Malaysian Cocoa Journal
Volume 13(2)/2021

METHOD DEVELOPMENT AND VALIDATION FOR QUANTITATIVE ANALYSIS OF MILK SOLID CONTENT IN CHOCOLATE

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Malaysian Cocoa J. (2021) 13(1): 137-147

ABSTRACT - *In this study, an efficient and rapid method for estimation of milk solid content in chocolate via quantification of butyric acid was developed and validated. The method was based on direct base-transesterification of chocolate sample without fat extraction prior to quantification using gas chromatography coupled with flame ionization detected (GC-FID). Good selectivity and sensitivity were obtained with LoD and LoQ at 1.41 ppm and 4.95 ppm respectively. The mean recovery for butyric acid was 90% with relative standard deviations of less than 10% and the expanded uncertainty measurements for elements were less than 25% (with a coverage factor of 2 with a confidence level of 95%). Finally, the developed method was successfully applied for routine analysis of milk chocolate and dark chocolate.*

Keywords: milk fat, butyric acid, measurement uncertainty, selectivity, linearity, GC-FID

INTRODUCTION

Chocolate is a food obtained by mixing of cocoa derived products namely cocoa liquor and cocoa butter with sugar, and for some types of chocolate, by adding milk or other ingredients. There are several types of chocolate which normally classified according to the proportion of cocoa solid contents and other ingredients used in a particular formulation, generally known as dark chocolate or milk chocolate. Dark chocolate or plain chocolate is a form of chocolate containing cocoa solids, cocoa butter, and sugar, without the milk components found as in milk chocolate. Milk chocolate is the same as dark chocolate, but with milk solids and fat replacing some of the cocoa liquor. Consumers are interested in the amount of milk fat in food for a variety of reasons; health, nutrition, weight loss, and more. Legislation is very strict about how much milk fat must be present. In some countries a high level of milk content must be labelled as household milk chocolate or its equivalent. There are specific regulations as to what can be marketed as 'chocolate' and what different varieties of chocolate are comprised of.

Inevitably, these regulations differ from country to country, though many have no concrete definition of what 'chocolate' is when sold to the consumer. In this respect, global legislative bodies especially CODEX Alimentarius Commission regulated a specification for chocolate as stipulated in CODEX STAN 87-1981, Rev.1-2016 (Anon, 2016). Meanwhile, EU Commission enforced and implemented the EC Directive 200/36/EC (Anon, 2000) under Cocoa and Chocolate Products Regulations 2003 to control the labeling and composition of chocolate products. In Malaysia, the law relating to the labelling of cocoa and chocolate products is governed by the Malaysian Food Act 1983 and Regulations (Anon 2019). The regulations apply to cocoa and cocoa products including chocolates intended for human consumption that are sold to consumers. The regulations set out detailed criteria which must be met before a product can be described using specific descriptions. Those regulations specify a range of reserved descriptions for chocolate products which have minimum requirements for levels (in percentage) of milk solid or milk fat content to be present in chocolates.

Due to products varieties with different levels of allowable milk fat content, and since those regulations do not cover aspects regarding methods of analysis for law enforcement, there is a need to develop reliable methods for the determination of milk fat content in milk chocolate, and in particular to demonstrate conformity for its intended use by giving a

measure of confidence that can be placed on the result. Method validation is an essential component of the measures that a laboratory should implement in order to produce reliable analytical data (Magnusson & Ornemark, 2014). For consistent interpretation of the measurement results, it is necessary to evaluate the confidence that can be placed in, therefore, the presentation of an analytical result which must be accompanied by indication of the data quality (Azevedo *et al.* 2009). Based on literature, several analytical methods have been developed to quantify the milk fat contents in foods. The most conventional method is based on the analysis of the amount of butyric acid (C4) using GC- in food products containing milk fat to be calculated (Ulbert 1997). Another widely applied alternative to determining butanoic acid is transesterification of a fat sample in the presence of a suitable IS, e.g. FID (Precht 1990; Molkentin & Precht 1998; Molkentin & Precht 2000), because C4 is only contained in milk fat from cows and other mammals, but not in animal adipose or vegetable fats. This enables to quantify butanoic acid as a methyl ester, which can be converted easily to express the analyte content as g butanoic acid 100 g^{-1} fat (Hadorn & Zurcher 1970; Schulte & Weber, 1989). However, most of the analysis conducted on the extracted fat or fat ready sample and not based on sample matrices. Fat from any food samples were extracted through solvent extraction before taken for analysis.

The aim of this study is to develop efficient and rapid analytical procedures through the process of direct *derivatization* or base-transesterification of chocolate sample, without fat extraction prior to quantification of butyric acid content (C4) and simultaneously to validate the method accordance to proper validation criteria namely limit of detection (LoD), limit of quantification (LoD), linearity, and uncertainty. Finally, the optimized method was then applied in a real sample monitoring programme carried out on chocolate collected from Malaysian local market.

MATERIALS AND METHODS

Instruments

The analyses were performed on Shimadzu Gas Chromatography GC-2010 (Japan) equipped with Flame Ionization Detector (FID) and auto sampler. The capillary column used was DB-23 30m. Acquisition was controlled by GC Solution Software V2.30.00SU7. The GC-FID was optimized using standard solution of reference material butyric acid methyl ester (C4) diluted in n-hexane as a diluents. A series of concentration ranging from 0.2 ppm to 20 ppm were prepared and 1 uL from each solution was injected using autosampler onto split mode (1:20) injector port controlled at 250ºC. The separation taken place in the chromatography column DB-23 30m x 0.25 mm coated with 0.25um (J&W Scientific 122-2332) Bonded crosslinked (50% cyanopropyl-methyl polysiloxane) under isothermal oven temperature program: initial temperature 50ºC for 1 min and increased to 175ºC at heating rate 25ºC/min before hold for 12 min.

Reagents and materials

Potassium hydroxide (KOH), n-hexane and methanol were of Analytical Reagent grade and purchased from Merck (Darmstadt, Germany). Standard Certified Reference material butyric acid methyl ester 99.65% ISO 17034 was purchased from LGC Dr Ehrenstorfer, Middlesex, UK. A transesterification reagent or base-catalyst 11.2g potassium hydroxide was dissolve in 100 mL methanol to obtain 2N KOH.

Standard solutions. A 100 ppm butyric acid methyl ester solution was prepared in n-hexane and further diluted to obtain a series of concentration ranging from 0.5, 1.0, 5.0, 10.0 and 50 ppm. Transfer about 1.5 mL of each concentration into individual autosampler vial and used for GC analysis.

Raw materials, Whole milk powder (WMP) was purchased from Fonterra Ltd, New Zealand. Cocoa butter and cocoa liquor were purchased from Guan Chong Cocoa Manufacturer Sdn Bhd, Johor.

References Chocolate samples (incurred samples).

Due to inavailability of reference chocolate sample with known milk fat content in the market, it is necessary in this study to establish the chocolate sample containing known value of milk fat. In this study, 6 types of milk chocolate

containing different amount of milk powder (MP) or milk fat at 0.1%, 0.5%, 1.0%, 5%, 10.5% and 29.3% were prepared in laboratory together with 1 sample dark chocolate (without MP) as a blank sample. The raw materials used (cocoa butter, cocoa liquor, sugar, whole milk powder, lecithin, vanillin) in the formulation, were purchased from the Pilot Plant of Cocoa Innovation and Technology Centre (CITC) Nilai, Malaysian Cocoa Board.

Samples derivatization

Base-catalyzed transesterification was performed at room temperature by reacting 0.5g of homogenized chocolate sample with a 2.5 mL n-hexane solvent.in a capped 15 mL centrifuge tube. The tube was vigorously vortexed/homogenized for 1 min. Then, 0.5 mL 2N KOH transesterification reagent was added and vortexed vigorously for another 2 min before the mixed solution was centrifuged at 12000 rpm at 4^{o}C for 5 min. The clear supernatant was transferred into a 2 mL autosampler vial and 1µL was injected onto GC-FID via autosampler.

Method validation criteria.

The validation parameters included in this study were linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) and Measurement Uncertainty (MU). The validation method followed the protocol EURACHEM guidelines on validation of analytical methods (Magnusson & Ornemark, 2014).

Selectivity and specificity depends on the selected analyte and possible interferences. It is always relates to "*the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour"* (Jorgen *et al*, 2001). In this work, the selectivity, specificity and linearity work were investigated by studying the GC-FID ability to identify methyl butyrate (butyric acid methyl ester as an analyte) present in chocolate sample. Altogether, its determined the ability of a method to demonstrate test results that are directly proportional to analyte concentration within a given range, and reported as a variance of the slope of the regression line *y=ax+b*. Acceptance criteria for linearity are that the correlation coefficient *R* is not less than 0.990 for the least squares method of analysis of the line.

The LOD indicates the lowest concentration that can be distinguished from noise, but not necessarily quantified, while LOQ is the lowest concentration of the analyte that can be determined with an acceptable level of repeatability, precision, and trueness. LOD was estimated from the calibration function for a signal equal to the net signal of blank and three times its standard deviation,

$$LOD = \left(\frac{3.sd_{blank}\ x\ C_{spiked}}{Signal_{spiked} - Signal_{blank}}\right),$$

while LOQ, was estimated from the calibration function for a signal equal to ten times of LOD, as mentioned by Dico *et al* (2015).

$$LOQ = \left(\frac{10.sd_{blank}\ x\ C_{spiked}}{Signal_{spiked} - Signal_{blank}}\right)$$

The uncertainty is a quantification of the doubt about the result and determined whether the measurement result is fitted for the intended purpose. There are various approaches related to measurement uncertainty, but the most common are known as Top-Down and Bottom-up approaches, whereby the overall uncertainty is obtained by identifying, quantifying and combining all individual contributions to uncertainty. In this study, the ''bottom-up'' approach was used and the measurement uncertainty was estimated using the data obtained during method validation. Based on this approach, it was found that uncertainty comprises two components – (i) precision *(P)* or repeatability and (ii) bias or recovery, were shown to represent the main source of combined standard uncertainty. On the other hand, uncertainties associated with calibration (uncertainties of weighing or diluting standards, glassware, temperature effect) were not so important. The relative expanded uncertainty was then calculated by using the coverage factor k = 2 at 95% confidence level.

The precision or the overall run to run variation of analytical procedure was performed during method development and validation studies with two different batches of calibration solutions, two different batches of reagents, two different

analysts, at two concentration levels, for two different types of chocolate samples. The precision assessments was determined based on relative standard deviation (RSD) of butyric acid content in 2 different types of chocolate samples prepared in laboratory which containing different amount of MP in their formulation. One sample contains 0.1% MP and another one is contains 1.0% MP.

Real samples of chocolate

Milk chocolate and dark chocolate containing various amount of cocoa solid were collected and purchased from local groceries shop for real sample monitoring purposes. Samples collected were labeled and stored in refrigerator at 5°C before taken for base-transesterification.

RESULTS AND DISCUSSION

Instrument Optimization

Result from this study found that the GC-FID was capable to detect C4 at the lowest concentration of 0.2 ppm and the targeted peak was appeared approximately after 1.48 min. acquisition time (retention time, t_R) as shown in Figure 1.

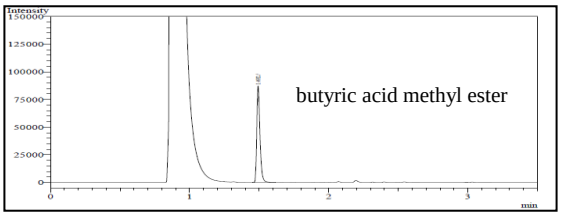

Figure 1. Peak of butyric acid methyl ester/butyrate methyl ester

Method Selectivity, specificity and linearity

In order to evaluate linearity of the method, six types of milk chocolate samples containing various amount of MP ranging from 0.1, 0.5, 1.0, 5.0, 10.5 and 29.3% MP were prepared and their butyric acid content quantified. Samples were gone through the base-transesterification steps prior injected GC-FID and the peak responses were recorded. A calibration graph, peak responses (intensity) versus concentration was plotted and found to be linear with a *R*-squared value better than 0.990. This result demonstrates linearity of this method over the specified range as shown in Figure 2.

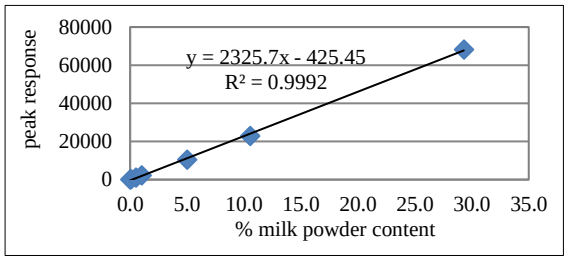

Figure 2 Linearity plot of peak intensity against chocolate samples

Limit of Detection (LoD) and Limit of Quantification (LoQ)

In order to determine this value, chocolate sample without MP which considered as a blank sample (in this case dark chocolate) was prepared together with spiked sample (dark chocolate with 5% MP) were analyzed and the results were summarized in Table 1. Result showed that the LoD and LoQ of butyric acid (C4) are 0.02% (or equivalent to 1.41 ppm) and 0.07% (or equivalent to 4.95 ppm) respectively.

Table 1 : LoD and LoQ of butyric acid in GC-FID (n=6)

	Signal of Blank Sample	Signal of Spiked sample at 5% MP	LoD (%)	LoQ (%)
Butyric acid (C4) in chocolate	27.5 ± 14.0	10416.1 ± 875.9	0.02% (1.41± 0.05 ppm)	0.07% (4.95± 0.17 ppm)

n=7	Conc.1 (0.1% milk powder) ppm			Conc.2 (1% milk powder) ppm			u(P) @ RSD_{pooled}
	Mean	sd	RSD	Mean	sd	RSD	
Butyric acid(C 4)	7.72	0.53	0.07	66.90	5.35	0.08	0.11

$$RSD_{pooled} = \sqrt{\frac{(\mathrm{RSD}_1) + (\mathrm{RSD}_2)}{(n_1 - 1) + (n_2 - 1)}} \qquad \text{(Eq. 1)}$$

Table 2 showed that the RSD observed from the 2 different concentrations were all in similar order of magnitude for butyric acid. In such cases, it is appropriate to pool the RSD for sample 0.1% and 1.0% MP using Equation 1 to obtain a sigle estimation of precision u(P) as 0.11. u(P) as 0.11.

Bias Study, Recovery

The bias of the analytical procedure was investigated using spiked samples of chocolate samples containing 0.1% and 1.0% MP. A 0.5g of each sample was spiked with 25 uL of 38.75 ppm methyl butyrate standard solution and undergone the whole entire analysis steps.

Precision study (repeatability), P

From 7 replications, the means and standard deviations of butyric acid content were calculated. The result and the RSD were summarized in Table 2.

The quantified butyric acid in spiked sample was compared with the unspiked sample. The ratio between spiked and unspiked sample is expressed as recovery. The recovery values obtained was further used in the calculation of standard uncertainty for recovery, μ(Rec) and relative standard uncertainty, (RSU) using Equation 2 and Equation 3 respectively as shown in Table 3. Additionally, a Student's t test was used to determine whether the mean recovery is significantly different from 1.

From the results obtained, it was found that the critical value t_{crit} were greater than the t_{cal} values, hence recoveries obtained in the validation data were not significantly different from 1, and hence no corrections need to be applied to the test results. Nonetheless, for the purpose of evaluating its significance to the measurement uncertainty estimate, the bias component has been included into the uncertainty budget. Another source of uncertainty that contributes to the uncertainty in bias is the purity of the reference standards from which the spiked samples are prepared. The purity of reference standards are given by the manufacturer (in the certificate of analysis) and the standard uncertainty, *u(purity)* was calculated using rectangular distribution. Finally, the Bias uncertainty is obtained by combining the RSU of each uncertainty contributors using equation 4, and the result is summarized in Table 3.

Other sources of uncertainty are adequately covered by the precision data and recovery data in the calculation of measurement uncertainty. Since balances, volumetric devices and environmental conditions were under regular control, and the verification were carried out over a longer period of time with variations in analyst, laboratory tools, and calibrations, it can be assumed, that the influences of the variability of most sources on the measurement uncertainty are covered by the within-laboratory precision.

Table 3: Results of Bias study (n=7).

Description	Mean of Conc. ppm (C_{mean})	Mean sd of Conc. ppm (C_{sd})	Mean Recovery $\left(\frac{C_{mean}}{C}\right)$	Standard uncertainty μ(Rec) (Eq. 2)	Relative std uncertainty (RSU) (Eq. 3)	RSU_{Pooled} (Eq. 4)
A) Recovery						
Spiked at 0.34 ppm ;						
i. 0.1% milk powder	7.26	0.35	0.900	0.016	0.018	0.016
ii. 1.0% milk powder	69.46	2.29	1.089	0.014	0.013	
B) Purity of butyric acid						
Purity of CRM : 99.65 ± 0.70% Manufacturer's cert. & standard uncertainty was assumed as rectangular distribution *u(purity)* = 0.004 was taken as uncertainty contributed to the standard uncertainty of purity					0.004	

$$\text{Standard uncertainty, } \mu(Rec) = Rec\ x \sqrt{\frac{(C_{sd})^2}{n\ x\ (C_{mean})^2}} \qquad \text{(Eq. 2)}$$

$$\text{Relative} \quad \text{Relative standard uncertainty (RSU)} = \sqrt{\frac{\mu(Rec)^2}{(Recovery)^2}} \qquad \text{(Eq. 3)}$$

$$\text{RSU}_{pooled} = \sqrt{\left(\frac{(n_1-1)(\mu(Rec1)^2)+(n_2-1)(\mu(Rec2)^2)}{(n_1-1)+(n_2-1)}\right)+\left(\frac{\mu_{(purity)}}{C_{purity}}\right)^2} \qquad \text{(Eq. 4)}$$

Combined standard uncertainty

During the in-house validation study of the analytical procedure the precision and the bias uncertainty sources had been thoroughly investigated. Both uncertainties were combined using Equation 5.

$$\frac{\mu(C_{butyric\ acid})}{C_{butyric\ acid}} = \sqrt{(precision)^2 + (bias)^2} \qquad \text{(Eq. 5)}$$

Finally, the expanded uncertainty U($C_{butyric\ acid}$) is calculated by multiplying the combined standard uncertainty with a coverage factor of 2 with a confidence level of 95% and the values are summarized in Table 4.

Table 4 Uncertainty components, combined std uncertainty and expanded uncertainty for the analysis of butyric acid in chocolate sample

Analyte	Precision	Bias	Combined Std Uncertainty	Expanded Uncertainty $U(C_{butyric\ acid})$
Butyric acid	0.11	0.016	0.111	$C_{butyric\ acid}$ x 0.111 x 2

Real samples monitoring

The developed and validated method was applied to routine monitoring analysis of butyric acid in various types of chocolate samples throughout the year 2018 and 2019. In total, 34 various types of chocolate samples were collected from local groceries shop which consisted of 14 milk chocolate samples and 20 dark chocolate samples. To calculate the amount of MP or milk fat in chocolate are based on the relative amount of butyric acid in milk chocolate. A series of authentic mixtures of MP or milk fat in chocolate ingredients were prepared to evaluate the use of butyric acid content as an index of the amount of milk powder in chocolate. In this study, a whole milk powder (WMP) was used and mixed with chocolate ingredients to obtain milk chocolate containing 0.1%, 0.5%, 1.0%, 5.0%, 15.0% and 29.3% milk powder. The fat content of cocoa liquor and WMP used in this study were pre-determined trice and the result found that the fat content were 51.85% and 25.06% respectively.

The butyric acid content of those samples was quantified based on the calibration solution of reference material of methyl butyrate. Once the butyric acid content in each samples determined, a linear graph of butyric acid content versus % MP was plotted and the slope of 0.000038 was obtained and taken as a universal conversion factor. This value should be used as a conversion factor to calculate the amount of MP presents in chocolate ingredients.

Table 5 shows the results for 14 samples of milk chocolate inclusive 6 chocolate samples with known value of MP. The table gives the percentages of butyric acid content and expected amount of MP used in the formulation. Besides that, the result for chocolate samples A, B, C, D, E and F with known value of MP added in the formulation shows good recovery at almost 100%. This indicates that the developed method using butyric acid as a marker to quantify milk fat content in milk chocolate can be used for surveillance purposes. Meanwhile, this method also can be used as a tool to distinguish between milk chocolate and dark chocolate. Table 6 shows the result for 20 samples of dark chocolate which indicated that all samples contain a traces amount of MP in their ingredients. The MP content lies between 0.22% - 4.86% which are comply with specification described in Malaysian Food Regulations (Anonymous, 2010) specifications. Only 1 sample, Hershey dark chocolate was found to contain 12.17% MP which is higher as specified in Malaysian Food Regulations (Anonymous, 2010), however it should not give any problem according to Codex Stan 87-1981 or EU Regulations (EU Directive 200/36/EC).

Based on the result of this study, the developed method is suitable to do quantification work for determination of milk solid content in a mixture ingredient such as chocolate especially between different fat system, cocoa butter and milk fat in particular. The uses of methyl butanoate as a marker for determination of milk fat content, seems to be promising and also in line with Ulberth (1997) findings. Eventhough, this method has shows good recovery, precision and repeatability, but there are some limitations that need to be taken for consideration before this method being put in place. Due to inavailability of chocolate
reference material (with ingredients certified) and various types of MP with different amount of fat content exist in the market, the analysis approach used in this method is only suitable for in-house quality control purposes, whereby all important parameters or ingredients used in the chocolate should predetermined, i.e; the types of MP (either whole milk powder, skimmed milk powder, sweet whey powder), the fat content of

that particular MP, and the actual amount of dairy constituents used in the chocolate. This information are significant important, and without knowing those information will tends to produce false positive results.

Low amount of expected milk powder content for sample milk compound (LKM-CITC) as shown in Table 5 is most probably due to the high amount of skimmed milk powder or sweet whey powder in their formulation which are less fat content (Augustin & Margetts, 2003) compared to MP used in this study.

Table 5 Milk powder content in various milk chocolate samples

	Sample	Conc. of methyl butyrate (from cal.curve) y=mx	Conc of methyl butyrate in ppm (based on dil.factor = 5.4)	Conc of butyric acid, ppm (mg/kg)	Conc of butyric acid (g/0.5g sample)	Expected % MP in formulation, calculated based on Conversion factor of 0.00004
1	Milk chocolate LeBourne	141.15	762.19	656.47	0.00032824	8.21
2	Milk chocolate ESTANA	115.61	624.31	537.71	0.00026886	6.72
3	Milk chocolate Tesco	288.71	1559.02	1342.77	0.00067139	16.78
4	Creamy chocolate Whittakers	462.07	2495.19	2149.08	0.00107454	26.86
5	Dairy milk chocolate Cadbury	396.64	2141.83	1844.74	0.00092237	23.06
6	Milk couverture (LKM-CITC)	396.73	2142.35	1845.19	0.00092259	23.06
7	Milk compound (LKM-CITC)	7.23	39.06	33.64	0.00001682	0.42
8	Milk chocolate Hershey Creamy	312.63	1688.22	1454.05	0.00072702	18.18
9	Chocolate A (0.1% MP)	1.75	9.48	8.16	0.00000408	0.10 (Recovery = 100%)
10	Chocolate B (0.5% MP)	7.35	39.71	34.20	0.00001710	0.43 (Recovery = 86%)
11	Chocolate C (1.0% MP)	15.92	85.98	74.05	0.00003703	0.93 (Recovery = 93%)
12	Chocolate D (5.0% MP)	72.69	392.51	338.06	0.00016903	4.23 (Recovery = 85%)
13	Chocolate E (10.5% MP)	166.22	897.59	773.08	0.00038654	9.66 (Recovery = 92%)
14	Chocolate F (29.3% MP)	489.75	2644.65	2277.81	0.00113891	28.47 (Recovery = 97%)

Note: LKM CITC – Lembaga Koko Malaysia, Cocoa Innovation Technology Centre

Table 6 Milk powder content in various dark chocolate samples

	Sample	Conc of methyl butyrate (from cal.curve) y=mx	Conc of methyl butyrate in ppm (based on dil.factor = 5.4)	Conc of butyric acid, ppm (mg/kg)	Conc of butyric acid (g/0.5g sample)	Expected % MP in formulation, calculated based on Conversion factor of 0.00004
1.	Dark Chocolate Whittakers	5.99	32.32	27.84	0.00001392	0.35
2.	Dark chocolate 70.5% Callebaut	3.78	20.41	17.58	0.00000879	0.22
3.	Dark Estana	5.38	29.03	25.00	0.00001250	0.31
4.	Dark chocolate Selbourne	8.52	45.98	39.61	0.00001980	0.50
5.	Dark chocolate Ritter Sport	45.40	245.16	211.15	0.00010558	2.64
6.	Dark couverture (LKM-KKIP)	56.11	302.99	260.96	0.00013048	3.26
7.	Dark chocolate Vochelle	30.89	166.78	143.65	0.00007182	1.80
8.	Dark chocolate Vochelle	30.89	166.78	143.65	0.00007182	1.80
9.	Dark chocolate Whittakers	4.97	26.84	23.12	0.00001156	0.29
10.	Dark chocolate Vochelle	28.40	153.33	132.07	0.00006603	1.65
11.	Dark Estana	4.53	24.45	21.06	0.00001053	0.26
12.	Dark Chocolate Selbourne	6.14	33.17	28.57	0.00001428	0.36
13.	Dark chocolate Ritter Sport	41.74	225.39	194.13	0.00009706	2.43
14.	Dark couverture (LKM-KKIP)	48.15	259.99	223.93	0.00011196	2.80
15.	Dark chocolate 55% Tudor Gold	34.54	186.53	160.66	0.00008033	2.01
16.	Dark chocolate Hershey	209.34	1130.42	973.62	0.00048681	12.17
17.	Dark chocolate Vochelle	72.56	391.84	337.49	0.00016874	4.22
18.	Dark couverture Marvellous	83.67	451.81	389.14	0.00019457	4.86
19.	Dark Cho 55% Tudor Gold	34.50	186.30	160.46	0.00008023	2.01
20.	Dark couverture (LKM-CITC)	5.39	29.12	25.08	0.00001254	0.31

Note: LKM –KKIP : Lembaga Koko Malaysia, Kota Kinabalu Industrial Park
LKM-CITC : Lembaga Koko Malaysia, Cocoa Innovation and Technology Centre

CONCLUSION

In this study, the quantification of % milk solid or MP in chocolate using direct base-catalyzed transesterification technique prior to identification of butyric acid content using GC-FID was successfully developed, validated and applied for the routine analysis via a monitoring study. It is considered to be excellent, reliable and fast technique in analyzing milk solid/milk fat content in chocolate. The method showed good selectivity, linearity, limit of detection/quantification, recovery and precision which acceptable under the validation criteria of EURACHEM guidelines. The expanded uncertainty measurements (using the coverage factor k=2 at 95% confidence level) for chocolate were less than 25% in which the uncertainty associated to precision or repeatability strongly contributes to the total uncertainty. The proposed method was successfully applied for the routine analysis of milk fat or MP content in chocolate. Finally, the analysis technique presented here can be considered as time- and cost-efficient, suitable for a routine analysis in determining the milk solid or milk fat content presents in chocolate.

ACKNOWLEDGEMENT

This project was funded under Tabung Amanah LKM / Baki Science Fund PTJ 8826091 (L 15515)

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