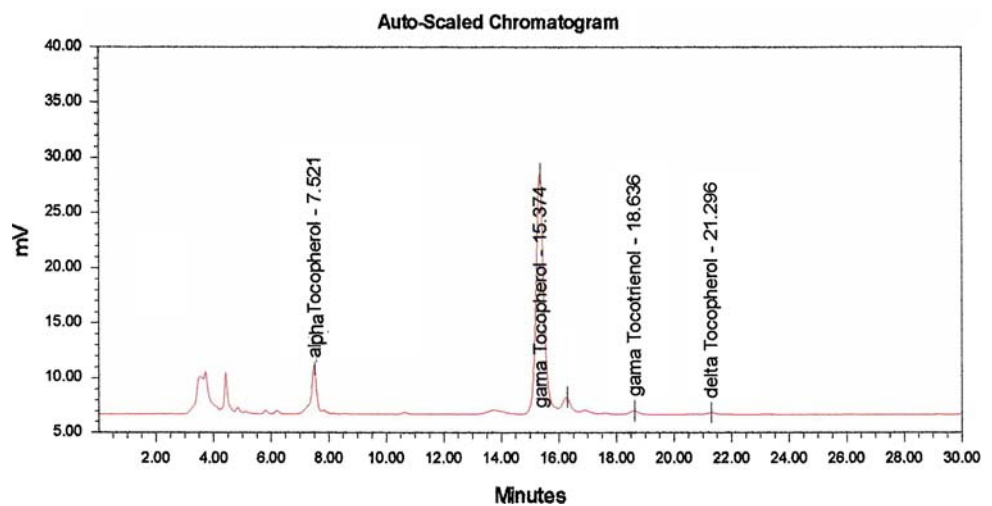


Fig. 3 Separation of tocopherols and tocotrienols in chocolate (control)



the PMF in the chocolate. In principle, fat, which is obtained from chocolate according to the saponification principle, is separated by the HPLC into vitamin E isomer fractions in triplicates. The predicted tocopherol and tocotrienol levels were shown in Fig. 5. Several independent

experiments were carried out to examine the adequacy of the predicted values by the model. The predicted tocopherol and tocotrienol levels were close to the experimental data. The responses and variable were fitted to each other and a good fit was obtained and no outlier was observed. The plot

Table 1 Tocopherol and tocotrienol composition of chocolate

PMF (%) in CB	0	5	10	15	20	21	22	23	24	25
PMF (%) in chocolate	0	1.4	2.8	4.2	5.6	5.9	6.2	6.4	6.7	7
α -T ₃ (ppm)	n.d.	0.16 ± 0.01 ^f	0.26 ± 0.01 ^e	0.53 ± 0.01 ^d	0.61 ± 0.01 ^c	0.61 ± 0.01 ^c	0.61 ± 0.02 ^c	0.62 ± 0.01 ^c	0.68 ± 0.01 ^b	0.74 ± 0.01 ^a

Each value in the table represents the mean ± standard deviation of three replications. Mean values within column with different superscript letters are significantly ($P < 0.05$) different

PMF palm mid-fraction, T tocopherol, T₃ tocotrienol, n.d. not detected

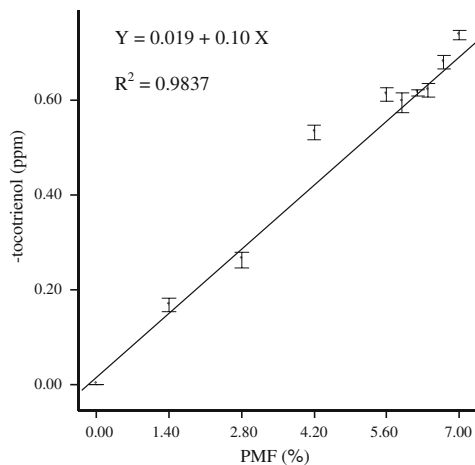


Fig. 4 The plot of α -tocotrienol concentrations (ppm) against different amount of palm mid-fraction (PMF%) in chocolate

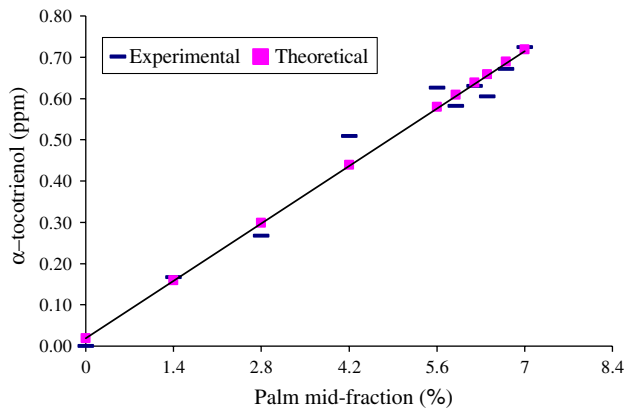


Fig. 5 The plot of PMF amount versus α -tocotrienol concentration in chocolate (experimental data). Theoretical data obtained from the equation in Fig. 4

of α -tocotrienol concentration (ppm) against PMF added (%) allowed the quantification of the addition up to 5%. The verification results showed a close correspondence between the theoretical and experimental values. The MD and SD of differences between the experimental and theoretical results for the α -tocotrienol were -0.003 and 0.039 respectively; this indicated that the regression model generated was capable of predicting the PMF concentration with

an appreciably high accuracy. It was found that the model fitted the theoretical data well ($P < 0.05$) with a small standard error (SE = 0.008) value.

Conclusion

The study indicated that α -tocotrienol could be used as an indicator to detect the PMF in chocolate and verified the previous study which has suggested the same indicator to be used to determine and quantify the addition of PMF in CB in model system. The models for the PMF concentration illustrated that the results were close to the observed experimental responses. This indicates that the generated models adequately explained the independent validation data and significantly represented the actual relationship between the process parameters. The regression model generated was capable of predicting the PMF concentration in chocolate with an appreciably high accuracy. In conclusion, adding the PMF to chocolate could be detected by a determination of the α -tocotrienol. The applied indicator performed well at the level of the statutory limit of 5% PMF addition on a chocolate basis. However, as preliminary work, this part focused on mono-additions, where only one type of foreign fat (PMF) was added to CB and several plots might be necessary to provide an optimal detection and quantification level. The identification of the nature of the foreign fat added (PMF) was possible because of the unique trends that its addition engendered, and because only one type of foreign fat was added in this experiment scheme. Current research is focusing on multi-indicator data using integrated statistical tools.

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