Q01122
Project Title:

Measurement of theobromine content in cocoa for determining cocoa solids content in chocolate and chocolate products

August 2011

Executive Summary

Cocoa and chocolate products are a multi million pound industry in the UK and throughout Europe. More chocolate is consumed in Europe than any other continent of the world. The amount of cocoa present in chocolate products is generally regarded as a guide to quality and attempts to quantify it accurately by analysis has been going on for over 100 years. The labelling and composition of chocolate products are controlled by the Cocoa and Chocolate Products (England) Regulations 2003 which implement the EC Directive 2000/36/EC. Identical provisions for labelling and composition are included in parallel regulations across the U.K. The regulations specify a range of reserved descriptions for chocolate products which have minimum requirements for levels of cocoa solids (and milk solids) to be present.

The measurement of cocoa solids in chocolate products has been carried out routinely by manufacturers and enforcement laboratories for many years. Over time the methods used have changed and been improved upon, but all of the methods have been based upon measurement of the naturally occurring alkaloids, theobromine and caffeine, present in cocoa. Direct measurement of alkaloid content enables the determination of total non-fat cocoa solids. In the last 50 years little systematic work has been done to establish whether the level of alkaloids has changed as a result of different methods of production or environmental factors.

The main objectives of the project were to optimise the method for theobromine determination, and apply the method to analyse the theobromine content, over a two year period, of around 200 samples of cocoa from around the world. The extended time period of analysis was to take account of seasonal variations in growing conditions and possible natural changes in alkaloid levels. A conversion factor could then be calculated from the average theobromine levels in cocoa, and compared with previously established factors to find out whether the levels of alkaloids have changed over the years.

At the start of the project, a ring trial between five different European labs was carried out to assess the comparability in the results for alkaloids and fat in six samples of cocoa liquor obtained when different analytical methods were used. The results of the ring trial showed that the methodology proposed by Durham for measurement of alkaloids was fit for purpose.

The levels of moisture in samples, as measured using Karl Fischer analysis, were also found to be variable and so corrections to alkaloid levels were made to take account of this. The fat content of the samples was also measured and the alkaloid levels calculated on the dry fat free part of the cocoa liquor.

The results showed that the levels of theobromine present were found to be generally lower than those found in survey work carried out over the last 50 years. This could be due to a combination of factors, including changes in the cocoa varieties being grown and an increase in use of hybrid varieties of cocoa that are hardy and high yielding, as well as changes in climate and cultivation methods. It must also be recognised that whilst in the past, analysts were probably measuring total alkaloids accurately; it was not always clear from the lack of detail in the references what measurements were being performed. Furthermore, the results indicate that the

conversion factor, calculated from theobromine content in the 200 samples of cocoa liquor, is higher than the factor currently used by enforcement laboratories to calculate the dry fat free cocoa solids.

The mean theobromine content of the cocoa liquor samples tested was 24,572 mg/kg which provided a conversion factor of 40.7. The conversion factor multiplied by the percentage of theobromine in the sample gives an indication of dry fat free cocoa solids in the food. The standard deviation of the results from 191 samples was 3,470 mg/kg and the range was 19803 – 39,168 mg/kg. This indicated that considerable caution must be taken when reporting on cocoa content of foods when the composition of the original cocoa liquor is unknown. Enforcement organisations will generally take the statistical variation of the results into account when reporting on predicted composition. Indication of adverse results on samples usually leads to further investigation of further samples and / or the source of raw materials used. Where the source of cocoa is known, it would be more appropriate to use a factor obtained for that geographical area. Persistent low results on a food would give cause for concern.

A comparison of the cocoa solids content calculated using the new factor of 40.7 and the current factor of 35.9 was carried out. It was found that the current factor gave lower results for cocoa solids than with the new calculated factor. The new factor also gave results closer to the expected amount than the current conversion factor, i.e. the calculation of cocoa levels in the controlled samples of manufactured chocolate were generally in better agreement with predictions using the new factor than with the current factor. Where total alkaloids are measured instead of theobromine alone a factor of 36.1 is proposed.

The manufacture of chocolate takes place by blending ingredients including cocoa liquor, sugar and flavourings. In some cases cocoa powder is added as well as additional cocoa fat, milk solids or vegetable fats. To validate the results of the cocoa liquor samples and determine the fitness for purpose of the new calculated factors, tests were carried out to measure the levels of alkaloids and fat in twenty samples of both the cocoa liquor and the finished chocolate made from the liquors. The results from the liquor were used to calculate the amount of dry fat free cocoa present in the chocolate. These results were compared with the amounts declared to be present. In general there was good agreement between the calculated amounts of cocoa liquor present and the actual declared amounts.

All of the in-house quality control checks carried out during the lifetime of the project were satisfactory. The paper has been extensively peer reviewed and the data and method were considered robust and fit for purpose. The method will be available to download through the Public Analyst journal website, http://www.apajournal.org.uk/index.html

Contents

Glossary

AOAC	Association of official analytical chemists
APA	Association of Public Analysts
AQC	Analytical quality control
BC	Barry Callebaut
CI	Confidence interval, at 95% level
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement
DAD	Diode array detector
DFFC	Dry fat free cocoa
FSA	Food Standards Agency
HPLC	High pressure liquid chromatography
IOCCC	International office of cocoa and chocolate and sugar confectionery
IQ Koln	Institut für Qualitätsförderung
JAOAC	Journal of Official Analytical Chemists
JAPA	Journal of Association of Public Analysts
KF	Karl Fischer
LCI	LebensmittelchemischeIsn Istitut
LOD	Limit of detection
sd	Standard deviation
UV	Ultra violet
VEMS	Validated Enforcement Methods

Definitions

Chocolate

The product obtained from cocoa products and sugars which contains not less than 35 per cent total dry cocoa solids, including not less than 18 per cent cocoa butter and not less than 14 per cent of dry non-fat cocoa solids.

Cocoa beans

The seeds of the cacao tree (*theobroma cacao*) fermented and dried.

Cocoa liquor (or mass)

Cocoa nib reduced to a paste by a mechanical process without losing any of its natural fat content.

Cocoa powder or cocoa

The product obtained by converting into powder cocoa beans which have been cleaned, shelled and roasted, and which contains not less than 20 per cent cocoa butter, calculated according to the weight of the dry matter, and not more than 9 per cent water.

Milk chocolate

The product obtained from cocoa products, sugars and milk or milk products which contain:

- not less than 20 per cent total dry cocoa solids;

- not less than 20 per cent dry milk solids obtained by partly or wholly dehydrating whole milk, semi-skimmed or skimmed milk, cream, or from partly or wholly dehydrated cream, butter or milk fat;
- not less than 2.5 per cent dry non-fat cocoa solids;
- not less than 5 per cent milk fat;
- not less than 25 per cent total fat (cocoa butter and milk fat).

Nib

Cocoa beans, roasted or unroasted, when cleaned, shelled and having a maximum residue of 5% shell or germ and a maximum content of 10% ash – these percentages to be based upon the weight of dry defatted matter.

Kibbled

The process of coarse grinding of a material such as cocoa nib

Introduction
Aims and Objectives

The objectives of this project were:

- to review and assess the published literature on alkaloid analysis of cocoa and chocolate products
- to collect 200 samples of cocoa from growers around the world and analyse them for levels of alkaloids and fat,
- to carry out a ring trial of methods used by enforcement laboratories and industry for measurement of alkaloids and fat,
- to optimise a method for analysis of alkaloids in the presence of common food additives in chocolate products,
- to measure alkaloids caffeine and theobromine, and fat content in chocolate samples and the liquor from which they were prepared,
- to carry out statistical analysis of the results and determine whether the levels of alkaloids (or theobromine) has changed significantly over time,
- to recommend a conversion factor for calculation of dry fat free cocoa solids from the measurement of alkaloids present in samples,
- to prepare a standard operating procedure (SOP) for analysis of alkaloids in cocoa and chocolate,
- to provide a reference for a method for measurement of cocoa butter in cocoa and chocolate products.

The review of the different methods of analysis for theobromine in cocoa identified a range of issues, and in many cases the precise description of samples tested was not clear. This is discussed in more detail in the Literature Review section. In order to collect the samples, help was sought from the cocoa manufacturers, Barry Callebaut, who supplies the majority of Europe and the world with raw materials for manufacturing of chocolate and chocolate products. It was agreed at the start of the project that samples of cocoa liquor (made from roasting and grinding of the beans) would be used for analysis of alkaloids, as this was a much more consistent starting product. The levels of moisture in the samples were variable, and so a measurement of water content was also carried out. Fats can be added or abstracted from the liquor during chocolate manufacture and so it was important to check the level of fat present in the liquor samples as well as the alkaloids.

The level of dry fat free cocoa present in a food is generally calculated from the amount of theobromine present. Theobromine is an alkaloid of the xanthine group of compounds, and this group also includes caffeine which also is present in cocoa, but at much lower levels. The term total alkaloids is often used during analysis of cocoa products, this refers to the sum of theobromine and caffeine content. Cocoa solids mainly consists of dry fat free cocoa and cocoa butter (fat obtained from the cocoa beans). The amounts of each of the components vary with the manufacturing process and the manufacturers' recipe. There are many thousands of food products that contain cocoa solids.

Calculation of total cocoa solids can also cause problems. Where milk solids are added to chocolate products, this adds a level of complexity to the measurements, as milk fat will interfere with the measurement of cocoa butter. Furthermore, simply

adding the fat-free dry cocoa and total fat together is not recommended as there is often more than one source of fat present. Vegetable fats, milk and lecithin are routinely added to many products, and these would need to be estimated before calculation of total cocoa solids can be made. A method was sourced, published by M. Buchgraber and S Androni, that has attempted to overcome the problem. This method was published in the report EUR 22666 EN by European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials, Geel, Belgium.

Background

Legislation

The labelling and composition of chocolate products are controlled by the Cocoa and Chocolate Products (England) Regulations 2003 which implement the EC Directive 2000/36/EC. Identical provisions for labelling and composition are included in parallel regulations across the U.K. The regulations specify a range of reserved descriptions for chocolate products which have minimum requirements for levels of cocoa solids (and milk solids) to be present. The specific names used to describe a range of cocoa and chocolate products are known as reserved descriptions. These chocolate products must carry labelling that declares the percentage of cocoa solids and, where appropriate, milk solids in the final food. Many methods have been used by enforcement laboratories over the last 100 years to check the accuracy of the declarations, and these were reviewed as part of this project. The conversion factor used to determine dry fat-free cocoa solids have also changed over the years. There is a need to review and update the factors, in order to have more accurate picture of the levels of the alkaloid marker, theobromine, in chocolate today. As a result of the experimental work described below, we propose a new modified approach to the measurement of dry cocoa solids content, and recommend a conversion factor calculated from these.

Cocoa Cultivation

Cocoa is the dried and partially fermented fatty seed of the cacao tree, *Theobroma cacao*. The seeds grow in pods generally 15 to 20cm long with a thick leathery outer skin. Each pod is filled with sweet pulp enclosing 30-50 large almond shaped beans, which are usually deep purple but can be white, pink or violet depending on the variety, and about 3cm in length. When the pods ripen (changing from a green to yellow colour or from red to orange depending on the variety) they are harvested. Harvesting occurs twice a year. The pods then are opened and the beans extracted with the pulp. The beans and pulp are heaped under banana leaves or placed in fermentation boxes for four to seven days. During this time the pulp ferments and liquefies, and a complex series of chemical reactions leads to the development of flavour pre-cursors which will give rise to the chocolate aromas during roasting. The liquefied pulp (sweating) drains away, leaving the fermented beans. The beans then are dried by the sunshine (or artificially) by spreading out and raking regularly to mix the product. The final moisture content is about 8 per cent.

Cocoa trees only grow successfully within a narrow latitude of the equator, with the majority of world production occurring in western Africa. The major producers in Africa are Ivory Coast, Ghana and Nigeria. Other producing areas are Indonesia, Brazil, Cameroon and Ecuador. There are three main 'types' of cocoa; Forastero, Criollo and Trinitario. The Forastero variety accounts for most of the production, particularly in West Africa. Criollo and Trinitario type cocoas are often lower yielding and/or more difficult to cultivate but they are grown for their specialty flavour characteristics, for example in some regions of South and Central America, the Caribbean, Java and Papua New Guinea. Trinatario is a hybrid of the other two types, and carries varying characteristics of each type.

The Chocolate Manufacturing Process

The manufacturing process involves roasting the beans at a temperature up to 140°C where the moisture content is at least halved. The seed coat (shell) is partially loosened and then removed by winnowing. The roasted nib is ground into a semi-plastic substance known as the 'cocoa liquor' or 'cocoa mass'. At this stage the product contains around 50% cocoa butter. The liquor is often "alkalised" which involves heating with a solution of potassium or calcium carbonate (1%). This is known as the Dutch process. The majority of the remaining moisture is removed by heating to 110°C.

The resulting product then is pressed to remove some of the cocoa butter and then pulverised into cocoa powder. The removal of the cocoa butter is necessary as the resultant slurry is too rich and fat separation occurs. The higher the remaining fat content, the darker the colour of the powder.

The project leader organised a visit to the research laboratories and manufacturing plant of Barry Callebaut (BC) in Brussels, to see the manufacturing process, from beans to the packaged chocolate product. The details of the project were also discussed with BC representatives during the visit, as BC would be supplying the cocoa liquor samples for the project. BC manufacture around 1000 tonnes of chocolate per day and are one of 3 major cocoa producers in Europe. They also supply cocoa to many of the major chocolate manufacturers around the world.

Cocoa beans are delivered to the factory in large sacks from the plantations. They are often blended followed by roasting and winnowing before the roasted nibs are milled in a rotary grinder. The friction produced is sufficient to melt the cocoa fat and liquefy the product to produce cocoa liquor (also known as cocoa mass). The raw ingredients used to make chocolate are cocoa and sugar. Milk solids are added if the final product is milk chocolate. Flavourings and emulsifiers are often added as minor ingredients.

The cocoa liquor is pressed to extract cocoa butter leaving a solid residue called press cake. Press cake is usually kibbled (broken or coarsely ground) to produce cocoa powder. The retained cocoa powder can be mixed with liquor and blended with varying amounts of cocoa fat and other ingredients to produce the different types of chocolate. Typical recipes are:

<u>Plain Dark Chocolate</u>
70% cocoa solids (cocoa liquor and cocoa butter),

29% sugar, vegetable lecithin as emulsifier and vanilla (vanillin) to flavour.

Milk Chocolate
40% cocoa solids (cocoa liquor and cocoa mass),
37% sugar,
20% whole milk powder, lactose, vegetable lecithin as emulsifier and vanilla (or vanillin) as flavouring.

White Chocolate
49% sugar, 33% cocoa butter, 18% whole dried milk and whey powder, vegetable lecithin and vanilla.

Different manufacturers use different variations of the above formulas.

Conching
The blended ingredients go through a grinding and refining process involving heavy rollers. This grinds down and blends the particles to a smooth paste and improves the texture. This is followed by conching. A conch is a container in which the refined and blended chocolate mass is continually kneaded and further smoothed. The frictional heat produced by this process keeps the chocolate liquid and eliminates acetic odours. The length of time given to the conching process determines the final smoothness and quality of chocolate. After the process is completed the chocolate is stored in heated tanks at about 46°C, ready for the final process.

Tempering
Cocoa butter exhibits up to 6 different crystal structures. The chocolate must go through a very precise cycle of heating and cooling to encourage the stable crystal formation needed to produce the desirable properties for nice tasting chocolate. One method is to use melted chocolate at about 46°C, cool to between 29-31°C and warm up again to between 30-32°C. It then can be held 'in temper' at this temperature until needed. The chocolate now is ready for use as couverture, for coating chocolates, chocolate biscuits and other coated products. Alternatively, it is poured into moulds and cooled for sale as the finished product such as solid chocolate bars. Every time it is allowed to harden and is re-melted it will have to be re-tempered again.

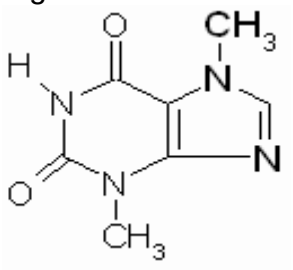
Well tempered chocolate has a shiny gloss, a good snap and a smooth tender melt on the tongue, coating the palate with long lasting flavour.

Literature Review

Compounds of interest

Theobromine ($C_7H_8N_4O_2$), Mol.wt. 180.17

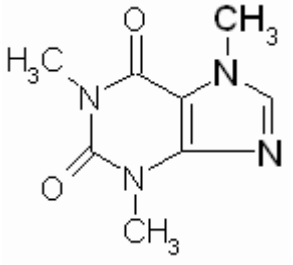
Figure 1



This is the characteristic chemical compound found in cocoa, which is responsible for the bitter taste and may also possess mild stimulation properties. It is a methylxanthine derivative, also known as xantheose, and is closely related to caffeine.

Caffeine ($C_8H_{10}N_4O_2$) Mol.wt. 194.19

Figure 2



Caffeine is also found naturally in cocoa and is a bitter xanthanoid and is also a stimulant and diuretic. It is also known as guaranine.

Historical Methods of Analysis

Various methods of extracting theobromine (and caffeine) from cocoa products have been used over the years. Whereas caffeine is highly soluble in water, theobromine is only sparingly soluble in cold water. However, theobromine is soluble in hot water and mineral acids or alkalis. Due to the nature of cocoa products, where a high fat content is present, some methods use a de-fatting stage prior to extraction of the alkaloids. Theobromine and caffeine are almost insoluble in fat and therefore this stage will have a negligible effect on any subsequent results. The many extraction techniques employed are matched by the many analysis techniques used to measure the active compounds present. A summary of the methods is given below.

- Nollet, Kreiser and Martin[1-2] describe methods where the product is de-fatted in petroleum ether and dried. The resulting solid then is extracted into hot water, filtered and injected onto an HPLC. The column was an RP-C18 using a mobile phase of methanol/water/acetic acid in the ratio of 20:79:1 and quantification is by UV measurement at 280nm.
- MacRae[3] describes a solid phase extraction method that doesn't rely on de-fatting. About 1g of finely ground product is extracted in water at 80°C for 1 hour. This mixture is cleaned up using a Sep-Pak C18 cartridge. The sugars and polar compounds are eluted with 10mL of water. The alkaloids are recovered using an aqueous/methanol/phosphate buffer at pH5.

- Pearson[4] describes a method where 2g of sample is weighed into a 500mL bottle and 270mL of chloroform added followed by 10mL of 10% ammonia solution. The mixture is stoppered and shaken for 5 minutes. Then, 12g of anhydrous sodium sulphate is added and the mixture shaken and allowed to stand overnight. The mixture is filtered and washed with 100mL of chloroform. The solvent is removed and the residue dried. To the residue, 50mL of water is added and boiled. The mixture is cooled and 1-2 drops of 0.1mol sodium hydroxide added to neutralise, using phenol red as indicator and 20mL of 0.1M silver nitrate added. The final mixture is titrated with 0.1M sodium hydroxide. The amount of theobromine present is calculated by using 1mL sodium hydroxide = 18.0mg theobromine.
- The AOAC method[5] involves weighing up to 4.5g of sample into a Teflon tube. The fat then is extracted using 2 portions of 30mL petroleum ether and centrifuging at 2000rpm for 10 minutes. The solvent is decanted and the tube dried. The residue is transferred to a 250mL conical flask with approximately 95mL of water. This then is heated at 100°C for 25 minutes and cooled. The mixture is transferred to a centrifuge tube and centrifuged for 5 minutes at 2000 rpm. The supernatant liquid then is ready for HPLC analysis. This method requires weights to be recorded at each step.
- AOAC has also published other methods.[7,8] These describe extraction using water and acetic acid, hot water and sulphuric acid and a further method for extraction from cocoa using solvent to de-fat followed by hot water alone.
- Davirieux[6] et al uses near-infrared spectrometry as a method for the determination of theobromine and caffeine. This is a rapid non-destructive method used to assess cocoa quality.
- Historically methods published in the Analyst [9,10] describe using chloroform in an alkaline mixture, evaporation and determination of total nitrogen, as well as a conversion of theobromine to caffeine, and analysis of the resulting total alkaloids.
- A further method published in the Analyst describes using a treatment of the chloroform extract with silver nitrate followed by titration[11].
- APA journal published a method (Chapman, Fogden and Urry, 1963)[12] which involves the measurement of total alkaloids by UV spectroscopy after adsorption onto Fuller's earth. The latter separates the active components from the food matrix
- A method by Palson et al[13] uses several water extractions in an ultrasonic bath. The volumes of water uses were very large. This method is generally used to produce alkaloid free cocoa.
- A method by Bigalli et al[14] recommended a relatively low temperature water extraction (45-55°C) followed by one or more extractions in water at 90-105°C. This method uses large amounts of sample and large volumes of water. As above, this was mainly for the removal of theobromine from cocoa to produce alkaloid free cocoa.
- The OICC[15] procedure uses boiling water and clarification of the resulting mixture with lead acetate. The excess acetate is removed and spectrophotometric measurements taken at 245, 272 and 306nm.
- A method carried out by NIST[16] involves melting the chocolate and adding β-hydroxyethyltheophylline as an internal standard. The sample is then de-fatted using hexane and dried under nitrogen. The sample is extracted into water using sonication and then filtered. This is then analysed using HPLC on a C18

column using isocratic elution with a mix of acetonitrile/water and acetic acid. The absorbances were measured at 274nm.

- The method currently used within many public analyst enforcement laboratories has been published in the VEMS catalogue of methods.[17] This uses an homogenised sample which is boiled gently in dilute hydrochloric acid. The mixture is cooled and mixed with Carrez clearing reagents. The mixture then is filtered prior to analysis on the HPLC using an RP 18e column at 40°C. Caffeine and theobromine are measured at 205nm.

Samples of chocolate can be either ground or melted before analysis, results in Table 1 show there is no difference between these preparation procedures. These results corroborate information provided by NIST and are published in AOAC method 970.20.

Previously Reported Levels of Alkaloids in Cocoa and Related Products

Historically, there have been many reported values for theobromine and total alkaloid (i.e. caffeine and theobromine) levels in dry fat free cocoa, cocoa powder, cocoa residue, and cocoa nib. The resulting % theobromine or % total alkaloids were found to vary depending on what was actually being tested and whether caffeine was incorporated into the measurement of alkaloids.

The first report looking at this topic was published in The Analyst in 1894 by Kunze [32]. A digestion of cocoa with acid was carried out, followed by precipitation of the alkaloids and proteins, before separating the alkaloids with barium hydroxide. The alkaloids were extracted with chloroform, and the residue from evaporation analysed for total nitrogen.

In 1921 Wadsworth analysed cocoa nib and cocoa powder by first mixing with water then extraction with tetrachlorethane. Alkaloids were precipitated with ether and total nitrogen determined. Results were quoted as 2.2 - 3.9% in cocoa nib and 3.0 - 3.6 in cocoa powder. Taking the mean of this range would equate to factors of 32.8 and 30.3.

In 1935 Moir and Hinks reviewed previous methods and developed a new method for cocoa nib. Samples were digested with ethanol and magnesium oxide, clarified with ferrocyanide reagents and filtered. Total alkaloids were determined on the filtered material by measuring total nitrogen. The average found was 3.10% in the dry fat free cocoa. This would equate to a conversion factor of 32.3

In 1950 Holmes extracted cocoa residue (after defatting) by mixing with hot water and magnesium oxide 3 times, treating with lead acetate and filtering each time. The filtrate is extracted with chloroform, and evaporated to dryness. The theobromine content is determined on the residue using silver nitrate method. The mean of ten results was 3.11% theobromine. This would equate to a conversion factor of 32.2.

In 1953 Gerritsma and Koers [27] made minor modifications to the extraction method of Holmes and quoted theobromine contents in cocoa powder residues as having a range of 1.50 – 3.12% and a mean of 2.54%, equating to a conversion factor 39.4

In 1963 a new method by Chapman and Fogden used hot water extraction, clarification with potassium ferrocyanide / zinc acetate, and filtration and adsorption of the alkaloids onto fullers earth to extract the alkaloids, and analyse by UV spectroscopy. Three samples of commercial cocoa and three samples of retail cocoa were analysed, results ranged from 2.73 – 3.25%, mean 3.0%. This would equate to a conversion factor of 33.3.

The variety, geography and type of bean all contribute to differing factors. Pusey[18] has reported a mean level of 3.48% theobromine in dry fat free cocoa solids. However, the calculated range was 2.57-5.26%.

Figures quoted in Pearson's Chemical Analysis of Foods 8th Edition: [19]

		Min	Max	Ave
Theobromine %	Cocoa nib	0.8	1.3	1.0
	Cocoa shell	0.2	0.9	0.5
	Commercial Cocoa	0.8	1.6	1.2
Caffeine %	Cocoa nib	0.1	0.7	0.4
	Cocoa shell	0.04	0.3	0.16
	Commercial Cocoa	0.04	0.3	0.2

In 1967 Hadorn and Kleinert [28] quoted theobromine contents in various blends as having a range of 2.95 – 3.22% mean 3.09%, giving a conversion factor of 32.4.

In 1997 Matissek [29] proposed that on fat free dry nibs the theobromine content was 2.85 – 3.63 and caffeine as 0.05 – 0.60%. Analysis of theobromine by HPLC. Using the theobromine content this would equate to a conversion factor of 30.9.

Tables published by the German Confectionery Industry [30] were based on a breakdown of geographical contents based on dry, defatted, fermented nib, and is given below.

	Theobromine%	Caffeine%
Ivory Coast	2.64-3.14	0.20-0.35
Ghana	2.65-3.21	0.21-0.29
Nigeria	2.65-3.44	0.10-0.28
Indonesia	1.83-3.12	0.21-0.83
Malaysia	2.32	0.29
Papua New Guinea	1.93-2.47	0.38-0.56
Cameroon	3.48	0.15
Ecuador	2.04-3.40	0.22-0.78
Venezuela	2.43-2.87	0.63-0.80

The range of theobromine values were found to be very variable for the nib samples tested, perhaps suggesting that geographical origin is an important factor in determining the alkaloid levels in cocoa.

The ICCO [20] quotes chocolate liquor as having 1.22% theobromine and 1.89 – 2.69% in cocoa with cocoa butter containing 0.008% theobromine. Caffeine also is present

and the level is dependent on the degree of fermentation and the type of cocoa beans. The caffeine content was found to vary between 0.1-0.5%.

Personal correspondence supplied by Alan Turner, expert consultant to the chocolate industry, suggested that a conversion factor of 38 should be used when analysing theobromine alone.

Project approach

In this project, the samples tested were cocoa liquor (mass), prepared from beans obtained from different parts of the world. The current methodology was an in-house modification of the method by Chapman and Fogden (1963), with final analysis by HPLC for quantification of theobromine and caffeine separately.

To test the difference between the current methodologies versus old methodology, a ring trial was carried out between four laboratories. Two of the laboratories used the classical UV method (Chapman and Fogden), while results from the other laboratories were obtained using HPLC.

Some Public analyst laboratories measure total alkaloids and use the factor from Chapman and Fogden (33.3). The factor used in Durham (35.9) was derived from this by correcting for measurement of theobromine alone rather than total alkaloids in the 1980's. The original data is no longer available in the laboratory as records were destroyed.

The literature review has highlighted several key issues to consider when comparing the historical data and the results reported in this project. Firstly, the samples tested in the past were not described very clearly; cocoa, cocoa residue, cocoa nib or cocoa powders were descriptions commonly used. A preliminary study will be carried out to examine the difference in alkaloid levels between cocoa nib and cocoa liquor to address this issue. Furthermore, the sample size tended to be quite small, and in many cases only 10 or less samples were analysed. This is not a statistically robust number with which to compare our results. The research described here will involve analysis of a large number of cocoa samples, from around the world collected over different growing seasons, to get a representative collection of data of the chocolate sold in the UK,

The review also highlighted the limitations of comparing historical data, which used different methods of analysis and factor calculations (e.g. using nitrogen content), to the data produced in this report, which specifically measures theobromine content. One therefore has to bear in mind all these issues when comparing the old and new factors.

Methodology

Collaboration on the Project

A vital part of this project was to obtain samples of cocoa from different parts of the world and consider whether different varieties of cocoa bean contained different levels of theobromine. It was also important to assess whether samples could be obtained from different growing seasons.

Arrangements were made with Barry Callebaut (BC), the major cocoa grinder in Europe to collaborate with the contractor on the project. A visit was organised to the factory in Belgium for 30th April 2008 which was attended by the Project Leader, and Senior Analyst. The Project Officer from the Food Standards Agency (FSA) also attended this meeting.

At the meeting it was agreed that BC would contact other cocoa grinders in Europe to request their cooperation in obtaining sufficient samples for the project and request their participation in a ring trial of methods currently used by the different companies and the Project Leaders' enforcement lab in the UK. The cocoa grinders ADM and Cargil agreed to provide samples in addition to those from BC and ensured the success of the project.

Cocoa Sampling

It was agreed with Barry Callebaut that the best samples to test would be the cocoa liquors rather than the nibs, as the latter were considered to produce more variable results. In addition, the industry uses the results from analysis of the liquor for their quality control checks on the product label declarations. Cocoa nib is the remaining part of the cocoa bean, often referred to as the kernel, once the husk has been removed after roasting. Cocoa liquor is prepared by finely grinding cocoa nibs, and typically contains between 50 percent and 60 percent by weight of cocoa fat. To check the difference between the two sample types, a small number of cocoa nibs were requested from BC. The ten cocoa nibs were prepared in-house, and analysed for moisture, fat, caffeine and theobromine content, and the factor calculated.

BC made arrangements for 200 samples of cocoa liquor to be sent to the contract laboratory for analysis. There are two cocoa crops each year, one of these is considered the main crop, producing the highest yields and the other the minor crop. It was agreed to analyse cocoa from main and minor crops as follows:

a. 152 samples from the main crop
b. 48 samples from the minor crop

About 200g was considered to be a representative sample of liquor and all samples would be labelled with the origin and an indication of whether it was main or minor crop.

It was planned that each of these groups would consist of (pure) geographical origin samples the proportions of which would mimic, as much as possible, the market. These samples would then be grouped together, for the analysis of the data, into four

global regions - West Africa, Pacific, Caribbean, and South America. The results were not as straight forward to evaluate because of the blending of beans from main and minor crops during the manufacturing process.

All samples were stored at ambient temperature.

Chocolate Sampling

In order to determine whether there were any effects on theobromine recovery from the final chocolate products five samples of chocolate at five different concentrations would be made up at BC (giving a total of 25 chocolate samples). Samples of both cocoa liquor, used to make up the chocolates, and the finished chocolate samples (min 400g) were to be sent to the contract laboratory for analysis. The five concentrations would range from 15/20% to 85% cocoa solids. In the end, 20 samples chocolate and cocoa liquors were supplied; however 6 of these were not made from cocoa liquor alone, as they had varying amounts of cocoa powder added. The composition of the cocoa powders was not available, nor were there any samples for testing. Therefore 14 samples of chocolate and corresponding cocoa liquors were used for comparisons. The final amounts of cocoa solids in the samples of chocolate varied from 7-70%.

Ring Trial

The cocoa manufacturer Barry Callebaut (BC) organised and distributed six homogenised cocoa liquor samples to each of the laboratories: BC, Durham, ADM and Cargill. The samples were analysed for fat, caffeine and theobromine, using the laboratories' own routine methods for extraction and analysis of the analytes. An added advantage of the laboratories using their own methods was that there would be an extensive range of experience available for the various tests. In order to make sure that laboratories were measuring accurately it was agreed that NIST Standard Reference Material 2384 (baking chocolate) be analysed alongside samples. This standard had certified reference values for theobromine and caffeine. The reference values are shown in Table 1.

The results obtained from the four laboratories were compared. This part of the project was carried out first to ensure consistency in the results for the different methods used. After the initial results were obtained an additional laboratory, Institut für Qualitätsförderung (IQ Koln), which specialised in chocolate and cocoa analysis, requested for samples to analyse in their laboratories. They analysed all samples using both UV (using the official IOCCC method) and HPLC analysis. These results are summarised in Appendix 1 (Tables 1, 1(a) and 1(b)), together with the initial ring trial results from June 2008 and the statistical analysis data.

Contractors Method

The Project Leader provided BC with a copy of a method used currently for measuring additives and flavourings in foods by HPLC, a copy is shown in Appendix 2. The method was suitable for routine measurement caffeine and theobromine in foods. The proposed approach was to modify the method and create an SOP which was a simplified version of this general method.

The routine additives method in use at the Project laboratory, DCC/F/0358, was modified slightly to improve separation of theobromine and caffeine from other additives that are permitted to be present in cocoa and chocolate products. This ensured that the main analytes of interest were separated from permitted sweeteners, preservatives and flavourings. Minor changes were made to the HPLC operating conditions to achieve this. Moisture levels were found to vary between different samples of cocoa liquor; results were corrected for moisture content to determine the true levels of theobromine and caffeine in the dry liquor. Moisture was measured using the standard Karl Fischer technique.

The final SOP, as detailed in Appendix 7, was used in the ring trial alongside other methods currently in use by cocoa manufacturers' own laboratories to measure levels of theobromine, caffeine and fat present in cocoa liquor samples.

Historical Data

In addition to the literature review of historical alkaloids data, additional information was identified from two different sources through private correspondence; Reinhard Matissek (LCI, Germany)**,** and Emile Cros (CIRAD, France). Research data from both sources seemed to indicate that the levels of alkaloids in cocoa varied, depending on influencing factors such as geographical origin and genotype of the cocoa, as well as cultivation and manufacturing conditions. It was decided that the data would not be added to the authentic data set obtained in this project, as the information was considered to be too old in the case of the Matissek review. The CIRAD data, presented in figures 4 and 5 (Appendix 3), showed a correlation in the theobromine/caffeine ratios between five different cocoa types (three single genotypes and two hybrid varieties). However, the information could not be incorporated into this project, because the research is still ongoing, and the results did not incorporate % fat calculations, as carried out in this project. The influence of variety on theobromine levels was investigated, and is discussed in the Results and Discussion section in this report.

Calculation of conversion factor to determine cocoa solids content

A conversion factor of 33.3 has been used by enforcement laboratories in the UK for many years to calculate the amount of dry fat free cocoa present in cocoa and chocolate products. Traditionally the factor was derived from the accepted average level of total alkaloids (theobromine and caffeine) present in cocoa. As outlined in the objectives, the aim is to recommend a new factor, taking into account theobromine and caffeine levels in cocoa today, compared with levels evaluated 50 years ago to give the current factors. The main problem in this project has been identifying what form of cocoa was used to measure this as some publications refer to analysis of cocoa powder, some to cocoa nib and others simply to cocoa. These descriptions have been inadequate for today's needs and so the current factor is in need of further definition. The work carried out in this project refers to the measurement of the levels of theobromine and caffeine, in prepared liquor samples obtained by grinding the roasted nibs. This approach was ratified by the collaborators from the commercial research laboratories as the best approach to ensure the authenticity of the results. Results obtained for theobromine and caffeine in liquor samples at the point of production are used to calculate the cocoa solids going into the foods. This in turn determines the declarations made on the labels.

The current factors have been calculated using the formulas below.

(1) Factor from theobromine $\frac{100}{\text{Mean theobromine (mg/kg)}} \times 10^4 = 35.9$

(2) % dry fat free cocoa = theobromine content (%) x 35.9

(3) Factor from total alkaloids $\frac{100}{\text{Mean total alkaloids (mg/kg)}} \times 10^4 = 33.3$

(4) % dry fat free cocoa = total alkaloids content (%) x 33.3

Results and discussion

Ring Trial

The aim of the ring trial was to assess the robustness of the contract laboratory's method against other established methods for extraction and analysis of alkaloids in cocoa. It was agreed from the outset that it would be most appropriate to seek participation from the laboratories in the three major European cocoa production companies, BC, ADM and Cargill, which routinely carry out the analysis of the chocolate as part of the manufacturing process. Each laboratory used their own methods for analysis of six cocoa liquor samples (melted and grated) from different regions of the world (Table 1).

One of the manufacturing laboratories (lab 3) used a method for total alkaloids by UV spectroscopy while the other three used HPLC to separate and quantify theobromine and caffeine separately. The outcome of the ring trial is detailed in Table 1, 1(a) and 1(b) of Appendix 1. The results for theobromine and total alkaloids on each of the 6 cocoa liquor samples were in close agreement. In the case of theobromine all of the results were within 12% of the robust mean and within 6% of the robust mean in the case of caffeine. Therefore the methods could be regarded as sufficiently robust to proceed.

After testing had been completed, an additional research laboratory, IQ Koln, submitted results from the analysis of the six cocoa liquor samples using both classical UV and HPLC methods. Their results of analysis showed that all six of the samples fell within the range of results reported by the other laboratories.

It was observed that the set of results for the theobromine analysis were not as statistically similar as for the results for caffeine and fat levels. This was due to the low level of caffeine in the samples compared to the theobromine content. Overall, it can be concluded that the contract laboratory method for extraction and measurement of alkaloids using HPLC, was fit for purpose, and produced equivalent results when compared to the other four labs. The results for fat analysis also showed good correlation between all laboratories.

In order to ensure accuracy of the measurements for theobromine and caffeine, the laboratories taking part in the ring trial all analysed the same standard reference

material and the results were compared with the certified values for NIST Standard Reference Material 2384 (baking chocolate). Again there was good agreement with the certified values which were supplied with the reference material.

A summary of ring trial results and statistical analysis is shown in Tables 1, 1(a) and 1(b) in Appendix 1.

Optimum sample type (Cocoa Nib Analysis)
Results of the analysis of the six cocoa nib samples (which were the beans prior to roasting and grinding), as shown in Table 3, indicated a wide variation in alkaloid levels. This confirmed trade colleagues' recommendations to assess cocoa liquor samples, which were regarded as a more reliable sample type for determining theobromine levels in cocoa. The nib samples were prepared by blending in a coffee grinder prior to analysis.

Cocoa sample analysis
The samples of cocoa liquor were analysed in batches, as received from BC over a period of two years. This was to ensure that beans from different growing seasons were included and also to collect a good population of samples from different continents. A total of 191 samples of cocoa liquor were analysed. The results obtained are summarised in Table 2 in Appendix 4. The results of laboratory AQC are summarised in Appendix 5. The AQC duplicate samples were generated and analysed randomly at a rate of 10% of the population or one with every batch where less than 10 were analysed. No data has been excluded and information from the AQC showed that the analysis was accurate and the information being collected was valid.

The results from the liquor analyses indicated that there was no clear link between alkaloid levels in the samples and the growing area or season in which they were harvested. There was some evidence of correlation of results when separated into different continents (Appendix 4 Figures 6-9).

Moisture content analysis
In order to calculate dry fat-free cocoa solids content it was necessary to measure the water content in the samples of liquor. The liquor samples were analysed by the Karl Fischer method, following an initial investigation which found this method to be more accurate in determining water content compared to oven drying. Comparison of methods in seven different cocoa liquor samples showed an average difference of 0.35%, with oven drying producing consistently higher results due to volatiles being removed from the samples as well as water. For this project, water analysis by Karl Fischer was used to ensure an accurate database of reference values. The results are incorporated into the final calculation of the factors in Appendix 4.

Statistical analysis
The project work was carried out mainly for the benefit of enforcement laboratories though it was necessary to work closely with producers in Europe. Research has showed that over 80% of chocolate made for the EU market is sourced from Africa. However, blending does occur at the grinding stage so a mixture could well be present in the finished products.

Enforcement laboratories will generally not know the precise origin of the cocoa used in the manufacture of the chocolate product. As a first action, it is recommended to use the results from this project to calculate an average conversion factor that will be used to calculate dry fat free cocoa.

In some cases premium chocolate declares the country of origin in the name of the food, eg Venezuela, Ecuador or Papua New Guinea. In these circumstances enforcement laboratories can take account of different results obtained from those continents and use a conversion factor that's more appropriate than an average factor.

All of the samples of cocoa liquor provided for analysis were assumed to be genuine and arising from the location indicated by the suppliers. For this reason it was deemed that no results could be excluded in the calculation of statistical data.

For the purposes of enforcement a simple statistical analysis approach was taken where an overall average factor was calculated. This was supported by calculation of range and standard deviation. Further calculations were done to assess the confidence intervals of the data. The results showed that whilst there was a fairly wide range between maximum and minimum figures, they were close to a normal distribution, Appendix 4, Figure 10. The chart in Figure 11 shows the relationships between the theobromine and caffeine contents which can vary depending upon the fermentation and production processes.

Summary of Cocoa Liquor Results

Mean Theobromine content	24,572 mg/kg (n=191)
Standard deviation	2,900 mg/kg
Confidence interval (95%)	24,161 – 24,988 mg/kg
Confidence interval (99%)	24,031 – 25,118 mg/kg
Range	17,700 – 31,329 mg/kg
Mean Caffeine content	3,165 mg/kg (n=191)
Standard deviation	1,268 mg/kg
Confidence interval (95%)	2,980 – 3,341 mg/kg
Confidence interval (99%)	2,923 – 3,397 mg/kg
Range	1,262 – 8,742 mg/kg
Mean Total alkaloids content	27,737 mg/kg (n=191)
Standard deviation	3,470 mg/kg
Confidence interval (95%)	27,240 – 28,229 mg/kg
Confidence interval (99%)	27,085 – 28,384 mg/kg
Range	19,803 – 39,168 mg/kg

Conversion factor

As discussed previously, the conversion factor will vary depending upon whether the laboratory measures theobromine or total alkaloids. For this reason factors have been calculated for both instances. The factors have been amended from those specified in equations (1)-(4).

(5) Factor from theobromine $\frac{100}{\text{Mean theobromine (mg/kg)}} \times 10^4 = 40.7$

(6) % dry fat free cocoa = theobromine content (%) x 40.7

(7) Factor from total alkaloids $\frac{100}{\text{Mean total alkaloids (mg/kg)}} \times 10^4 = 36.1$

(8) % dry fat free cocoa = total alkaloids content (%) x 36.1

For the purposes of enforcement the confidence interval will always be taken into consideration when considering whether to report against a declaration of cocoa solids. The range of factors calculated from the confidence intervals are

Theobromine (95%)	40.0 – 41.4
Theobromine (99%)	39.8 – 41.6
Total Alkaloids (95%)	35.4 – 36.7
Total Alkaloids (99%)	35.2 – 36.9

The factor currently used for conversion of theobromine to dry fat free cocoa solids is 35.9. This replaced the previous factor of 33.3, which was calculated around 50 years ago from average content of total alkaloids, analysed using UV spectrometry. Therefore it can be concluded that on average the amount of theobromine observed has changed and now appears to be naturally present at lower levels.

Analysis of Chocolate Samples

The aim of this exercise was to firstly assess whether there were any effects on theobromine recovery from chocolate, and secondly to compare the current factor (35.9) against the new calculated factor (40.7), and assess which gave the closest results for dry fat-free cocoa solids content to the levels declared by BC, who prepared the chocolate samples.

Each of the chocolate samples was made from corresponding cocoa liquors (paired results in the table). The total dry fat free cocoa was calculated from the total theobromine content of the chocolate divided by the total theobromine content of the dry cocoa liquor. The total cocoa solids were then calculated by adding the total fat in the chocolate. This will be reliable unless non-cocoa fat is present and corrections must be allowed for.
DFFC = total theobromine in sample / theobromine in dry fat free cocoa liquor x 100

The results are shown in Table 5 of Appendix 6 and it was clear that there was good correlation between the theoretical amount of cocoa liquor present and the amount calculated from analysis. There was a small but consistent positive bias on the amount found compared to the declaration of 2-3%. It was clear from the results that the fat present in the chocolate was not only from the cocoa liquor added but was supplemented, presumably by other cocoa fat to obtain a product with the acceptable properties.

As outlined in the methodology section, 20 different samples of chocolate products with known levels of cocoa were analysed blind, alongside the corresponding cocoa liquors that were used in their manufacture. The results are shown in Table 6 of Appendix 4. Calculations were carried out for cocoa content from measurements of theobromine levels in the cocoa liquors and the chocolate products and these were compared with declared values and with results that would have been reported using the current and new conversion factors.
i.e.
dry fat free theobromine in the sample / dry fat free theobromine in liquor x 100, and
dry fat free theobromine in sample x 35.9, and
dry fat free theobromine in sample x 40.7, and
dry fat free theobromine in sample x (40.7 + 5), and
dry fat free theobromine in sample x 40.7 – 5).

Following analysis it was established that six of the chocolate samples had additional ingredients present, including cocoa powder that would interfere with the interpretation of the results. The results from these samples were excluded from the exercise. This came to light only when sample details were checked.

In 18 cases out of 20 the new factor gave better results than the current factor for the cocoa liquor (which should give 100% as it is all cocoa). In comparing results for the chocolate products the new factor gave results closer to the expected amount in 17 cases out of 20. Cocoa contents varied from 7 – 70%. These results are detailed in Table 6.

In practice Public Analysts would rely upon the factor to calculate the amount of dry fat free cocoa in a food from theobromine. It has been shown that using the new factor, results were found to be closer to the declared amount added. However, the difficulties in interpretation of results based upon average factors needs to be considered, and the Public Analyst will take these limitations into account in the reporting of samples. Allowances for natural variation and statistical errors are a normal part of the interpretation and adverse reports will generally not be issued if there is sufficient doubt over the results. In many cases further investigations are recommended where manufacturers are requested to provide evidence of compliance testing in support of a defence. The investigating officer will decide in consultation with the Public Analyst whether the defence is sufficient evidence of the facts.

Effect of variety on results

A growing number of cocoa and chocolate products are now being marketed as high quality, speciality products, being derived from named varieties of cocoa bean. These are generally made from the high quality Criollo bean. Other quality products are made using the variety Trinatario which is a hybrid of the Criollo and Forestero beans. The cocoa liquor samples analysed during the course of this project were not identified as belonging to any particular variety of bean. Consideration was therefore given to the possibility that the levels of theobromine and caffeine were different in the different varieties of bean. Discussions with colleagues working with plantations in St Lucia and Trinidad showed that it would be difficult to obtain pure genotype samples for the analysis of alkaloids. Specialist varieties of cocoa bean are sometimes grown in part of the plantation that also grows other varieties. Cross fertilisation of varieties

through pollen transfer is a recognised problem. Trinatario was originally a distinct hybrid of Criollo and Forestero but now, through years of cross pollination it contains varying amounts of genetic information from both plants. There is therefore a likelihood that samples obtained from adjacent trees could contain different amounts of genetic traits that would affect any interpretation of results. It was finally concluded that without genetic information on the beans used to manufacture the cocoa liquor any results obtained using this approach could be heavily flawed. Genetic analysis was outside the scope of this project. Information from Matissek, shown in Appendix 3 demonstrates that there is likely to be varying amounts of alkaloids in the different varieties and that there are overlaps where genetic changes may have occurred.

Measurement of total nitrogen

The early research which provided the basis for the current factor involved extraction of total alkaloids, after precipitation of interfering substances and analysis of total nitrogen using the Kjeldahl method[9,23,32]. In order to compare the new factor calculated in this project, and the current factor, it was agreed to carry out analysis for total nitrogen on 24 liquor samples taken at random from the collection. The results of this analysis showed that there was no correlation between total nitrogen and theobromine content due to interference from proteins and other nitrogen containing compounds naturally present. The results are shown in Appendix 9. Due to the time limitation of the project, it was not possible to carry out a more comprehensive examination into this issue.

Conclusions

The project contains a summary of a literature review of methods for analysing cocoa and chocolate. Samples for testing were obtained from the three main cocoa producers in Europe. Analysis was carried out for theobromine, caffeine, fat and moisture. These were used to calculate a conversion factor for reporting dry fat free cocoa in cocoa and chocolate products for the purposes of food enforcement. Results of analysis from 191 cocoa liquor samples from a variety of sources around the world showed that the amounts of alkaloids present were generally lower than results in previous reports published over the last 100 years. The overall average conversion factor for theobromine content to dry fat free cocoa solids was found to be 40.7, and if total alkaloids are measured the average conversion factor was 36.1.

There was some correlation seen in the theobromine levels from cocoa of different geographical origin, by both country and continent. Conversion factors for different countries may therefore be assigned to enable more accurate DFFC content to be calculated. However, this would be more difficult to calculate in some samples due to the unknown origin of the cocoa used, and also may not be relevant in cases of production of bulk chocolate where cocoa beans of different origin is mixed.

The use of theobromine and total alkaloids for calculation of dry fat free cocoa in chocolate samples both gave results that were fit for purpose and in general agreement with the declarations given by the manufacturer providing the samples. It is therefore concluded that it makes no difference as to whether calculations are made from theobromine or total alkaloid measurements, as long as the relevant factor is used.

The standard operating procedure in Appendix 7 may be used to measure theobromine and caffeine by HPLC, and use the results used to calculate dry fat free cocoa in foods. It has been shown that no additives that are permitted to be present in cocoa and chocolate products will interfere with the chromatography using the method as described.

The addition of cocoa fat, and other vegetable fats to liquor in the manufacture of cocoa and chocolate products should be recognised. A reference method is available for measurement of cocoa butter equivalents in cocoa and chocolate products[31]. The method will allow the analyst to estimate amounts of non-cocoa fats present. These non-cocoa fats must not be included in the calculation of total cocoa solids, and so it is strongly recommended that fat, and cocoa fat are measured alongside alkaloids in the final calculation of DFFC.

Public Analysts in the UK and other food enforcement analysts should take note of the findings in this report. The proposed method of testing has been shown to give results for theobromine and total alkaloids that are in close agreement with other routine methods currently in use by cocoa manufacturers. A collaborative trial has not been commissioned and this should be considered as possible future work.

Acknowledgements

ADM for provision of authentic samples of cocoa liquor and participation in the ring trial

Alan Turner, consultant to the chocolate industry for providing historical information

Barry Callebaut for provision of authentic samples of cocoa liquor, chocolate and participation in the ring trial

Cargil for provision of authentic samples of cocoa liquor and participation in the ring trial

CIRAD for advice on interpretation of results particularly in relation to genotypes

Cocoa Research Association Ltd for advice on technical details

Food Standards Agency for the funding of this project

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APPENDIX 1
SUMMARY OF RING TRIAL DATA

Table 1 - Ring Trial Data

SUMMARY OF RESULTS FROM THE RING TRIAL (JUNE 2008)

ORIGIN	SAMPLE LABREF		THEOBROMINE				CAFFEINE				TOTAL ALKALOID				FAT			
			Durham	Lab 2	Lab 3	Lab 4	Durham	Lab 2	Lab 3	Lab 4	Durham	Lab 2	Lab 3	Lab 4	Durham	Lab 2	Lab 3	Lab 4
VENEZUELA	20090187	A	1.18	1.17	-	1.22	0.26	0.27	-	0.24	1.45	1.44	1.54	1.46	50.5	50.82	50.47	50.64
		B	1.18	1.26	-	1.26	0.26	0.29	-	0.25	1.44	1.55	1.57	1.51	50.3	51.15	50.45	50.94
PAPUA NEW GUINEA	20090188	A	1.04	1.07	-	1.10	0.15	0.16	-	0.14	1.19	1.23	1.30	1.24	51.3	52.83	52.05	52.67
		B	1.02	1.11	-	1.05	0.15	0.16	-	0.13	1.16	1.27	1.31	1.18	51.0	52.84	52.03	52.32
ECUADOR	20090189	A	1.15	1.18	-	1.20	0.19	0.21	-	0.19	1.35	1.39	1.44	1.39	51.5	52.38	51.77	52.09
		B	1.15	1.27	-	1.21	0.19	0.22	-	0.18	1.34	1.49	1.43	1.39	51.4	52.35	51.72	51.91
SAN TOME	20090190	A	1.19	1.23	-	1.25	0.10	0.11	-	0.10	1.30	1.34	1.40	1.35	50.0	50.12	50.73	47.94
		B	1.17	1.39	-	1.25	0.10	0.12	-	0.10	1.27	1.51	1.41	1.35	50.0	50.53	50.76	47.61
MADAGASCAR	20090191	A	1.12	1.15	-	1.18	0.16	0.18	-	0.15	1.28	1.33	1.41	1.33	51.6	52.33	51.68	51.67
		B	1.11	1.23	-	1.15	0.16	0.18	-	0.15	1.27	1.41	1.41	1.30	51.6	52.13	51.43	51.72
TANZANIA	20090192	A	1.20	1.29	-	1.20	0.16	0.17	-	0.13	1.36	1.46	1.48	1.33	56.0	55.94	55.06	55.87
		B	1.16	1.26	-	1.22	0.15	0.17	-	0.13	1.32	1.43	1.49	1.35	55.9	56.36	55.56	55.74
NIST 2384	MELTED	A	1.11				0.11				1.22				50.4			
	GRATED	B	1.10				0.11				1.21				50.6			

Reference values for NIST CRM 2384

Theobromine %	1.16	±	0.11
Caffeine %	0.106	±	0.05
Fat (Extractable) %	51.4	±	1.10

NOTE: Lab 3 measured total alkaloids by spectrophotometric method

Table 1(a) Statistical analysis of ring trial data

ORIGIN	THEOBROMINE				CAFFEINE				TOTAL ALKALOID				FAT			
	Max	Min	Ave		Max	Min	Ave		Max	Min	Ave		Max	Min	Ave	
VENEZUELA	1.26	1.17	1.21		0.29	0.24	0.26		1.57	1.44	1.49		51.15	50.30	50.69	
PAPUA NEW GUINEA	1.11	1.02	1.06		0.16	0.13	0.15		1.31	1.16	1.23		52.84	51.00	52.20	
ECUADOR	1.27	1.15	1.19		0.22	0.18	0.20		1.49	1.34	1.40		52.38	51.40	51.91	
SAN TOME	1.39	1.17	1.25		0.12	0.10	0.11		1.51	1.27	1.36		50.76	47.61	49.32	
MADAGASCAR	1.23	1.11	1.16		0.18	0.15	0.16		1.41	1.27	1.34		52.33	51.43	51.76	
TANZANIA	1.29	1.16	1.22		0.17	0.13	0.15		1.49	1.32	1.39		56.36	55.06	55.80	

Table 1(b) Further Ring Trial Data Results from IQ Koln

ORIGIN	THEOBROMINE	CAFFEINE	Caffeine + Theobromine	TOTAL ALKALOID	FAT
VENEZUELA	1.201	0.259	1.460	1.527	48.73
	1.197	0.261	1.458	1.536	48.98
PAPUA NEW GUINEA	1.034	0.146	1.180	1.241	52.30
	1.036	0.147	1.183	1.282	52.49
ECUADOR	1.172	0.191	1.363	1.442	51.47
	1.179	0.191	1.370	1.443	51.73
SAN TOME	1.183	0.100	1.283	1.419	50.19
	1.188	0.106	1.294	1.425	49.97
MADAGASCAR	1.121	0.158	1.279	1.381	51.36
	1.129	0.154	1.283	1.385	51.48
TANZANIA	1.212	0.144	1.356	1.442	55.66
	1.213	0.147	1.360	1.443	55.35

APPENDIX 2
DCC/F/0358

THE DETERMINATION OF ADDITIVES AND FLAVOURINGS IN FOODS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (GENERAL METHOD)

1. SCOPE AND FIELD OF APPLICATION

This method describes a procedure for the determination of artificial sweeteners, preservatives and flavourings in soft drinks, cakes and confectionery, as well as caffeine and theobromine in chocolate and chocolate products.

2. DEFINITION

For the purpose of this method, the additives and flavourings content of foods and drinks are those extracted and determined under the conditions specified.

3. PRINCIPLE

The analytes are extracted by the appropriate method:

(a) soft drinks are analysed directly after appropriate dilutions;
(b) foods are acidified and extracted with an aqueous methanol solution;
(c) chocolate products are extracted with dilute acid.

After filtration the additive and/or flavouring content of the solution is determined by HPLC.

4. HEALTH & SAFETY

4.1 EYE PROTECTION SHOULD NORMALLY BE WORN AT ALL TIMES.

4.2 METHANOL IS HIGHLY FLAMMABLE AND TOXIC BY INHALATION OR IF SWALLOWED. KEEP CONTAINER TIGHTLY CLOSED. AVOID CONTACT WITH SKIN. KEEP AWAY FROM SOURCES OF IGNITION. USE ONLY IN A DESIGNATED FLAME FREE AREA.

4.3 ACETONITRILE IS HIGHLY FLAMMABLE AND TOXIC BY INHALATION, IN CONTACT WITH SKIN AND IF SWALLOWED. KEEP AWAY FROM SOURCES OF IGNITION. TAKE OFF IMMEDIATELY ANY CONTAMINATED CLOTHING. IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE. USE ONLY IN A DESIGNATED FLAME FREE AREA.

4.4 CAFFEINE IS TOXIC IF SWALLOWED. AVOID CONTACT WITH SKIN AND EYES. IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE.

4.5 QUININE SULPHATE IS TOXIC IF SWALLOWED. AVOID CONTACT WITH SKIN AND EYES. IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE.

5. PRE-TRAINING REQUIREMENTS

5.1 Use of analytical balance.

5.2 Use of volumetric glassware.

5.3 Use of pH meter.

5.4 Use of filtration apparatus.

5.5 Use of HPLC system.

6. REAGENTS

6.1 Analytical Reagent (AR) grade reagents are suitable unless otherwise stated.

Water should be deionised, distilled or of similar quality.

6.2 Methanol, HPLC grade.

6.3 Acetonitrile, HPLC grade. Degas and filter through a 0.45 µm organic filter.

6.4 Sodium dihydrogen orthophosphate.

6.5 Caffeine.

6.6 Acesulfame K.

6.7 Aspartame.

6.8 Benzoic acid.

6.9 Sorbic acid.

6.10 Saccharin.

6.11 Theobromine.

6.12 Quinine sulphate.

6.13 Methylparaben (Methyl 4-Hydroxybenzoate).

6.14 Ethylparaben (Ethyl 4-Hydroxybenzoate).

6.15 Propylparaben (Propyl 4-Hydroxybenzoate)

6.16 Hydrochloric acid (S.G. 1.18).

6.17 Sodium hydroxide.

6.18 0.1mol/L sodium hydroxide. Weigh 4.0g of sodium hydroxide (6.17) and dissolve in water, allow to cool, and dilute to 1 litre.

6.19 Phosphate buffer. Accurately weigh 3.12g of sodium dihydrogen orthophosphate (6.4) and make up to 1 litre with water. Adjust to Ph 5.0 with 0.1mol/L sodium hydroxide (6.18). Filter through a 0.45 µm filter (7.3)

6.20 50Ml/100Ml methanol. Dilute equal volumes of methanol (6.2) and water as required.

6.21 3mol/L Hydrochloric acid. Add 267Ml of hydrochloric acid (6.16) to water and dilute to 1 litre.

6.22 0.5mol/L Hydrochloric acid. Add 44.5Ml of hydrochloric acid (6.16) to water and dilute to 1 litre.

6.23 Stock standard additive and flavouring solutions (Shelf life 3 months, except sorbic acid and aspartame – prepare fresh when needed).

6.23.1 Caffeine 1000mg/L. Weigh 0.5g, accurate to 0.001g, of caffeine (6.5). Transfer quantitatively into a 500Ml volumetric flask with water. Dissolve in water, dilute to volume with water and mix.

6.23.2 Acesulfame K 1000mg/L. Weigh 0.5g, accurate to 0.001g, of acesulfame K (6.6). Transfer quantitatively into a 500Ml volumetric flask with water. Dissolve in water, dilute to volume with water and mix.

6.23.3 Aspartame 5000mg/L. Weigh 0.5g, accurate to 0.001g, of aspartame (6.7). Transfer quantitatively into a 100Ml volumetric flask with water. Dissolve in water, dilute to volume with water and mix.

6.23.4 Saccharin 1000mg/L. Weigh 0.5g, accurate to 0.001g, of saccharin (6.10). Transfer quantitatively into a 500Ml volumetric flask with water. Dissolve in water, dilute to volume with water and mix.

6.23.5 Quinine sulphate 1000mg/L. Weigh 0.5g, accurate to 0.001g, of quinine sulphate (6.12) into a 100Ml beaker, dissolve in

water with heating, cool and transfer quantitatively into a 500Ml volumetric flask with water. Dilute to volume with water and mix.

6.23.6 Theobromine 500mg/L. Weigh 0.25g, accurate to 0.001g, of theobromine (6.11) into a 400Ml beaker, dissolve in boiling water, cool and transfer quantitatively into a 500Ml volumetric flask with water. Dilute to volume with water and mix.

6.23.7 Benzoic acid 1000mg/L. Weigh 0.5g, accurate to 0.001g, of benzoic acid (6.8) into a 100Ml beaker, dissolve in about 50Ml of methanol (6.2) transfer quantitatively to a 500Ml volumetric flask with methanol and dilute to volume with water and mix.

6.23.8 Sorbic acid 1000mg/L. Weigh 0.5g, accurate to 0.001g, of sorbic acid (6.9) into a 100Ml beaker, dissolve in about 50Ml of methanol (6.2), transfer quantitatively to a 500Ml volumetric flask with methanol and dilute to volume with water and mix.

6.23.9 Mixed Paraben standard 1000mg/L. Weigh 0.5g, accurate to 0.001g, of each Parabens (6.13, 6.14, 6.15,) into separate 100Ml beakers, dissolve each in about 50Ml of methanol (6.2), transfer quantitatively to a 500Ml volumetric flask with methanol and dilute to volume with water and mix.

6.24 Working standard solutions

Using pipettes measure 1.0, 2.5, 5.0 and 10.0Ml (2, 5, 10 and 20Ml of theobromine) of each stock standard required, into separate 100mL volumetric flasks, dilute to volume with water and mix. This gives working standard solutions of concentrations 10, 25, 50 and 100mg/L of each additive except aspartame (50, 125, 250 and 500mg/L). Working standard solutions should be prepared fresh on the day of use.

6.25 Stock standard control solutions

STANDARD MATERIAL USED TO PREPARE STANDARD CONTROL SOLUTIONS MUST BE FROM A DIFFERENT COMMERCIAL SOURCE TO THOSE USED TO PREPARE STANDARD CALIBRATION SOLUTIONS.

6.25.1 Caffeine 10,000mg/L. Weigh 0.5g accurate to 0.001g of caffeine. Transfer quantitatively to a 50mL volumetric flask with 20mL of methanol and dissolve. Dilute to volume with water and mix.

6.25.2 Acesulfame K 10,000mg/L. Weigh 0.5g accurate to 0.001g of acesulfame K. Transfer quantitatively to a 50mL volumetric

flask with 20mL of methanol and dissolve. Dilute to volume with water and mix.

6.25.3 Benzoic Acid 10,000mg/L. Weigh 0.5g accurate to 0.001g of benzoic acid. Transfer quantitatively to a 50mL volumetric flask with 20mL of methanol and dissolve. Dilute to volume with water and mix.

The shelf life of these solutions is 3 months when stored at 5°C.

6.26 Working standard control solution

Using a pipette, measure 2.5Ml of each stock standard control solution (6.25.1, 6.25.2 and 6.25.3) into the same 250Ml volumetric flask, dilute to volume with water and mix.

This gives a mixed, working standard control solution containing 100mg/L of each additive.

The working standard control solution should be prepared fresh on the day of use.

6.27 Zinc acetate dihydrate.

6.28 Glacial acetic acid.

6.29 Potassium ferrocyanide trihydrate.

6.30 Clearing reagents 1 and 2

1. Dissolve 21.9g, accurate to 0.1g, zinc acetate dihydrate (6.27) in water containing 3g of glacial acetic acid (6.28) and make up to 100mL with water.

2. Dissolve 10.6g, accurate to 0.1g, potassium ferrocyanide trihydrate (6.29) in water and make up to 100mL with water.

7. APPARATUS

7.1 Normal laboratory glassware and apparatus.

7.2 Analytical balance of appropriate accuracy as specified.

7.3 0.45µm disposable syringe filters or 0.45µm sample filter kit (millipore or equivalent).

7.4 Solvent filter system with 0.45µm membrane filters.

7.5 Ultra sonic bath.

7.6 High Performance Liquid Chromatography (HPLC) system ideally with Diode Array Detector and integrating device which allows the measurement of peak heights or areas.

7.7 Glass microfibre filters, at least 1.6 µm GFA or equivalent.

7.8 HPLC Chromatographic column such as Merck Lichrocart Purospher RP-18e, 5µm, 250 x 4mm, fitted with a Purospher RP-18e, 5µm 4 x 4mm guard column, equivalent columns may be used, provided they give satisfactory resolutions.

The following HPLC conditions have been found to be suitable. The conditions can be modified if necessary to achieve suitable resolution of the additives and flavourings of interest.

Mobile phase A (6.3), Mobile phase B (6.19)

Gradient time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	8.0	92.0
5.00	10.0	90.0
22.50	19.3	80.7
30.00	21.5	78.5
35.00	45.0	55.0
40.00	45.0	55.0
40.01	8.0	92.0
45.00	8.0	92.0

Flow rate: 1.0Ml/min
Injection volume: 5µL
Column Temperature: 40°C
Detector Wavelengths: 205nm for theobromine, saccharin, benzoic acid, caffeine and aspartame
231nm for acesulfame K and quinine sulphate
256nm for sorbic acid and parabens

It is suggested that to help maintain column efficiency, it is flushed with a mixture of water, acetonitrile (about 50:50) for about 30 minutes prior to instrument shutdown. Column performance will be maintained through use of a guard column.

8. **PROCEDURE**

Preparation of the test sample

8.1 Soft drinks.

8.1.1 Degas, if necessary, by sonication in an ultrasonic bath (7.5) for 5 minutes or by filtration. Dilute if necessary, with water so

that the sample test solution is within the range of working standards (6.24).

8.2 Foods – blend or homogenise to make a homogenous sample.

8.2.1 Weigh 9 – 10g, accurate to 0.001g, of sample into a 100Ml beaker and mix with 5Ml of 3mol/L hydrochloric acid (6.21). (If problems are encountered filtering the sample (8.2.4) take 5g).

8.2.2 Add about 50Ml of 50Ml/100Ml methanol (6.20), mix and transfer quantitatively to a 100Ml volumetric flask. Dilute to volume with 50Ml/100Ml methanol.

8.2.3 Shake for 1 minute.

8.2.4 Filter the solution through a filter paper (7.7) then through a 0.45µm syringe filter (7.3).

8.3 Chocolate and chocolate products (for theobromine analysis) (grate or blend if necessary).

8.3.1 Weigh about 1g, accurate to 0.001g, of dark chocolate, 2 – 3g of milk chocolate or 3g cake into a 100Ml beaker.

8.3.2 Add 25Ml of water and 2Ml of 0.5mol/L hydrochloric acid (6.22) and mix.

8.3.3 Bring to the boil on a hotplate and then transfer the beaker to a water bath for 15 minutes.

8.3.4 Transfer the solution to a 50Ml volumetric flask and allow to cool.

8.3.5 Add 1Ml of each clearing reagent (6.28), make to volume with water and mix.

8.3.6 Allow the solution to stand for about 30 minutes and then filter through a filter paper (7.7) and a syringe filter (7.3).

8.4 Chromatography

Set up the HPLC system (7.6) according to the manufacturers instructions. The instrument must be fitted with a suitable column (7.8). The operating conditions must be adjusted so as to achieve sufficient separation of the additives of interest to enable identification. The specific operating conditions are detailed in Appendix 1.

Typical chromatographic separations are shown in Figure 3 at the end of this method.

8.5 Inject a suitable volume e.g. 5µL of the test solution and run the chromatographic separation.

8.6 Determine the peak areas (by electronic integration) at the appropriate wavelength (see 7.8.1). If a diode array detector is not available then separate runs at each wavelength may be required.

8.7 Preparation of calibration curve.

8.7.1 Successively analyse each working standard solution (6.24) according to 8.5 to 8.6.

8.7.2 Plot a calibration curve of analyte concentration against peak area for each analyte of interest.

8.7.3 When fresh stock standards are prepared a new calibration curve is analysed.

8.8 Calibration check.

8.8.1 Provided that the calibration curve (8.7.2) is linear and the HPLC conditions remain ostensibly unaltered, a single working standard solution of each analyte may be used to check the calibration curve.

8.8.2 Analyse working standard 50mg/L according to 8.5 to 8.6. Carry out a duplicate injection of the working standard solution.

8.8.3 The calibration check standard is deemed satisfactory if the mean concentration is within ±5% of the expected value (i.e. 50mg/L) when extrapolated from the stored calibration graph.

8.8.4 If the calibration check standard meets the requirements (8.8.3) then the stored calibration graph may be used to calculate the analyte concentration. Otherwise a fresh stock standard must be prepared.

9. CALCULATION

9.1 If a calibration curve is used, determine the concentration © of each analyte in the test solution directly from the calibration graph.

9.2 If a diode array detector is available, the identity of sample peaks can be confirmed if necessary.

9.3 The concentration of each analyte in the sample, expressed in mg/L or mg/kg, may be calculated according to the following formula:

$$\text{Analyte in the sample (mg/L or mg/kg)} = \frac{C \times V}{m}$$

Where C = concentration in mg/L of the analyte component from the graph

V = final volume, in Ml of test solution

m = mass (or volume), in g (or Ml) of test portion taken for analysis

10. EXPRESSION OF RESULTS

Record the identity of the analyte and its concentration, expressed as mg/L or mg/kg as appropriate, to the nearest 1mg/kg or 1mg/L.

11. INTERPRETATION

The amount of fat free dry cocoa in chocolate products may be calculated from the theobromine content according to the following formula.

Fat free dry cocoa (g/100g) = theobromine (g/100g) x 35.9

12. DISPOSAL

No specific problems.

13. REFERENCES

14. ANALYTICAL QUALITY ASSURANCE

14.1 Performance Characteristics

14.1.1 L.O.D

	liquids direct	or	solid foods*
Acesulfame K	0.5mg/L		10mg/kg
Theobromine	0.5mg/L		25mg/kg
Saccharin	1.2mg/L		15mg/kg
Benzoic Acid	1.0mg/L		10mg/kg
Caffeine	0.5mg/L		10mg/kg
Sorbic Acid	0.5mg/L		10mg/kg
Aspartame	5.0mg/L		50mg/kg
Methyl Parabens	5.0mg/L		50mg/kg
Quinine Sulphate	5.0mg/L		50mg/kg
Ethyl Paraben	5.0mg/L		50mg/kg
Propyl Paraben	5.0mg/L		50mg/kg

*Assuming 10g sample diluted to 100mL (8.2.1) for solid foods and 1g diluted to 50mL for theobromine (8.3.1)

14.1.2 Bias

	Recovery Mean		Recovery Standard Deviation	
	Foods	Drinks	Foods	Drinks
Acesulfame K	101.5%	105.5%	3.6%	3.7%
Theobromine	99.2%	-	2.8%	-
Saccharin	99.8%	100.7%	4.0%	1.6%
Benzoic Acid	-	98.9%	-	1.7%
Caffeine	-	104.3%	-	3.3%
Sorbic Acid	95.8%	92.1%	6.4%	8.4%
Aspartame	94.0%	99.7%	4.4%	2.9%
Methyl Paraben	94.6%	-	5.7%	-
Quinine Sulphate	-	101.9%	-	9.5%
Ethyl Paraben	92.7%	-	8.9%	-
Propyl Paraben	91.0%	-	10.6%	-

(Soft drinks spiked at a level of 50mg/L except aspartame 250mg/L). Foods spiked at a level 25mg/L except Aspartame 100mg/L. Theobromine spiked at a level of 50mg/L in chocolate)

14.1.3 Precision

	Absolute difference Standard Deviation	
	Foods (mg/kg)	Drinks (mg/L)
Acesulfame K	4.8 (10 samples in the range 89 to 100)	1.4 (15 samples in the range 25 to 100)
Theobromine	31.6 (10 samples in the range 823 to 3117)	-
Saccharin	4.4 (10 samples in the range 76 to 530)	1.1 (15 samples in the range 43 to 183)
Benzoic Acid	-	2.4 (18 samples in the range 78 to 138)
Caffeine	-	1.2 (14 samples in the range 12 to 91)
Sorbic Acid	7.0 (10 samples in the range 132 to 1189)	0.8 (7 samples in the range 100 to 189)

Aspartame	8.6 (12 samples in the range 60 to 400)	1.9 (10 samples in the range 25 to 1362)
Methyl Paraben	6.1 (10 samples in the range 368 to 896)	
Quinine Sulphate	-	1.7 (14 samples in the range 38.0 to 82)
Ethyl Paraben	4.6 (10 samples in the range 369 to 876)	
Propyl Paraben	6.0 (10 samples in the range 340 to 834)	

14.2 Internal Quality Control

14.2.1 Instrument Calibration : Refer to instrument manual.

14.2.2 Blank determination : Take an appropriate blank through the procedure.

14.2.3 Standard Control : Each batch of samples should include analysis of an in-house standard control material.

14.2.4 Repeatability Check : At least every tenth sample should be analysed in duplicate and the difference between the results should conform to the performance characteristics.

Figure 3 Chromatogram of Food Additives

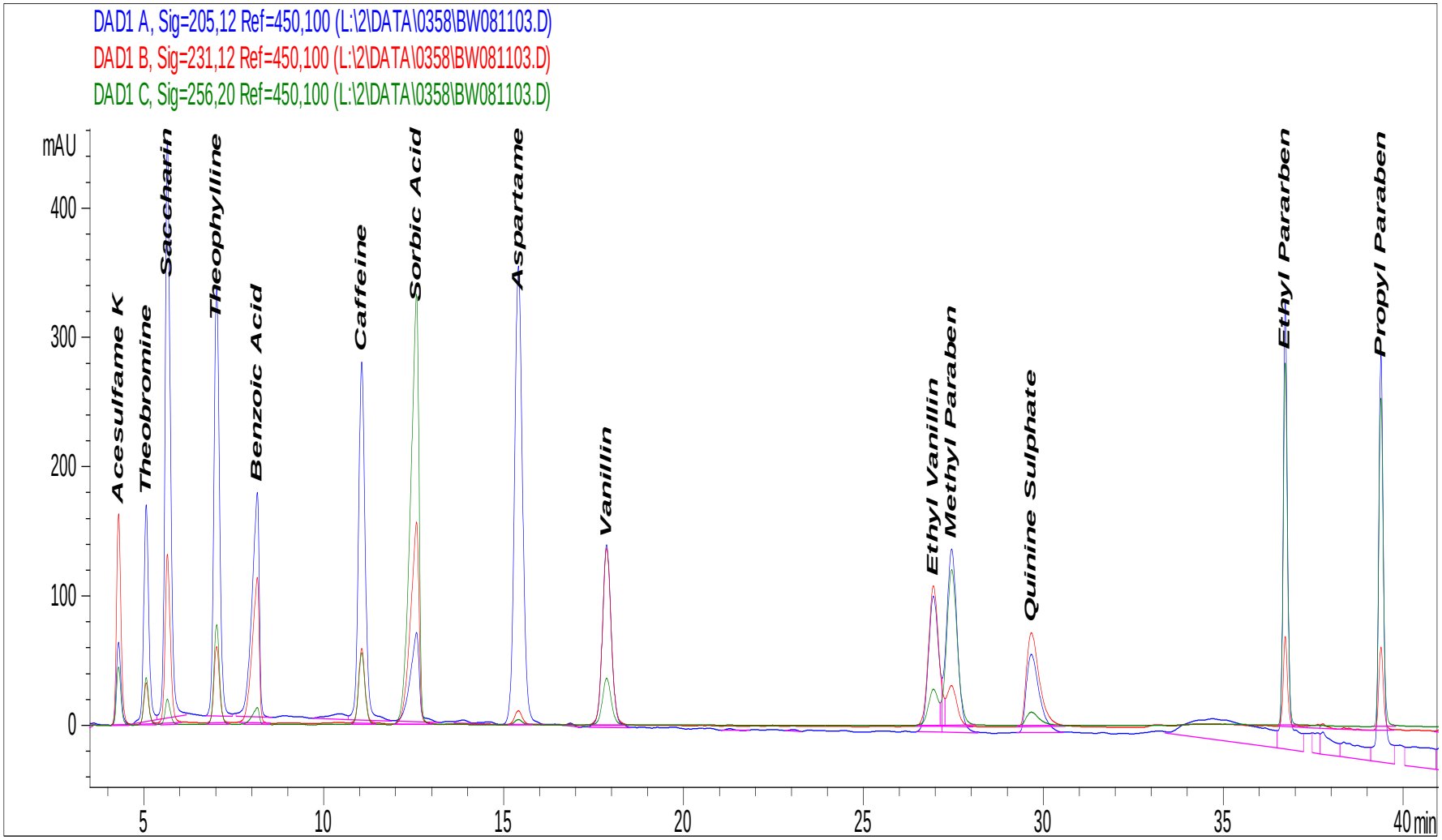


Figure 4 Genotypes of Pure and Hybrid Origin

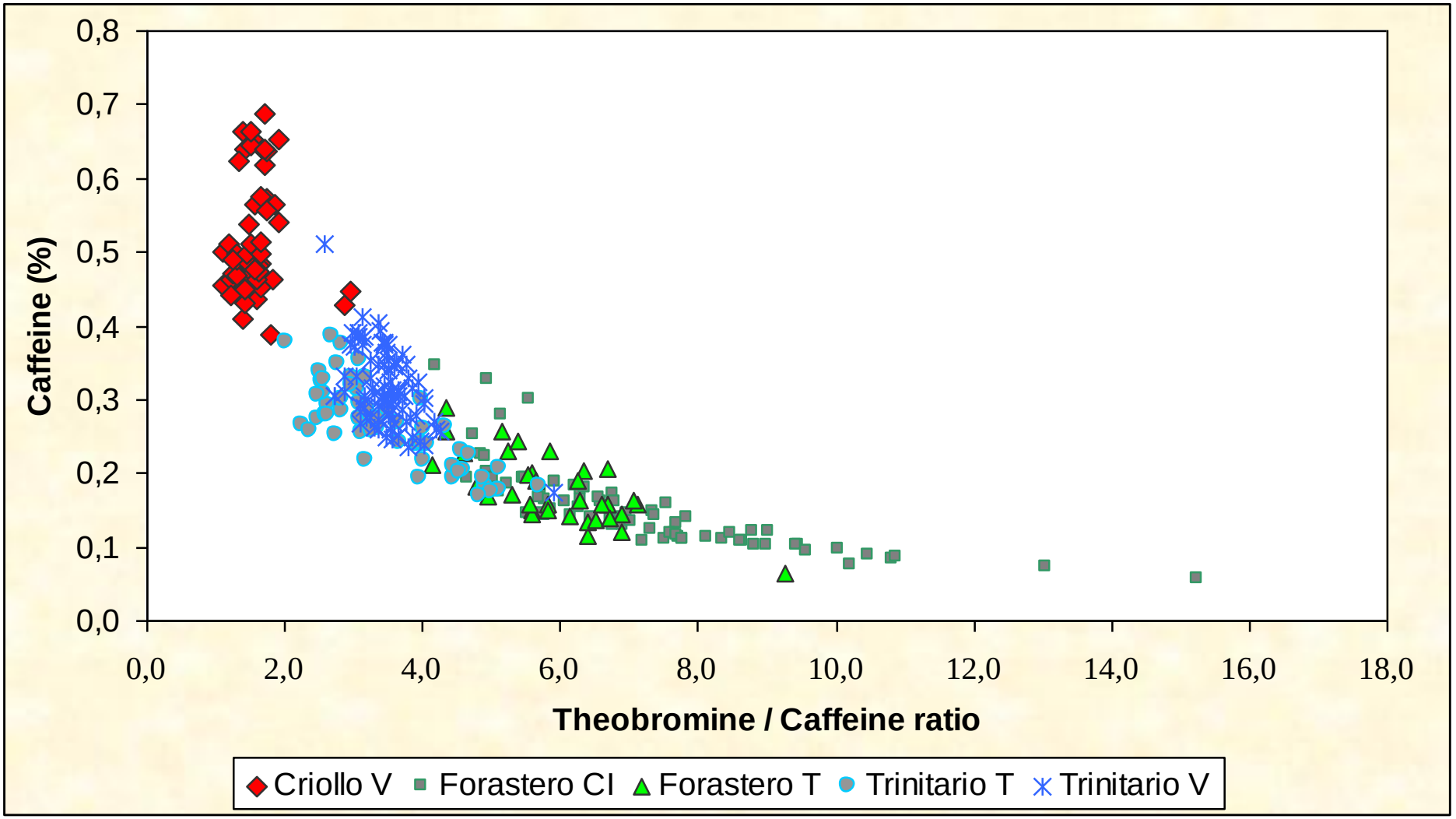
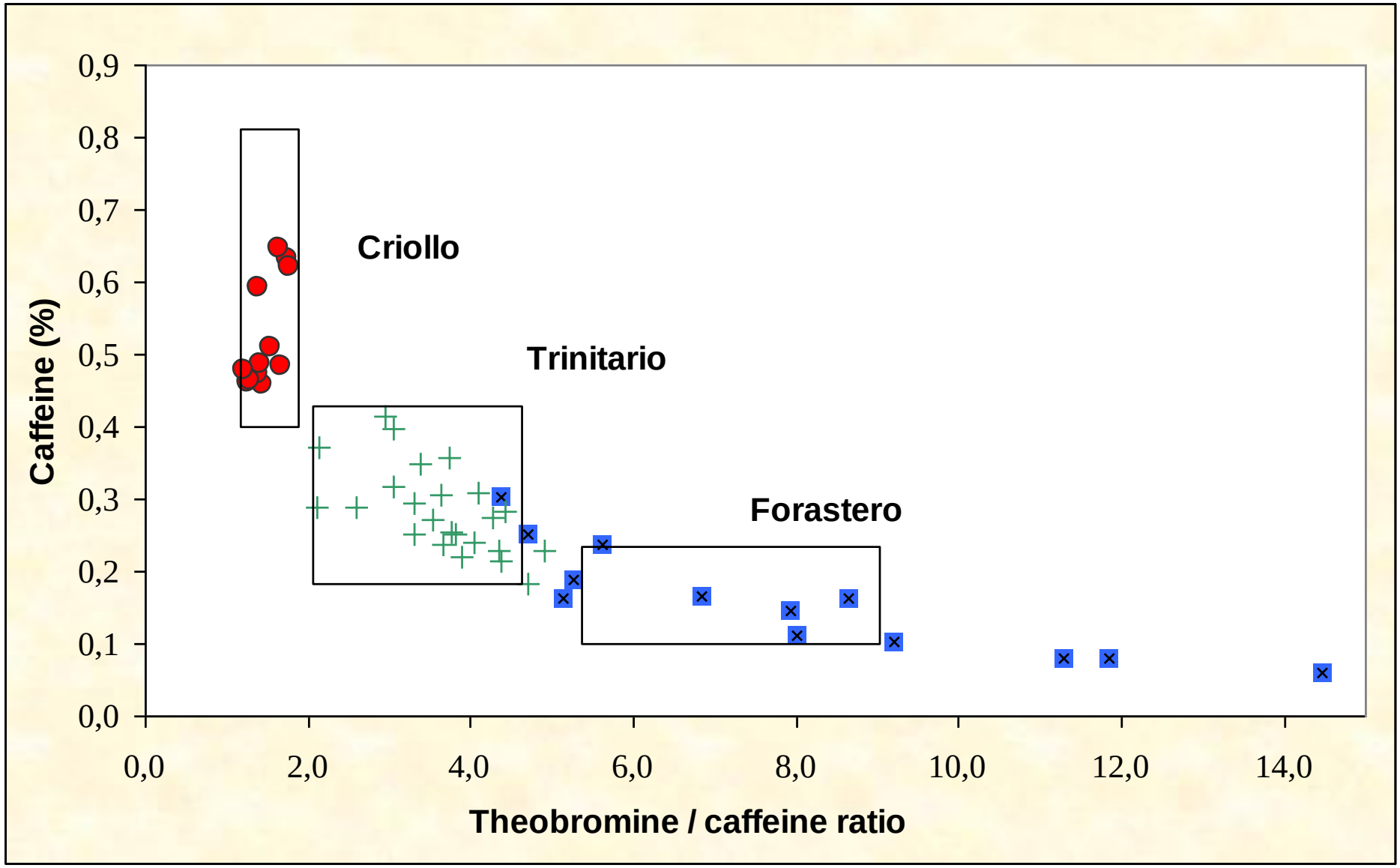


Figure 5 Genotypes of Pure Origin



APPENDIX 4

Table 2 Cocoa Liquor Results

Summary of the results of analysis on 191 samples of cocoa liquor obtained from a range of countries worldwide.

Country	Moisture KF	Fat	Caffeine	Theobromine	Total Alkaloids	Caffeine on Dry Fat Free Matter	Theobromine on Dry Fat Free Matter	Total Alkaloids on Dry Fat Free Matter	Factor (Theobromine)	Factor (Total Alkaloids)
	%	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg		
BOLIVIA	2.20	54.80	1,756	10,377	12,133	4,084	24,133	28,216	41.44	35.44
BOLIVIA	1.80	53.80	2,002	11,246	13,248	4,509	25,329	29,838	39.48	33.51
BOLIVIA	2.10	53.90	1,514	11,469	12,983	3,441	26,066	29,507	38.36	33.89
BRAZIL	3.30	42.80	2,279	11,793	14,072	4,228	21,879	26,108	45.71	38.30
BRAZIL	3.40	49.70	4,100	14,270	18,370	8,742	30,426	39,168	32.87	25.53
CAMEROON	1.60	51.90	1,094	10,584	11,678	2,353	22,761	25,114	43.93	39.82
COLUMBIA	3.00	55.40	1,918	10,359	12,277	4,611	24,901	29,512	40.16	33.88
COLUMBIA	3.10	58.60	2,300	11,094	13,394	6,005	28,966	34,971	34.52	28.59
CONGO	1.10	53.80	776	11,085	11,861	1,721	24,579	26,299	40.69	38.02
COSTA RICA	1.10	58.50	1,853	9,416	11,269	4,587	23,307	27,894	42.91	35.85
DOMINICAN REPUBLIC	1.40	54.80	1,198	11,619	12,817	2,735	26,527	29,263	37.70	34.17
DOMINICAN REPUBLIC	1.20	52.50	1,674	12,820	14,494	3,616	27,689	31,305	36.12	31.94
ECUADOR	1.20	52.50	1,687	10,581	12,268	3,644	22,853	26,497	43.76	37.74
ECUADOR	1.20	50.70	1,531	11,041	12,572	3,183	22,954	26,137	43.56	38.26
ECUADOR	1.30	51.30	1,681	11,170	12,851	3,546	23,565	27,112	42.44	36.88
ECUADOR	0.90	55.20	2,177	10,583	12,760	4,959	24,107	29,066	41.48	34.40
ECUADOR	1.20	54.40	2,020	10,786	12,806	4,550	24,293	28,842	41.16	34.67
ECUADOR	1.00	51.80	1,749	11,491	13,240	3,706	24,345	28,051	41.08	35.65
ECUADOR	1.00	52.40	1,207	11,382	12,589	2,590	24,425	27,015	40.94	37.02
ECUADOR	1.60	51.80	1,962	11,459	13,421	4,210	24,590	28,800	40.67	34.72
ECUADOR	2.10	51.00	2,067	11,539	13,606	4,407	24,603	29,011	40.64	34.47
ECUADOR	1.30	50.80	1,893	11,793	13,686	3,952	24,620	28,572	40.62	35.00
ECUADOR	1.10	52.40	1,812	11,452	13,264	3,897	24,628	28,525	40.60	35.06
ECUADOR	0.60	54.70	2,021	11,012	13,033	4,521	24,635	29,157	40.59	34.30
ECUADOR	0.90	57.40	3,636	10,298	13,934	8,719	24,695	33,415	40.49	29.93
ECUADOR	1.40	51.30	1,906	11,699	13,605	4,030	24,734	28,763	40.43	34.77
ECUADOR	3.50	50.30	2,086	11,557	13,643	4,515	25,015	29,530	39.98	33.86
ECUADOR	1.80	50.90	1,864	11,861	13,725	3,941	25,076	29,017	39.88	34.46

ECUADOR	2.90	51.50	1,950	11,548	13,498	4,276	25,325	29,601	39.49	33.78
ECUADOR	1.10	53.30	2,032	11,616	13,648	4,456	25,474	29,930	39.26	33.41
ECUADOR	1.30	55.80	1,277	10,941	12,218	2,977	25,503	28,480	39.21	35.11
ECUADOR	4.20	49.50	2,120	11,934	14,054	4,579	25,775	30,354	38.80	32.94
ECUADOR	1.00	54.40	2,109	11,573	13,682	4,729	25,948	30,677	38.54	32.60
ECUADOR	1.40	53.40	1,976	11,759	13,735	4,372	26,015	30,387	38.44	32.91
ECUADOR	1.30	55.40	2,507	11,415	13,922	5,790	26,363	32,152	37.93	31.10
ECUADOR	1.20	55.10	2,418	12,000	14,418	5,533	27,460	32,993	36.42	30.31
EQUADOR	3.50	50.20	1,727	9,046	10,773	3,730	19,538	23,268	51.18	42.98
GHANA	1.30	53.30	947	10,784	11,731	2,086	23,753	25,839	42.10	38.70
GHANA	0.80	52.80	1,004	11,067	12,071	2,164	23,851	26,015	41.93	38.44
GHANA	1.00	53.80	1,017	10,844	11,861	2,250	23,991	26,241	41.68	38.11
GHANA	1.00	54.30	1,007	10,800	11,807	2,253	24,161	26,414	41.39	37.86
GHANA	1.20	53.70	967	10,991	11,958	2,144	24,370	26,514	41.03	37.72
GHANA	0.70	53.70	1,072	11,130	12,202	2,351	24,408	26,759	40.97	37.37
GHANA	3.20	55.20	989	10,887	11,876	2,377	26,171	28,548	38.21	35.03
GHANA	3.30	54.60	754	11,386	12,140	1,791	27,045	28,836	36.98	34.68
GRENADA	1.30	52.50	1,950	10,271	12,221	4,221	22,232	26,452	44.98	37.80
HAITI	1.40	53.70	1,830	11,674	13,504	4,076	26,000	30,076	38.46	33.25
HAITI	0.80	52.00	1,904	13,468	15,372	4,034	28,534	32,568	35.05	30.71
HAITI	0.90	53.90	1,944	13,153	15,097	4,301	29,100	33,400	34.36	29.94
HAITI	2.80	53.40	1,473	13,697	15,170	3,363	31,272	34,635	31.98	28.87
INDIA	3.30	53.50	1,348	12,375	13,723	3,120	28,646	31,766	34.91	31.48
INDONESIA	1.70	53.30	2,398	10,779	13,177	5,329	23,953	29,282	41.75	34.15
INDONESIA	2.00	53.00	2,380	11,071	13,451	5,289	24,602	29,891	40.65	33.45
IVORY COAST	2.10	50.60	807	8,560	9,367	1,706	18,097	19,803	55.26	50.50
IVORY COAST	1.00	51.10	840	8,831	9,671	1,754	18,436	20,190	54.24	49.53
IVORY COAST	1.90	53.10	802	8,374	9,176	1,782	18,609	20,391	53.74	49.04
IVORY COAST	2.00	52.80	836	8,513	9,349	1,850	18,834	20,684	53.10	48.35
IVORY COAST	1.00	48.90	921	9,463	10,384	1,838	18,888	20,727	52.94	48.25
IVORY COAST	2.30	53.30	742	8,458	9,200	1,671	19,050	20,721	52.49	48.26
IVORY COAST	0.90	47.70	841	9,820	10,661	1,636	19,105	20,741	52.34	48.21
IVORY COAST	2.60	52.50	839	8,698	9,537	1,869	19,372	21,241	51.62	47.08
IVORY COAST	2.40	53.30	834	8,791	9,625	1,883	19,844	21,727	50.39	46.03
IVORY COAST	2.10	53.40	790	8,881	9,671	1,775	19,957	21,733	50.11	46.01
IVORY COAST	2.50	53.00	794	8,902	9,696	1,784	20,004	21,789	49.99	45.90
IVORY COAST	2.20	51.90	829	9,240	10,069	1,806	20,131	21,937	49.68	45.59

IVORY COAST	2.90	46.70	636	10,427	11,063	1,262	20,688	21,950	48.34	45.56
IVORY COAST	2.30	52.30	801	9,520	10,321	1,764	20,969	22,733	47.69	43.99
IVORY COAST	0.90	47.10	1,076	11,314	12,390	2,069	21,758	23,827	45.96	41.97
IVORY COAST	0.90	53.30	836	10,009	10,845	1,825	21,854	23,679	45.76	42.23
IVORY COAST	3.90	47.20	772	10,712	11,484	1,579	21,906	23,485	45.65	42.58
IVORY COAST	1.70	53.50	905	9,836	10,741	2,020	21,955	23,975	45.55	41.71
IVORY COAST	1.80	53.20	1,020	9,947	10,967	2,267	22,104	24,371	45.24	41.03
IVORY COAST	0.90	51.10	863	10,612	11,475	1,798	22,108	23,906	45.23	41.83
IVORY COAST	1.20	53.60	815	10,062	10,877	1,803	22,261	24,064	44.92	41.56
IVORY COAST	1.90	54.20	1,035	10,011	11,046	2,358	22,804	25,162	43.85	39.74
IVORY COAST	0.80	50.50	898	11,125	12,023	1,844	22,844	24,688	43.78	40.51
IVORY COAST	3.70	48.70	1,003	11,010	12,013	2,107	23,130	25,237	43.23	39.62
IVORY COAST	2.50	52.30	924	10,669	11,593	2,044	23,604	25,648	42.37	38.99
IVORY COAST	2.50	53.40	902	10,433	11,335	2,045	23,658	25,703	42.27	38.91
IVORY COAST	1.20	49.00	948	11,796	12,744	1,904	23,687	25,590	42.22	39.08
IVORY COAST	1.10	50.10	995	11,577	12,572	2,039	23,723	25,762	42.15	38.82
IVORY COAST	2.50	52.60	910	10,699	11,609	2,027	23,829	25,855	41.97	38.68
IVORY COAST	2.80	52.60	919	10,664	11,583	2,061	23,910	25,971	41.82	38.50
IVORY COAST	0.70	49.00	1,073	12,056	13,129	2,133	23,968	26,101	41.72	38.31
IVORY COAST	0.80	51.20	958	11,546	12,504	1,996	24,054	26,050	41.57	38.39
IVORY COAST	1.00	49.00	1,116	12,079	13,195	2,232	24,158	26,390	41.39	37.89
IVORY COAST	1.20	51.10	943	11,584	12,527	1,977	24,285	26,262	41.18	38.08
IVORY COAST	0.60	53.00	869	11,310	12,179	1,873	24,375	26,248	41.03	38.10
IVORY COAST	2.40	49.60	1,048	11,764	12,812	2,183	24,508	26,692	40.80	37.46
IVORY COAST	2.50	53.30	938	10,837	11,775	2,122	24,518	26,640	40.79	37.54
IVORY COAST	3.10	53.30	881	10,730	11,611	2,021	24,610	26,631	40.63	37.55
IVORY COAST	1.10	54.50	992	10,970	11,962	2,234	24,707	26,941	40.47	37.12
IVORY COAST	1.10	55.10	916	10,893	11,809	2,091	24,870	26,961	40.21	37.09
IVORY COAST	1.00	51.60	1,068	11,843	12,911	2,253	24,985	27,238	40.02	36.71
IVORY COAST	0.80	53.70	1,078	11,481	12,559	2,369	25,233	27,602	39.63	36.23
IVORY COAST	2.70	53.70	945	11,006	11,951	2,167	25,243	27,411	39.61	36.48
IVORY COAST	2.70	52.30	1,018	11,569	12,587	2,262	25,709	27,971	38.90	35.75
IVORY COAST	2.60	52.40	1,085	11,673	12,758	2,411	25,940	28,351	38.55	35.27
IVORY COAST	2.10	54.50	1,056	11,352	12,408	2,433	26,157	28,590	38.23	34.98
IVORY COAST	2.70	52.20	1,043	11,820	12,863	2,313	26,208	28,521	38.16	35.06
IVORY COAST	1.20	54.70	997	11,561	12,558	2,261	26,215	28,476	38.15	35.12
IVORY COAST	1.30	54.30	982	11,669	12,651	2,212	26,282	28,493	38.05	35.10

IVORY COAST	1.20	54.60	998	11,617	12,615	2,258	26,283	28,541	38.05	35.04
IVORY COAST	3.10	55.20	831	11,058	11,889	1,993	26,518	28,511	37.71	35.07
IVORY COAST	1.40	54.40	998	11,742	12,740	2,258	26,566	28,824	37.64	34.69
IVORY COAST	1.20	54.50	1,022	11,810	12,832	2,307	26,659	28,966	37.51	34.52
IVORY COAST	1.20	55.80	962	11,667	12,629	2,237	27,133	29,370	36.86	34.05
IVORY COAST	1.10	55.60	1,003	11,988	12,991	2,316	27,686	30,002	36.12	33.33
IVORY COAST	1.20	55.80	1,009	12,026	13,035	2,347	27,967	30,314	35.76	32.99
IVORY COAST	1.10	56.30	998	12,069	13,067	2,343	28,331	30,674	35.30	32.60
JAMAICA	3.40	53.60	1,668	11,046	12,714	3,879	25,688	29,567	38.93	33.82
JAMAICA	3.40	54.10	1,629	11,370	12,999	3,833	26,753	30,586	37.38	32.69
MADAGASCAR	0.70	49.90	1,804	8,913	10,717	3,652	18,043	21,694	55.42	46.09
MADAGASCAR	1.10	50.00	1,434	9,986	11,420	2,933	20,421	23,354	48.97	42.82
MADAGASCAR	1.60	50.30	1,620	10,599	12,219	3,368	22,035	25,403	45.38	39.36
MADAGASCAR	0.80	49.70	1,373	11,496	12,869	2,774	23,224	25,998	43.06	38.46
MADAGASCAR	1.30	53.10	1,752	10,592	12,344	3,842	23,228	27,070	43.05	36.94
MADAGASCAR	2.40	51.60	1,592	11,181	12,773	3,461	24,307	27,767	41.14	36.01
MADAGASCAR	1.10	52.60	1,238	11,697	12,935	2,674	25,263	27,937	39.58	35.79
MADAGASCAR	1.70	51.50	1,655	11,976	13,631	3,536	25,590	29,126	39.08	34.33
MEXICO	1.40	51.80	846	10,796	11,642	1,808	23,068	24,876	43.35	40.20
MEXICO	1.10	52.70	922	10,788	11,710	1,996	23,351	25,346	42.83	39.45
MEXICO	1.60	51.70	853	11,001	11,854	1,827	23,557	25,383	42.45	39.40
MEXICO	1.30	52.60	916	11,779	12,695	1,987	25,551	27,538	39.14	36.31
N/A	0.70	53.70	945	11,435	12,380	2,072	25,077	27,149	39.88	36.83
PAPUA NEW GUINEA	1.20	52.70	1,618	9,002	10,620	3,510	19,527	23,037	51.21	43.41
PAPUA NEW GUINEA	0.50	53.70	1,430	9,225	10,655	3,122	20,142	23,264	49.65	42.98
PAPUA NEW GUINEA	1.80	54.10	1,677	9,129	10,806	3,803	20,701	24,503	48.31	40.81
PAPUA NEW GUINEA	1.00	53.00	1,956	9,613	11,569	4,252	20,898	25,150	47.85	39.76
PAPUA NEW GUINEA	1.80	49.10	1,462	10,403	11,865	2,978	21,187	24,165	47.20	41.38
PAPUA NEW GUINEA	0.80	54.50	1,769	9,507	11,276	3,957	21,268	25,226	47.02	39.64
PAPUA NEW GUINEA	1.40	53.70	1,892	9,664	11,556	4,214	21,523	25,737	46.46	38.85
PAPUA NEW GUINEA	1.30	52.90	1,924	9,935	11,859	4,201	21,692	25,893	46.10	38.62
PAPUA NEW GUINEA	3.70	49.00	1,435	10,310	11,745	3,034	21,797	24,831	45.88	40.27
PAPUA NEW GUINEA	1.30	53.50	1,923	9,924	11,847	4,254	21,956	26,210	45.55	38.15
PAPUA NEW GUINEA	1.10	54.20	1,761	9,920	11,681	3,940	22,192	26,132	45.06	38.27
PAPUA NEW GUINEA	1.20	49.80	1,393	10,892	12,285	2,843	22,229	25,071	44.99	39.89
PAPUA NEW GUINEA	2.50	51.30	1,511	10,394	11,905	3,271	22,498	25,768	44.45	38.81
PAPUA NEW GUINEA	1.40	51.10	1,559	10,875	12,434	3,282	22,895	26,177	43.68	38.20

PAPUA NEW GUINEA	1.70	51.40	1,511	10,892	12,403	3,222	23,224	26,446	43.06	37.81
PAPUA NEW GUINEA	3.50	50.60	1,460	10,847	12,307	3,181	23,632	26,813	42.32	37.30
PERU	2.00	55.50	3,284	9,416	12,700	7,727	22,155	29,882	45.14	33.46
PERU	1.50	52.50	1,186	10,665	11,851	2,578	23,185	25,763	43.13	38.82
PERU	1.10	53.70	1,592	10,744	12,336	3,522	23,770	27,292	42.07	36.64
PERU	1.00	53.20	1,894	11,032	12,926	4,135	24,087	28,223	41.52	35.43
PERU	1.20	54.80	1,724	11,770	13,494	3,918	26,750	30,668	37.38	32.61
PERU	1.10	55.40	2,107	11,874	13,981	4,844	27,297	32,140	36.63	31.11
PERU	3.80	53.10	1,535	12,147	13,682	3,561	28,183	31,745	35.48	31.50
PERU	0.90	55.60	2,266	13,167	15,433	5,209	30,269	35,478	33.04	28.19
SAN TOME	2.60	50.00	1,041	11,913	12,954	2,196	25,133	27,329	39.79	36.59
SAO TOME	3.70	46.70	912	10,641	11,553	1,839	21,454	23,292	46.61	42.93
SAO TOME	1.40	52.00	708	11,917	12,625	1,519	25,573	27,092	39.10	36.91
SAO TOME	3.30	50.80	925	12,156	13,081	2,015	26,484	28,499	37.76	35.09
SAO TOME	1.00	55.80	715	11,470	12,185	1,655	26,551	28,206	37.66	35.45
ST DOMINGO	3.20	46.50	1,642	11,149	12,791	3,264	22,165	25,429	45.12	39.32
ST DOMINGO	3.30	46.80	2,002	12,847	14,849	4,012	25,745	29,758	38.84	33.60
ST DOMINGO	3.40	48.70	1,960	12,659	14,619	4,092	26,428	30,520	37.84	32.77
ST DOMINGO	3.00	53.60	2,061	12,791	14,852	4,749	29,472	34,221	33.93	29.22
ST.DOMINGO	3.20	55.50	1,705	12,070	13,775	4,128	29,225	33,354	34.22	29.98
ST.DOMINGO	3.10	55.10	1,824	12,784	14,608	4,364	30,584	34,947	32.70	28.61
ST.DOMINGO	3.30	54.70	2,068	12,954	15,022	4,924	30,843	35,767	32.42	27.96
ST.DOMINGO	3.00	54.50	1,728	13,132	14,860	4,066	30,899	34,965	32.36	28.60
ST.DOMINGO	3.00	54.70	1,615	13,076	14,691	3,818	30,913	34,730	32.35	28.79
TANZANIA	1.80	53.00	1,223	11,415	12,638	2,706	25,254	27,960	39.60	35.77
TANZANIA	1.20	54.30	1,412	11,258	12,670	3,173	25,299	28,472	39.53	35.12
TANZANIA	1.20	54.20	954	11,498	12,452	2,139	25,780	27,919	38.79	35.82
TANZANIA	1.00	56.40	1,221	11,055	12,276	2,866	25,951	28,817	38.53	34.70
TANZANIA	1.10	54.70	1,138	11,793	12,931	2,575	26,681	29,256	37.48	34.18
TANZANIA	3.60	53.90	1,059	11,576	12,635	2,492	27,238	29,729	36.71	33.64
TANZANIA	3.20	52.80	1,595	12,382	13,977	3,625	28,141	31,766	35.54	31.48
TANZANIA	3.30	53.80	1,123	12,150	13,273	2,618	28,322	30,939	35.31	32.32
TANZANIA	1.00	54.20	1,610	12,706	14,316	3,594	28,362	31,955	35.26	31.29
TANZANIA	2.60	56.00	1,562	12,050	13,612	3,773	29,106	32,879	34.36	30.41
TANZANIA	0.50	56.00	1,428	13,485	14,913	3,283	31,000	34,283	32.26	29.17
TOGO	0.70	52.70	1,025	11,998	13,023	2,200	25,747	27,946	38.84	35.78
UGANDA	3.20	53.30	1,197	11,603	12,800	2,752	26,674	29,425	37.49	33.98

UGANDA	2.80	55.80	1,119	12,379	13,498	2,703	29,901	32,604	33.44	30.67
UGANDA	3.10	55.50	1,142	12,515	13,657	2,758	30,229	32,988	33.08	30.31
UGANDA	3.10	56.20	1,120	12,751	13,871	2,752	31,329	34,081	31.92	29.34
UNITED STATES	0.90	55.50	1,468	11,563	13,031	3,367	26,521	29,888	37.71	33.46
UNITED STATES	0.80	55.50	1,771	11,623	13,394	4,053	26,597	30,650	37.60	32.63
UNITED STATES	0.90	55.80	1,594	11,575	13,169	3,681	26,732	30,413	37.41	32.88
UNITED STATES	1.00	54.50	1,482	12,105	13,587	3,330	27,202	30,533	36.76	32.75
VENEZUELA	1.30	49.00	1,882	8,797	10,679	3,787	17,700	21,487	56.50	46.54
VENEZUELA	1.40	49.80	2,302	10,764	13,066	4,717	22,057	26,775	45.34	37.35
VENEZUELA	1.90	53.80	1,376	10,384	11,760	3,106	23,440	26,546	42.66	37.67
VENEZUELA	1.60	52.80	1,928	10,895	12,823	4,228	23,893	28,121	41.85	35.56
VENEZUELA	1.00	50.80	2,170	11,701	13,871	4,502	24,276	28,778	41.19	34.75
VENEZUELA	1.50	51.50	2,650	11,589	14,239	5,638	24,657	30,296	40.56	33.01
VENEZUELA	2.30	53.30	1,467	10,963	12,430	3,304	24,691	27,995	40.50	35.72
VENEZUELA	2.70	50.50	2,622	11,849	14,471	5,603	25,318	30,921	39.50	32.34

Table 2(a) Statistical analysis of cocoa liquor results

	Caffeine on Dry Fat Free Matter	Theobromine on Dry Fat Free Matter	Total Alkaloids on Dry Fat Free Matter
	mg/kg	mg/kg	mg/kg
Mean	3,165	24,572	27,737
sd	1,268	2,900	3,470
max	8,742	31,329	39,168
min	1,262	17,700	19,803
CI 95%	3,341	24,988	28,229
	2,980	24,161	27,240
CI 99%	3,397	25,118	28,384
	2,923	24,031	27,085

CI is the confidence interval of the results

Factors calculated from the average levels of alkaloids:

Theobromine -

Mean	Max	Min	95% CI		99% CI	
40.70	31.92	56.50	40.02	41.39	39.81	41.61

Total Alkaloids –

Mean	Max	Min	95% CI		99% CI	
36.05	25.53	50.50	35.42	36.71	35.23	36.92

Table 3 Cocoa Nib Analysis

Country	Sample name	Moisture KF	Fat	Caffeine	Theobromine	Total Alkaloids	Caffeine on Dry Fat Free Matter	Theobromine on Dry Fat Free Matter	Total Alkaloids on Dry Fat Free Matter	Factor (Theobromine)	Factor (Total Alkaloids
		%	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg		
TANZANIA	COCOA BEANS 3: TANZANIA, MAIN CROP 2009, PROD.DATE: 04-12-09	1.30	52.00	1315	13158	14473	2816	28176	30991	35.5	32.27
N/A	COCOA BEANS 1:50% IVORY COAST, 50% CAMEROEN, MAIN CROP 2009, PROD.DATE: 06-12-09	1.80	52.80	909	10842	11751	2002	23881	25883	41.9	38.64
TOGO	COCOA BEANS 4: TOGO, MAIN CROP 2009, PROD.DATE: 06-12-09	1.40	48.80	959	11575	12534	1926	23243	25169	43.0	39.73
HAITI	COCOA BEANS: NO.24 – SAINT DOMINQUE	2.30	39.90	2144	12869	15013	3709	22265	25974	44.9	38.50
GHANA	COCOA BEANS 2: GHANA, MAIN CROP 2009, PROD.DATE: 04-12-09	1.30	51.40	1004	10355	11359	2123	21892	24015	45.7	41.64
TANZANIA	COCOA BEANS: NO.23 – TANZANIA	2.90	38.40	1084	12608	13692	1847	21479	23325	46.6	42.87
TANZANIA	COCOA BEANS: NO.26 – TANZANIA	3.30	42.90	1290	10464	11754	2398	19450	21848	51.4	45.77
PAPUA NEW GUINEA	COCOA BEANS: NO.25 – PAPUA NEW GUINEA	2.50	40.70	1783	9572	11355	3139	16852	19991	59.3	50.02
PAPUA NEW GUINEA	COCOA BEANS: NO.27 – PAPUA NEW GUINEA	3.30	42.40	1884	7687	9571	3470	14157	17626	70.6	56.73
VENEZUELA	COCOA BEANS: NO.22 – VENEZUELA	3.00	34.80	2024	7617	9641	3254	12246	15500	81.7	64.52

Graphical Representation of Results
Only countries where 4 or more results have been obtained are included in the chart.

Figure 6 Conversion factor from theobromine by country

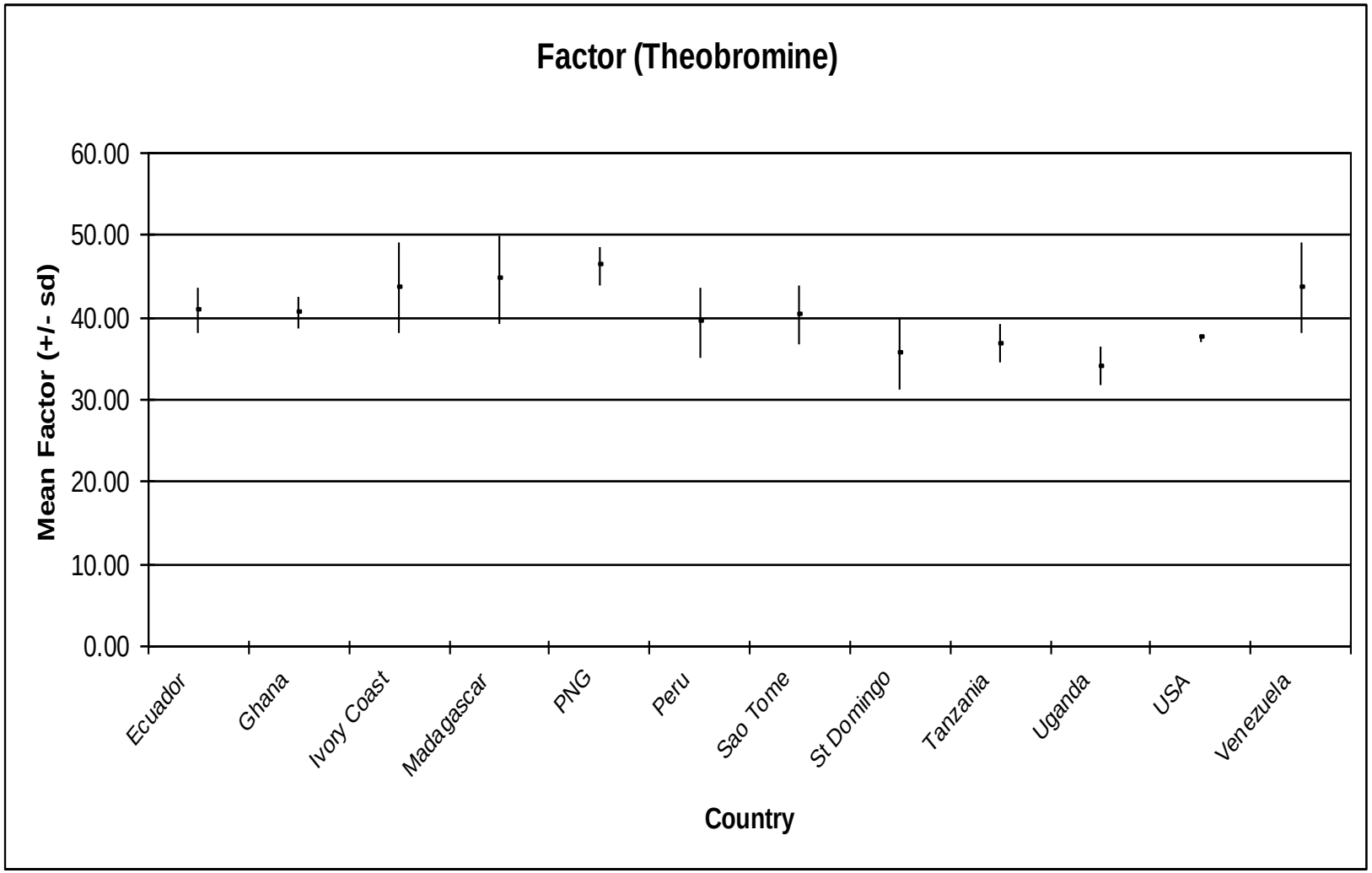


Figure 7 Conversion factor from total alkaloids by country

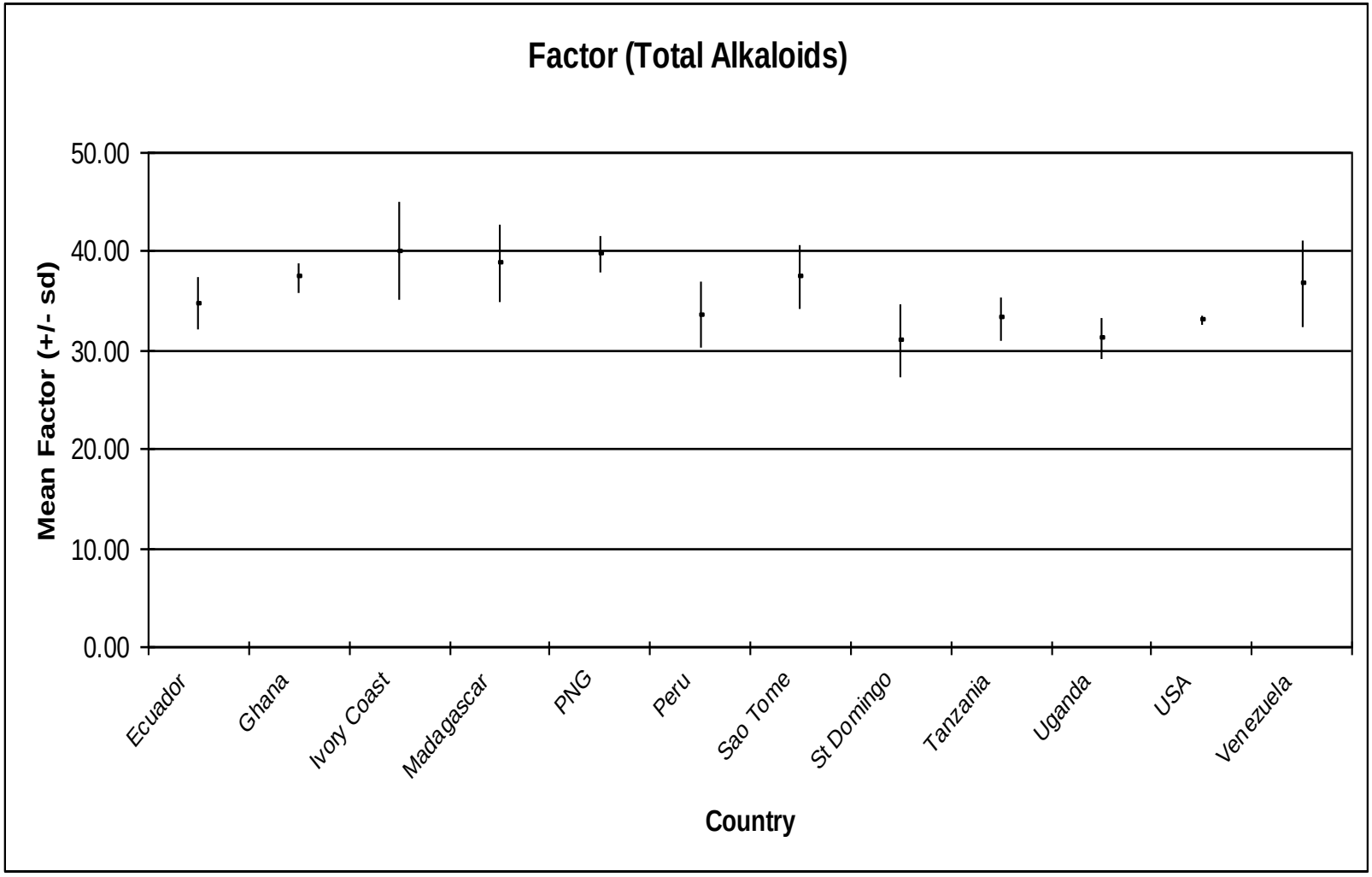
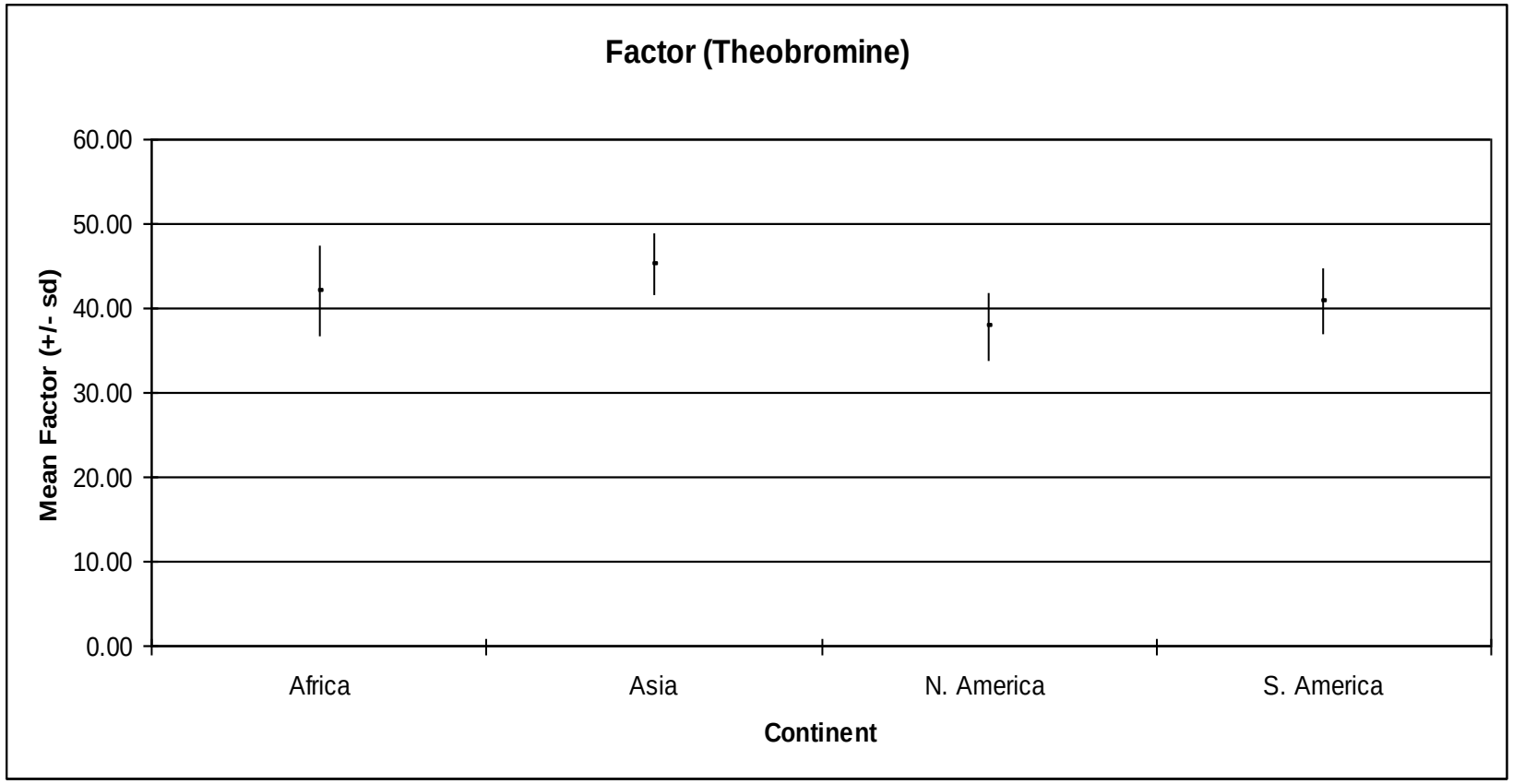


Figure 8 Conversion factor from theobromine by continent



Summary

		Factor (theobromine)		Theobromine in DFFC (mg/kg)	
Continent	**Samples (n)**	**Mean**	**CI (95%)**	**Mean**	**CI (95%)**
Africa	97	41.9	40.8-43.0	24,255	23,660-24,851
South America	48	40.7	39.5-41.8	24,791	24,141-25,442
N. & Central America	27	37.6	36.0-39.2	26,887	25,784-27,990
Asia & Indonesia	19	45.1	43.3-46.8	22,345	21,383-23,308

Figure 9 Conversion factor from total alkaloids by continent

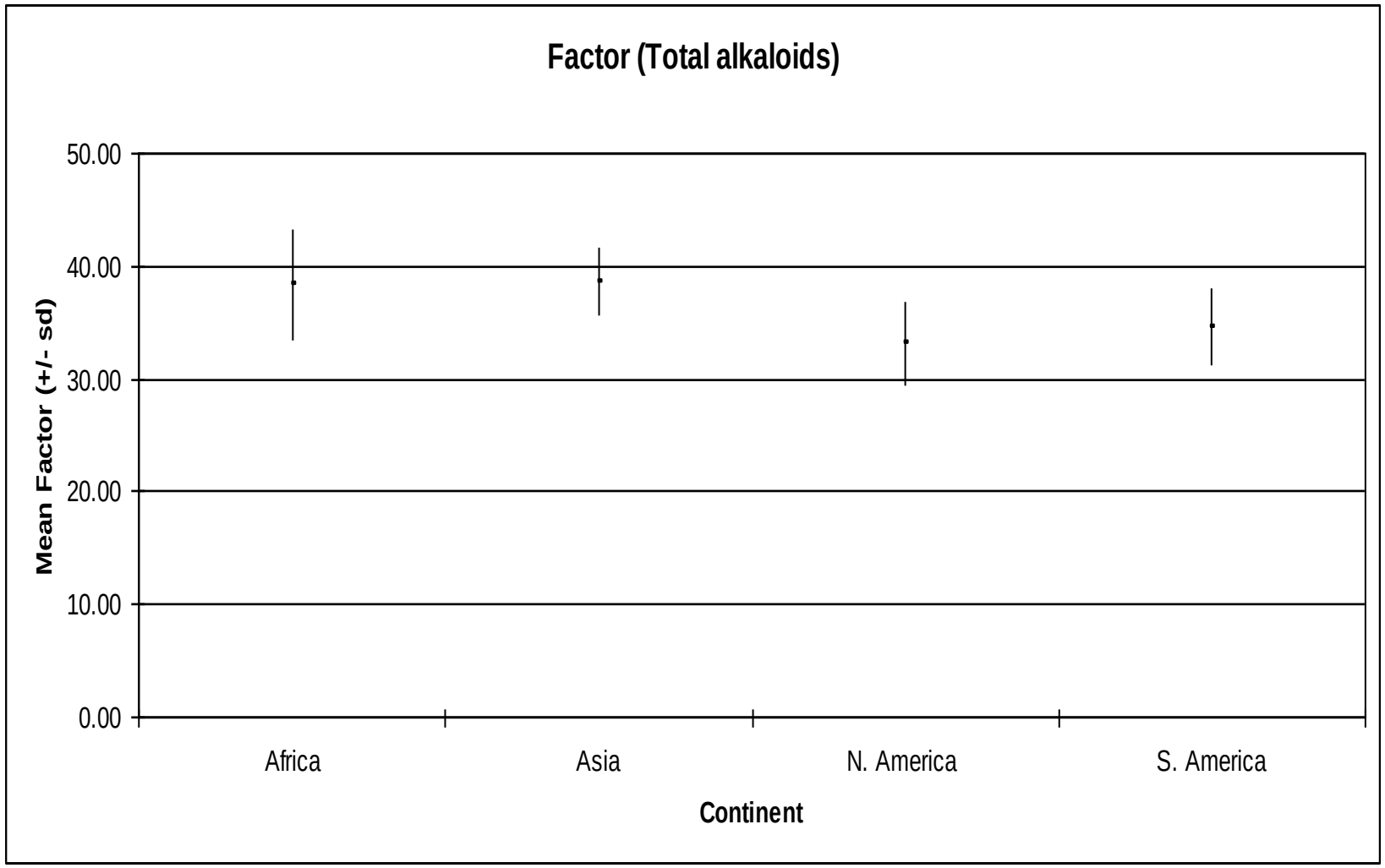


Figure 10
Gaussian Plot of Results for Theobromine in Dry Fat Free Cocoa

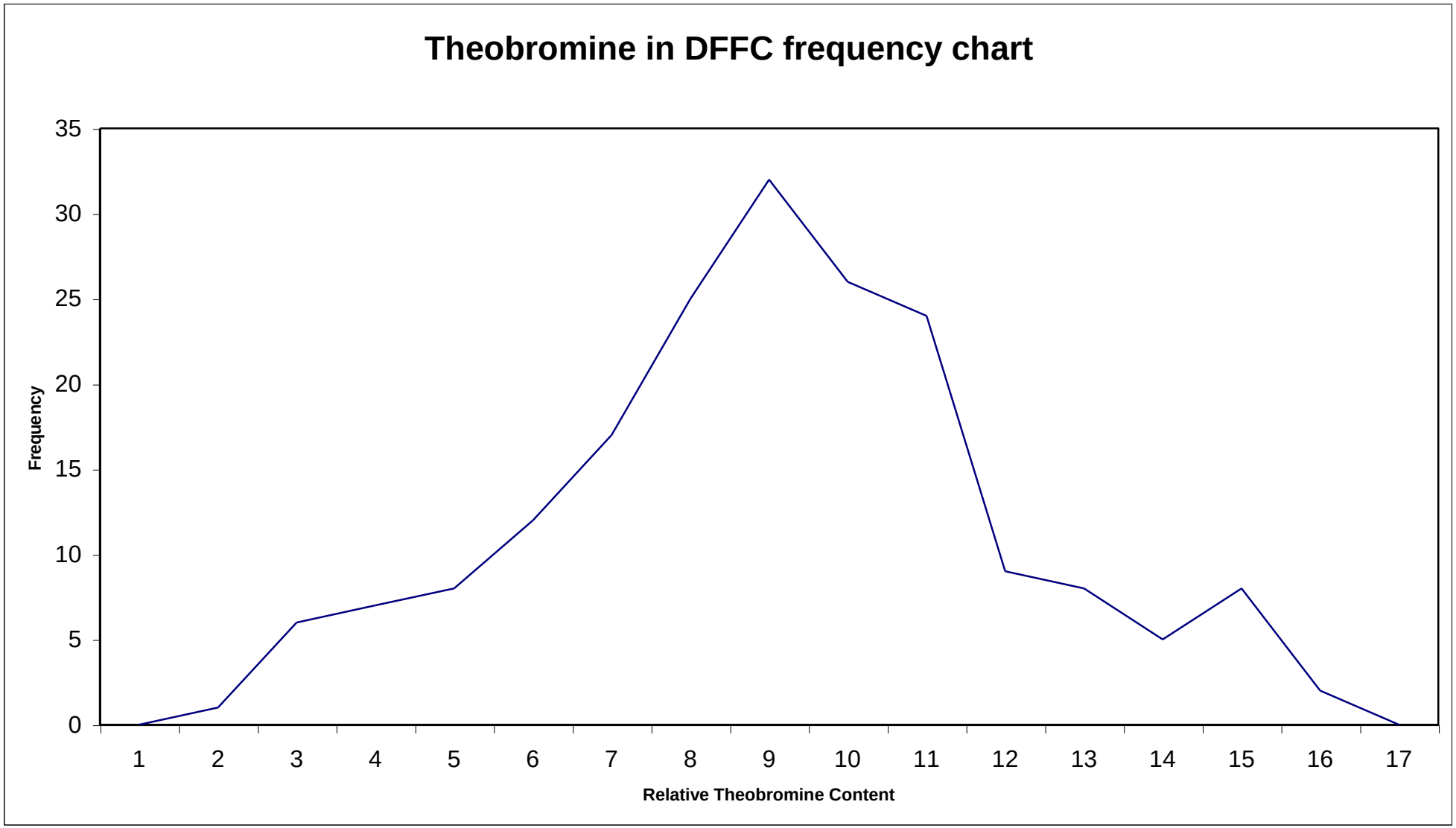


Figure 11 Ratio of Dry Fat Free Theobromine to Caffeine content of cocoa liquors by Country

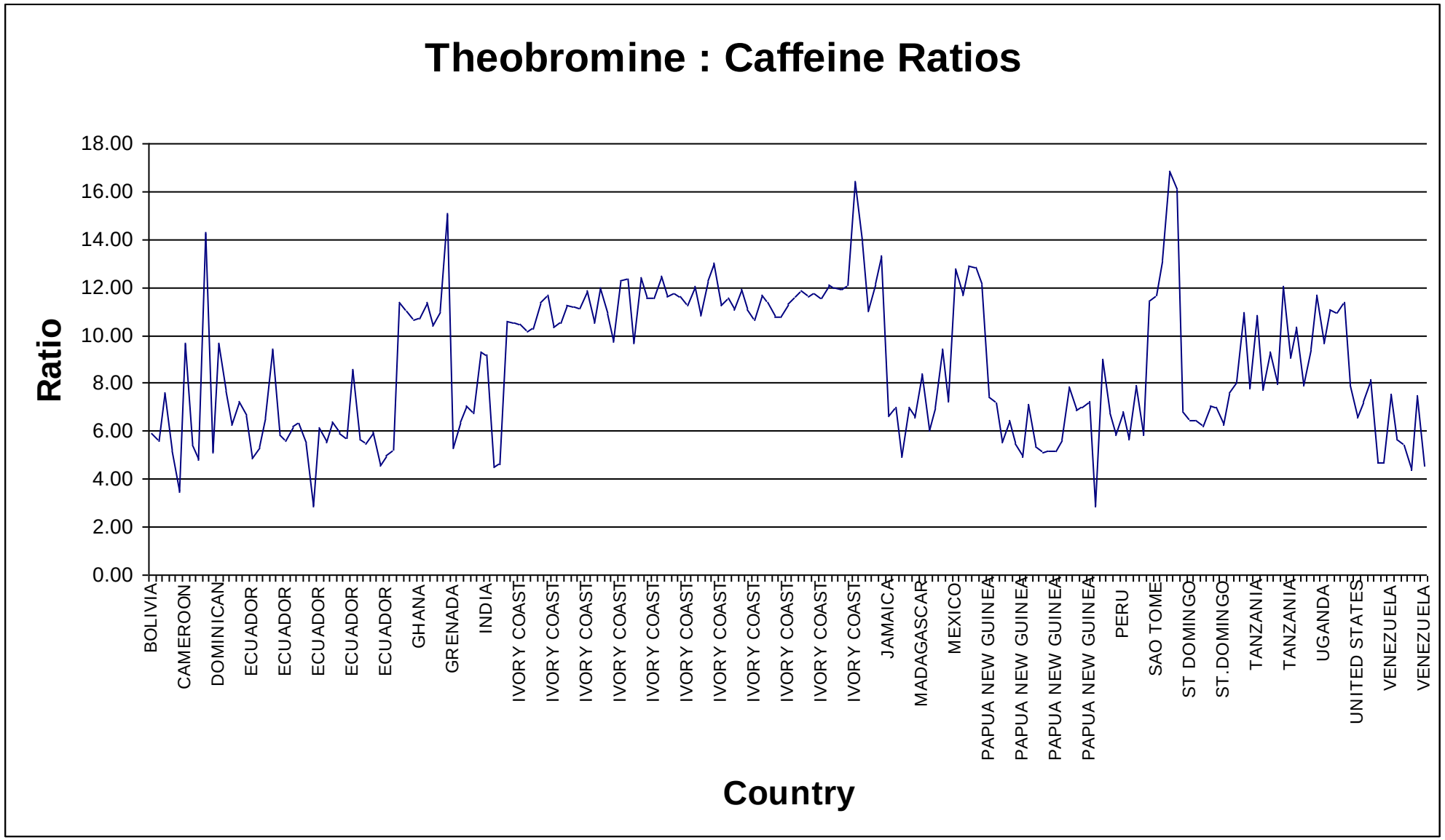


Table 4 Laboratory AQC

Samples - Fat

AQC_NAME	RESULT	AQC	RESULT	STATUS	TARGET_SD	SAMPNUM
AQC DIFFERENCE	50.48	50.30	-0.19	OK	0.250	20090187
AQC DIFFERENCE	51.32	51.02	-0.30	OK	0.250	20090188
AQC DIFFERENCE	51.46	51.44	-0.02	OK	0.250	20090189
AQC DIFFERENCE	49.98	50.00	0.02	OK	0.250	20090190
AQC DIFFERENCE	51.64	51.58	-0.06	OK	0.250	20090191
AQC DIFFERENCE	55.97	55.89	-0.07	OK	0.250	20090192
AQC DIFFERENCE	53.32	53.14	-0.19	OK	0.250	20090640
AQC DIFFERENCE	52.39	52.73	0.34	OK	0.250	20090768
AQC DIFFERENCE	53.38	53.38	0.00	OK	0.250	20090778
AQC DIFFERENCE	50.53	50.50	-0.02	OK	0.250	20090788
AQC DIFFERENCE	54.48	54.02	-0.46	OK	0.250	20090798
AQC DIFFERENCE	46.51	46.30	-0.21	OK	0.250	20091328
AQC DIFFERENCE	49.04	49.64	0.60	WARNING	0.250	20091338
AQC DIFFERENCE	36.22	35.74	-0.48	OK	0.250	20092032
AQC DIFFERENCE	55.62	55.50	-0.12	OK	0.250	20100082
AQC DIFFERENCE	55.06	54.99	-0.07	OK	0.250	20100092
AQC DIFFERENCE	50.02	49.92	-0.10	OK	0.250	20100259
AQC DIFFERENCE	53.38	53.26	-0.12	OK	0.250	20100265
AQC DIFFERENCE	50.32	50.47	0.15	OK	0.250	20100959
AQC DIFFERENCE	53.31	53.28	-0.03	OK	0.250	20100969
AQC DIFFERENCE	52.69	52.58	-0.11	OK	0.250	20101804
AQC DIFFERENCE	53.78	53.59	-0.19	OK	0.250	20101814
AQC DIFFERENCE	48.96	48.72	-0.25	OK	0.250	20101824
AQC DIFFERENCE	53.65	53.71	0.06	OK	0.250	20101834
AQC DIFFERENCE	53.31	53.30	-0.01	OK	0.250	20101844
AQC DIFFERENCE	33.65	33.49	-0.16	OK	0.250	20101897
AQC DIFFERENCE	35.95	36.25	0.30	OK	0.250	20101907
AQC DIFFERENCE	31.12	30.79	-0.33	OK	0.250	20101917
AQC DIFFERENCE	27.87	27.74	-0.13	OK	0.250	20101927
AQC DIFFERENCE	52.59	52.59	0.00	OK	0.250	20102078
AQC DIFFERENCE	55.61	55.56	-0.05	OK	0.250	20102088
		Average	**-0.07**			
		sd	**0.22**			

APPENDIX 5

LGC SAMPLE

AQC_NAME	RESULT	STATUS	TARGET	TARGET_SD	SAMPNUM
AQC STD LGCQC1003	29.67	OK	29.50	0.75	20090187
AQC STD LGCQC1003	29.73	OK	29.50	0.75	20090641

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AQC_NAME	RESULT	STATUS	TARGET	TARGET_SD	SAMPNUM
AQC STD NIST2384	50.41	OK	51.40	0.55	20090187
AQC STD NIST2384	50.59	OK	51.40	0.55	20090188
AQC STD NIST2384	51.65	OK	51.40	0.55	20090647
AQC STD NIST2384	51.84	OK	51.40	0.55	20090766
AQC STD NIST2384	52.23	OK	51.40	0.55	20090776
AQC STD NIST2384	51.20	OK	51.40	0.55	20090786
AQC STD NIST2384	50.54	OK	51.40	0.55	20090796
AQC STD NIST2384	50.43	OK	51.40	0.55	20091326
AQC STD NIST2384	51.56	OK	51.40	0.55	20091346
AQC STD NIST2384	51.93	OK	51.40	0.55	20092032
AQC STD NIST2384	52.19	OK	51.40	0.55	20100075
AQC STD NIST2384	51.83	OK	51.40	0.55	20100085
AQC STD NIST2384	52.53	WARNING	51.40	0.55	20100252
AQC STD NIST2384	51.32	OK	51.40	0.55	20100262
AQC STD NIST2384	51.72	OK	51.40	0.55	20100958
AQC STD NIST2384	51.75	OK	51.40	0.55	20100968
AQC STD NIST2384	52.04	OK	51.40	0.55	20101803
AQC STD NIST2384	50.75	OK	51.40	0.55	20101813
AQC STD NIST2384	51.97	OK	51.40	0.55	20101823
AQC STD NIST2384	51.72	OK	51.40	0.55	20101833
AQC STD NIST2384	51.60	OK	51.40	0.55	20101843
AQC STD NIST2384	52.03	OK	51.40	0.55	20101896
AQC STD NIST2384	50.33	OK	51.40	0.55	20101906
AQC STD NIST2384	51.86	OK	51.40	0.55	20101916
AQC STD NIST2384	52.03	OK	51.40	0.55	20101926
AQC STD NIST2384	51.96	OK	51.40	0.55	20102076
AQC STD NIST2384	52.10	OK	51.40	0.55	20102086
	Average	**51.56**			
	sd	**0.64**			

Samples - Caffeine

AQC_NAME	RESULT	AQC	Rel %	STATUS	TARGET_SD	SAMPNUM
AQC DUPLICATE	2622	2607	-0.57	NO LIMIT	0.000	20090187
AQC DUPLICATE	1511	1474	-2.45	NO LIMIT	0.000	20090188
AQC DUPLICATE	1950	1933	-0.87	NO LIMIT	0.000	20090189
AQC DUPLICATE	1041	1028	-1.25	NO LIMIT	0.000	20090190
AQC DUPLICATE	1592	1608	1.01	NO LIMIT	0.000	20090191
AQC DUPLICATE	1562	1530	-2.05	NO LIMIT	0.000	20090192
AQC DUPLICATE	1535	1544	0.59	NO LIMIT	0.000	20090631
AQC DUPLICATE	1705	1721	0.94	NO LIMIT	0.000	20090647
AQC DUPLICATE	1043	1093	4.79	OK	3.000	20090766
AQC DUPLICATE	794	783	-1.39	OK	3.000	20090776
AQC DUPLICATE	829	843	1.69	OK	3.000	20090786
AQC DUPLICATE	1073	1074	0.09	OK	3.000	20090796
AQC DUPLICATE	815	824	1.10	OK	3.000	20090805
AQC DUPLICATE	1960	1993	1.68	OK	3.000	20091336
AQC DUPLICATE	1197	1104	-7.77	WARNING	3.000	20091346
AQC DUPLICATE	2266	2243	-1.02	OK	3.000	20100082
AQC DUPLICATE	2418	2470	2.15	OK	3.000	20100092
AQC DUPLICATE	1434	1444	0.66	OK	3.000	20100259
AQC DUPLICATE	1976	1974	-0.11	OK	3.000	20100265
AQC DUPLICATE	1620	1620	0.04	OK	3.000	20100959
AQC DUPLICATE	947	976	3.05	OK	3.000	20100969
AQC DUPLICATE	922	925	0.33	OK	3.000	20101804
AQC DUPLICATE	1189	1221	2.69	OK	3.000	20101823
AQC DUPLICATE	1923	1920	-0.16	OK	3.000	20101830
AQC DUPLICATE	1025	1024	-0.10	OK	3.000	20101842
AQC DUPLICATE	1064	1055	-0.85	OK	3.000	20101902
AQC DUPLICATE	962	985	2.39	OK	3.000	20101912
AQC DUPLICATE	1012	1023	1.09	OK	3.000	20101922
AQC DUPLICATE	1070	1083	1.22	OK	3.000	20101932
AQC DUPLICATE	198	198	0.00	OK	3.000	20101933
AQC DUPLICATE	1009	1009	0.00	OK	3.000	20102086
		Average	**0.22**			
		sd	**2.14**			

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AQC_NAME	RESULT	STATUS	TARGET	TARGET_SD	SAMPNUM
AQC STD NIST2384	1101.00	OK	1060	25	20090187
AQC STD NIST2384	1101.00	OK	1060	25	20090192
AQC STD NIST2384	1025.00	OK	1060	25	20090647
AQC STD NIST2384	1015.00	OK	1060	25	20090776
AQC STD NIST2384	1017.00	OK	1060	25	20090786
AQC STD NIST2384	1053.00	OK	1060	25	20090796
AQC STD NIST2384	1010.00	OK	1060	25	20090805
AQC STD NIST2384	1068.00	OK	1060	25	20091346
AQC STD NIST2384	1076.00	OK	1060	25	20100075
AQC STD NIST2384	1059.00	OK	1060	25	20100085
AQC STD NIST2384	1020.30	OK	1060	25	20100252
AQC STD NIST2384	1060.40	OK	1060	25	20100262
AQC STD NIST2384	1041.50	OK	1060	25	20100958
AQC STD NIST2384	1025.00	OK	1060	25	20100968
AQC STD NIST2384	1020.00	OK	1060	25	20101803
AQC STD NIST2384	1012.00	OK	1044	25	20101813
AQC STD NIST2384	1015.00	OK	1044	25	20101823
AQC STD NIST2384	1020.00	OK	1044	25	20101833
AQC STD NIST2384	1049.00	OK	1044	25	20101843
AQC STD NIST2384	1035.00	OK	1044	25	20101896
AQC STD NIST2384	1079.00	OK	1044	25	20101906
AQC STD NIST2384	1043.00	OK	1044	25	20101916
AQC STD NIST2384	1025.00	OK	1044	25	20101926
AQC STD NIST2384	1035.00	OK	1044	25	20102076
AQC STD NIST2384	1047.00	OK	1044	25	20102086
Average	**1042.09**				
sd	**26.9**				

Samples - Theobromine

AQC_NAME	RESULT	AQC	Rel %	STATUS	TARGET_SD	SAMPNUM
AQC DUPLICATE	11849	11798	-0.43	NO LIMIT	0.00	20090187
AQC DUPLICATE	10394	10169	-2.16	NO LIMIT	0.00	20090188
AQC DUPLICATE	11548	11478	-0.61	NO LIMIT	0.00	20090189
AQC DUPLICATE	11913	11693	-1.85	NO LIMIT	0.00	20090190
AQC DUPLICATE	11181	11125	-0.50	NO LIMIT	0.00	20090191
AQC DUPLICATE	12050	11624	-3.54	NO LIMIT	0.00	20090192
AQC DUPLICATE	12147	12245	0.81	NO LIMIT	0.00	20090631
AQC DUPLICATE	12070	12151	0.67	NO LIMIT	0.00	20090647
AQC DUPLICATE	11820	12140	2.71	NO LIMIT	0.00	20090766
AQC DUPLICATE	8902	8866	-0.40	NO LIMIT	0.00	20090776
AQC DUPLICATE	9240	9291	0.55	NO LIMIT	0.00	20090786
AQC DUPLICATE	12056	11881	-1.45	NO LIMIT	0.00	20090796
AQC DUPLICATE	10062	10321	2.57	NO LIMIT	0.00	20090805
AQC DUPLICATE	12659	13113	3.59	NO LIMIT	0.00	20091336
AQC DUPLICATE	11603	11521	-0.71	NO LIMIT	0.00	20091346
AQC DUPLICATE	13167	12849	-2.42	OK	3.00	20100082
AQC DUPLICATE	12000	12096	0.80	OK	3.00	20100092
AQC DUPLICATE	9986	10065	0.79	OK	3.00	20100259
AQC DUPLICATE	11759	11681	-0.66	OK	3.00	20100265
AQC DUPLICATE	10599	10614	0.15	OK	3.00	20100959
AQC DUPLICATE	10784	11060	2.56	OK	3.00	20100969
AQC DUPLICATE	10989	10796	-1.76	OK	3.00	20101803
AQC DUPLICATE	10958	11055	0.89	OK	3.00	20101823
AQC DUPLICATE	9924	9848	-0.77	OK	3.00	20101830
AQC DUPLICATE	11998	11937	-0.51	OK	3.00	20101842
AQC DUPLICATE	11961	11812	-1.25	OK	3.00	20101902
AQC DUPLICATE	11156	11209	0.48	OK	3.00	20101912
AQC DUPLICATE	11137	11144	0.06	OK	3.00	20101922
AQC DUPLICATE	11561	11515	-0.40	OK	3.00	20101932
AQC DUPLICATE	2084	2101	0.82	OK	3.00	20101933
AQC DUPLICATE	12026	12112	0.72	OK	3.00	20102086
		Average	**-0.04**			
		sd	**1.57**			

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AQC_NAME	RESULT	STATUS	TARGET	TARGET_SD	SAMPNUM
AQC STD NIST2384	11053	OK	11600	550	20090187
AQC STD NIST2384	11033	OK	11600	550	20090192
AQC STD NIST2384	11553	OK	11600	550	20090647
AQC STD NIST2384	11107	OK	11600	550	20090776
AQC STD NIST2384	11339	OK	11600	550	20090786
AQC STD NIST2384	11495	OK	11600	550	20090796
AQC STD NIST2384	10832	OK	11600	550	20090805
AQC STD NIST2384	11537	OK	11600	550	20091346
AQC STD NIST2384	11751	OK	11600	550	20100075
AQC STD NIST2384	11699	OK	11600	550	20100085
AQC STD NIST2384	10738	OK	11600	550	20100252
AQC STD NIST2384	11041	OK	11600	550	20100262
AQC STD NIST2384	10953	OK	11600	550	20100958
AQC STD NIST2384	10918	OK	11600	550	20100968
AQC STD NIST2384	10888	OK	11235	350	20101803
AQC STD NIST2384	10940	OK	11235	350	20101813
AQC STD NIST2384	10972	OK	11235	350	20101823
AQC STD NIST2384	11005	OK	11235	350	20101833
AQC STD NIST2384	11048	OK	11235	350	20101843
AQC STD NIST2384	11362	OK	11235	350	20101896
AQC STD NIST2384	10960	OK	11235	350	20101906
AQC STD NIST2384	10908	OK	11235	350	20101916
AQC STD NIST2384	11014	OK	11235	350	20101926
AQC STD NIST2384	11489	OK	11235	350	20102076
AQC STD NIST2384	11524	OK	11235	350	20102086
Average	**11166**				
sd	**297**				

Table 5 Chocolate and Cocoa Liquor Results

Sample name	Moisture KF	Fat	Caffeine	Theobromine	Total Alkaloids	Caffeine in DFFC	Theobromine in DFFC	Total Alkaloids in DFFC	DFFC (Theobromine)	DFFC (Total Alkaloids)	Total Dry Cocoa Liquor	Declar-ation
	%	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	%	%	%	%
CHOCOLATE 6A: 09344-23/07	0.40	26.30	480	5076	5556				20.3	20.4	43.2	40.89
COCOA LIQUOR 6B: 09344-23/07 - AFRICA	1.30	52.80	1035	11484	12519	2255	25020	27275				
CHOCOLATE 7A: 09346-24/04	1.20	36.00	221	1517	1738				7.4	7.4	14.8	12.02
COCOA LIQUOR 7B: 09346-24/04 - PAPUA NEW GUINEA	1.40	49.70	1355	10071	11426	2771	20595	23366				
CHOCOLATE 8A: 09346-23/01	0.50	35.30	1143	7575	8718				35.9	35.7	71.9	68.7
COCOA LIQUOR 8B: 09346-23/01 - MADAGASCAR	1.20	50.40	1613	10222	11835	3333	21120	24452				
CHOCOLATE 9A: 09348-24/05	0.60	30.70	180	1612	1792				6.5	6.6	14.4	10.68
COCOA LIQUOR 9B: 09348-24/05 - AFRICA	1.30	53.90	962	11156	12118	2147	24902	27049				
CHOCOLATE 11A: 09348-24/04	0.90	38.60	250	2285	2535				8.9	9.1	20.1	17.09
COCOA LIQUOR 11B: 09348-24/04 - AFRICA	1.40	54.60	991	11256	12247	2252	25582	27834				
CHOCOLATE 12A: 09349-22/05	1.10	31.10	214	2029	2243				8.1	8.2	18.2	14.94
COCOA LIQUOR 12B: 09349-22/05 - AFRICA	1.00	55.00	1006	11059	12065	2286	25134	27420				
CHOCOLATE 13A: 09349-22/06	0.40	28.40	194	1719	1913				6.7	6.9	15.3	11.44
COCOA LIQUOR 13B: 09349-22/06 - AFRICA	1.00	55.00	1029	11221	12250	2339	25502	27841				
CHOCOLATE 14A: 09349-24/07	0.80	31.20	190	1525	1715				6.0	6.2	13.8	10.91
COCOA LIQUOR 14B: 09349-24/07 - AFRICA	0.90	55.10	1012	11137	12149	2300	25311	27611				
CHOCOLATE 15A: 09351-24/08 09352-24/01	0.40	34.10	241	2150	2391				8.8	8.9	19.7	17.23
COCOA LIQUOR 15B: 09351-24/08 09352-24/01 - AFRICA	1.00	54.70	1052	10829	11881	2375	24445	26819				
CHOCOLATE 16A: 09351-22/08	0.20	28.10	173	1582	1755				6.5	6.6	14.5	11.44
COCOA LIQUOR 16B: 09351-22/08 - AFRICA	1.10	54.70	1042	10778	11820	2357	24385	26742				
CHOCOLATE 17A: 09361-24/01	0.40	27.90	119	1163	1282				4.4	4.5	9.9	7.21

COCOA LIQUOR 17B: 09361-24/01 - AFRICA	1.10	54.90	1087	11525	12612	2470	26193	28664				
CHOCOLATE 18A: 09361-24/05	0.60	28.30	198	2043	2241				7.8	7.8	17.3	15.09
COCOA LIQUOR 18B: 09361-24/05 - AFRICA	1.00	55.00	1084	11559	12643	2464	26270	28734				
CHOCOLATE 19A: 09361-07/08	0.30	29.00	208	2137	2345				8.2	8.2	18.2	15.49
COCOA LIQUOR 19B: 09361-07/08 - AFRICA	1.00	54.90	1070	11561	12631	2426	26215	28642				
CHOCOLATE 20A: 09361-24/0204	0.70	28.90	198	2084	2282				7.6	7.7	17.1	15.09
COCOA LIQUOR 20B: 09361-24/0204 - AFRICA	1.00	55.00	1038	12048	13086	2359	27382	29741				

Table 6 DFFC - Comparison of results using current and proposed new factors

No	Theobromine %	Declared DFFC %	DFFC (Liquor) %	DFFC (current factor) %	DFFC (new av factor) %	DFFC +1sd %	DFFC -1sd %	
1	1972.435	7.08	8.3	7.0	8.1	7.2	9.2	Included 2.3% cocoa powder
1a	23870			85.3	97.4	86.8	111.0	
2	5026	10.4	22	17.9	20.5	18.3	23.4	included 8% cocoa powder 10-12% fat
2a	22827			81.5	93.2	83.0	106.2	
3	8511	45.07	33.3	30.4	34.7	31.0	39.6	included 3.63% cocoa powder 10-12% fat
3a	25588			91.4	104.4	93.0	119.0	
4	14542	50.17	56.9	51.9	59.4	52.9	67.6	Included 13.49% cocoa powder 10-12% fat
4a	25558			91.3	104.3	92.9	118.9	
5	14357	25.61	58.4	51.3	58.6	52.2	66.8	Included 21.51% cocoa powder 10-12% fat
5a	24605			87.9	100.4	89.5	114.4	
6	6925	40.89	27.7	24.7	28.3	25.2	32.2	
6a	25020			89.4	102.1	91.0	116.4	
7	2416	12.02	11.7	8.6	9.9	8.8	11.2	
7a	20595			73.6	84.1	74.9	95.8	
8	11799	68.7	55.9	42.1	48.2	42.9	54.9	
8a	21120			75.4	86.2	76.8	98.2	
9	2346	10.68	9.4	8.4	9.6	8.5	10.9	+4.46% cocoa powder 10-12% fat
9a	24902			88.9	101.6	90.6	115.8	
10	8812	41.18	35.4	31.5	36.0	32.0	41.0	
10a	24914			89.0	101.7	90.6	115.9	
11	3777	17.09	14.8	13.5	15.4	13.7	17.6	
11a	25582			91.4	104.4	93.0	119.0	
12	2993	14.94	11.9	10.7	12.2	10.9	13.9	
12a	25134			89.8	102.6	91.4	116.9	
13	2414	11.44	9.5	8.6	9.9	8.8	11.2	
13a	25502			91.1	104.1	92.7	118.6	
14	2243	10.91	8.9	8.0	9.2	8.2	10.4	
14a	25311			90.4	103.3	92.0	117.7	
15	3282	17.23	13.4	11.7	13.4	11.9	15.3	
15a	24445			87.3	99.8	88.9	113.7	
16	2206	11.44	9	7.9	9.0	8.0	10.3	
16a	24385			87.1	99.5	88.7	113.4	
17	1622	7.21	6.2	5.8	6.6	5.9	7.5	
17a	26193			93.5	106.9	95.2	121.8	
18	2873	15.09	10.9	10.3	11.7	10.4	13.4	
18a	26270			93.8	107.2	95.5	122.2	
19	3023	15.49	11.5	10.8	12.3	11.0	14.1	
19a	26215			93.6	107.0	95.3	121.9	
20	2960	15.09	10.8	10.6	12.1	10.8	13.8	
20a	27382			97.8	111.8	99.6	127.4	

a = liquor sample

Chocolate samples 1,2,3,4, 5 and 9 were not made from pure cocoa liquors

STANDARD OPERATING PROCEDURE (SOP) 003 **APPENDIX 7**

THE DETERMINATION OF THEOBROMINE AND CAFFEINE IN COCOA AND CHOCOLATE PRODUCTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Version 1.0, December 2010

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Date November 2010

Approved by Authenticity Methodology Working Group (AMWG) **Date** 5 August 2011

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THE DETERMINATION OF THEOBROMINE AND CAFFEINE IN DRY FAT FREE COCOA AND CHOCOLATE PRODUCTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. BACKGROUND

Regulations are in force across Europe specifying minimum amounts of cocoa in a range of products with reserved descriptions. The alkaloids theobromine and caffeine are naturally present in cocoa. Cocoa content of food has been measured over the last 50 years using a conversion factor calculated from analysis of alkaloids in genuine cocoa samples. Changes in production methods and climate may have resulted in significant changes in alkaloid levels in cocoa. The standard operating procedure was produced as part of FSA project Q01122. An updated conversion factor was calculated from 191 samples of cocoa liquor, analysed using the method provided in this protocol.

2. PURPOSE

There is a need to produce a method that can measure theobromine and caffeine levels quickly and accurately in a wide range of cocoa samples.

3. SCOPE

This method describes a procedure for the determination of caffeine and theobromine in chocolate and chocolate products including drinking chocolate, milk chocolate, plain chocolate, chocolate cake. A simple and rapid procedure for extraction of alkaloids and analysis by HPLC is described.

A separate method is available for the measurement of cocoa butter equivalents in milk chocolate, which is necessary for the calculation of total cocoa solids in milk chocolate products.

4. PRINCIPLE OF THE METHOD

The alkaloids are extracted from cocoa and chocolate products with dilute acid. After clarification and filtration the alkaloids in the solution are determined by HPLC. The following additives, which may interfere with the analysis of theobromine or caffeine if present in the food, are also extracted: saccharin, benzoic acid, aspartame, acesulfame K, quinine sulphate, sorbic acid, vanillin, ethyl vanillin and 3 parabens.

5. HEALTH & SAFETY

5.1 EYE PROTECTION SHOULD NORMALLY BE WORN AT ALL TIMES.

5.2 METHANOL IS HIGHLY FLAMMABLE AND TOXIC BY INHALATION OR IF SWALLOWED. KEEP CONTAINER TIGHTLY CLOSED. AVOID CONTACT WITH SKIN. KEEP AWAY FROM SOURCES OF IGNITION. USE ONLY IN A DESIGNATED FLAME FREE AREA.

5.3 ACETONITRILE IS HIGHLY FLAMMABLE AND TOXIC BY INHALATION, IN CONTACT WITH SKIN AND IF SWALLOWED. KEEP AWAY FROM SOURCES OF IGNITION. TAKE OFF IMMEDIATELY ANY CONTAMINATED CLOTHING. IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE. USE ONLY IN A DESIGNATED FLAME FREE AREA.

5.4 CAFFEINE IS TOXIC IF SWALLOWED. AVOID CONTACT WITH SKIN AND EYES. IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE.

6. **REAGENTS**

6.1 Analytical Reagent (AR) grade reagents are suitable unless otherwise stated.

6.2 Water should be deionised, distilled or of similar quality.

6.3 Acetic acid, glacial.

6.4 Acetonitrile, HPLC grade. Degas and filter through a 0.45 µm organic filter. (Mobile phase A for HPLC).

6.5 Caffeine.

6.6 Hydrochloric acid, 11mol/L

6.7 Methanol, HPLC grade.

6.8 Potassium ferrocyanide trihydrate.

6.9 Sodium dihydrogen orthophosphate.

6.10 Sodium hydroxide.

6.11 Theobromine.

6.12 Zinc acetate dihydrate.

6.13 Sodium hydroxide, 0.1mol/L. Weigh 4.0g of sodium hydroxide (5.10) and dissolve in water, allow to cool and dilute to 1 litre.

6.14 Phosphate buffer. Accurately weigh 3.12g of sodium dihydrogen orthophosphate (6.9) and make up to 1 litre with water in a volumetric flask. Adjust to pH 5.0 with 0.1mol/L sodium hydroxide (6.13). Filter through a 0.45 µm filter (7.4). (Mobile phase B for HPLC).

6.15 Hydrochloric acid, 0.5mol/L. Add 44.5mL of hydrochloric acid (6.6) to water and dilute to 1 litre in a volumetric flask.

6.16 Stock standard caffeine 1000mg/L. Weigh 0.5g, accurate to 0.001g, of caffeine (6.5). Transfer quantitatively into a 500mL volumetric flask with water. Dissolve in water, dilute to volume with water and mix by inversion 6 times.

6.17 Stock standard theobromine 500mg/L. Weigh 0.25g, accurate to 0.001g, of theobromine (6.11) into a 400mL beaker, dissolve in boiling water, cool and transfer quantitatively into a 500mL volumetric flask with water and mix by inversion 6 times.

6.18 Working standard solutions

Using pipettes measure 1.0, 2.5, 5.0 and 10.0mL of caffeine stock standard and 2, 5, 10 and 20mL of theobromine stock standard into a 100mL volumetric flask, dilute to volume with water and mix by inversion 3 times. This gives working standard solutions of concentrations 10, 25, 50 and 100mg/L of each additive. Working standard solutions should be prepared fresh on the day of use.

6.19 Stock standard control solutions

STANDARD MATERIAL USED TO PREPARE STANDARD CONTROL SOLUTIONS MUST BE FROM A DIFFERENT COMMERCIAL SOURCE TO THOSE USED TO PREPARE STANDARD CALIBRATION SOLUTIONS.

6.19.1 Caffeine 10,000mg/L

Weigh 0.5g accurate to 0.001g of caffeine. Transfer quantitatively to a 50mL volumetric flask with 20mL of methanol and dissolve. Dilute to volume with water and mix by inversion 6 times.

6.19.2 Theobromine 10,000mg/L

Weigh 0.5g accurate to 0.001g of theobromine. Transfer quantitatively to a 50mL volumetric flask with 20mL of methanol and dissolve. Dilute to volume with water and mix by inversion 6 times. The shelf life of these solutions is 3 months when stored at 5°C.

6.20 Working standard control solution

Using a pipette, measure 2.5mL of each stock standard control solution into a 250mL volumetric flask, dilute to volume with water and mix by inversion 6 times.

This gives a mixed, working standard control solution containing 100mg/L of each alkaloid.

The working standard control solution should be prepared fresh on the day of use.

6.21 Clearing reagents 1 and 2

1. Dissolve 21.9g, accurate to 0.1g, zinc acetate dihydrate (6.12) in water containing 3g of acetic acid (6.3) and make up to 100mL with water.

2. Dissolve 10.6g, accurate to 0.1g, potassium ferrocyanide trihydrate (6.8) in water and make up to 100mL with water.

7. APPARATUS

7.1. Normal laboratory glassware and apparatus.

7.2. Analytical balance of appropriate accuracy as specified.

7.3. 0.45µm disposable syringe filters or 0.45µm sample filter kit (Millipore or equivalent).

7.4. Solvent filter system with 0.45µm membrane filters.

7.5. Ultrasonic bath.

7.6. High Performance Liquid Chromatography (HPLC) system ideally with Diode Array Detector and integrating device which allows the measurement of peak heights or areas.

7.7. Glass microfibre filters, at least 1.6 µm (GFA or equivalent).

7.8. HPLC Chromatographic column such as Merck Lichrocart Purospher RP-18e, 5µm, 250 x 4mm, fitted with a Purospher RP-18e, 5µm 4 x 4mm guard column. Equivalent columns may be used provided they give satisfactory resolutions.

7.8.1. The following HPLC conditions have been found to be suitable. The conditions can be modified if necessary to achieve suitable resolution of the additives and flavourings of interest.

Mobile phase A (6.4), Mobile phase B (6.14)

Gradient time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	8.0	92.0
5.00	10.0	90.0
22.50	19.3	80.7
30.00	21.5	78.5
35.00	45.0	55.0
40.00	45.0	55.0
40.01	8.0	92.0
45.00	8.0	92.0

Flow rate: 1.0mL/min
Injection volume: 5µL
Column Temperature: 40°C
Detector Wavelengths: 205nm for theobromine, saccharin, benzoic acid, caffeine and aspartame
231nm for acesulfame K and quinine sulphate
256nm for sorbic acid and parabens

It is suggested that to help maintain column efficiency, it is flushed with a mixture of water and acetonitrile (about 50:50) for about 30 minutes prior to instrument shutdown. Column performance will be maintained through use of a guard column.

8. **PROCEDURE**

8.1. Chocolate and chocolate products grate, melt or blend if necessary.

8.1.1. Weigh about 1g, accurate to 0.001g, of dark chocolate, 2 - 3g of milk chocolate or 3g cake into a 100mL beaker.

8.1.2. Add 25mL of water and 2mL of 0.5mol/L hydrochloric acid (6.15) and mix by inversion 3 times.

8.1.3. Bring to the boil on a hotplate and then transfer the beaker to a boiling water bath for 15 minutes.

8.1.4. Transfer the solution to a 50mL volumetric flask and allow to cool.

8.1.5. Add 1mL of each clearing reagent (6.21), make to volume with water and mix by inversion 3 times.

8.1.6. Allow the solution to stand for about 30 minutes and then filter through a filter paper (7.7), rejecting the first 5mL and then through a syringe filter (7.3) for HPLC analysis. The filtration rate depends upon the amount of precipitated solids present. Solutions are stable for at least 24 hours.

8.2. Chromatography

Setup the HPLC system (7.6) according to the manufacturer's instructions. The instrument must be fitted with a suitable column (7.8). The operating conditions must be adjusted so as to achieve sufficient separation of the additives of interest to enable identification. Typical chromatographic separations are shown in Appendix 1.

8.3. Inject a suitable volume e.g. 5µL of the test solution and run the chromatographic separation.

8.4. Determine the peak areas (by electronic integration) at the appropriate wavelength (see 7.8.1). If a diode array detector is not available then separate runs at each wavelength may be required.

8.5. Preparation of calibration curve.

8.5.1. Successively analyse each working standard solution (6.18) according to steps 8.3 - 8.4.

8.5.2. Plot a calibration curve of analyte concentration against peak area for each analyte of interest.

8.5.3. When fresh stock standards are prepared a new calibration curve is analysed.

8.6. Calibration check.

8.6.1. Provided that the calibration curve is linear and the HPLC conditions remain ostensibly unaltered, a single working standard solution of each analyte may be used to check the calibration curve.

8.6.2. Analyse working standard 50mg/L according to steps 8.3 – 8.4. Carry out a duplicate injection of the working standard solution.

8.6.3. The calibration check standard is deemed satisfactory if the mean concentration is within ±5% of the expected value (i.e. 50mg/L) when extrapolated from the stored calibration graph.

8.6.4. If the calibration check standard meets the requirements then the stored calibration graph may be used to calculate the analyte concentration. Otherwise a fresh stock standard must be prepared.

9. CALCULATION

9.1. If a calibration curve is used, determine the concentration (C) of each analyte in the test solution directly from the calibration graph.

9.2. If a diode array detector is available, the identity of sample peaks can be confirmed if necessary.

9.3. The concentration of each analyte in the sample, expressed in mg/L or mg/kg, may be calculated according to the following formula:

$$\text{Analyte in the sample (mg/L or mg/kg)} = \frac{C \times V}{m}$$

Where C = concentration in mg/L of the analyte component from the graph

V = final volume, in mL of test solution

m = mass (or volume), in g (or mL) of test portion taken for analysis

10. EXPRESSION OF RESULTS

Record the identity of the analyte and its concentration, expressed as mg/L or mg/kg as appropriate, to the nearest 1mg/kg or 1mg/L.

11. INTERPRETATION

The amount of fat free dry cocoa in chocolate products may be calculated from the theobromine content according to the following formula.

Fat free dry cocoa (g/100g) = theobromine (g/100g) x 40.7

12. DISPOSAL

No specific problems.

13. REFERENCES

Validated Enforcement Method VEMS 0358 – Determination of additives and flavourings in food by HPLC, APA Publication for internal use
IRRM Report Detection and Quantification of Cocoa Butter Equivalents in Milk chocolate, EUR 22666 EN, M. Buchgrabber, S. Androni [31]

14. ANALYTICAL QUALITY ASSURANCE

14.1. Performance Characteristics

14.1.1. L.O.D

	liquids direct	or solid foods*
Theobromine	0.5mg/L	25mg/kg
Caffeine	0.5mg/L	10mg/kg

*Assuming 1g diluted to 50mL for theobromine (8.3.1)

14.1.2. Bias

	Recovery Mean		Recovery Standard Deviation	
	Foods	Drinks	Foods	Drinks
Theobromine	99.2%	-	2.8%	-
Caffeine	-	104.3%	-	3.3%

Theobromine spiked at a level of 50mg/L in chocolate)

14.1.3. Precision

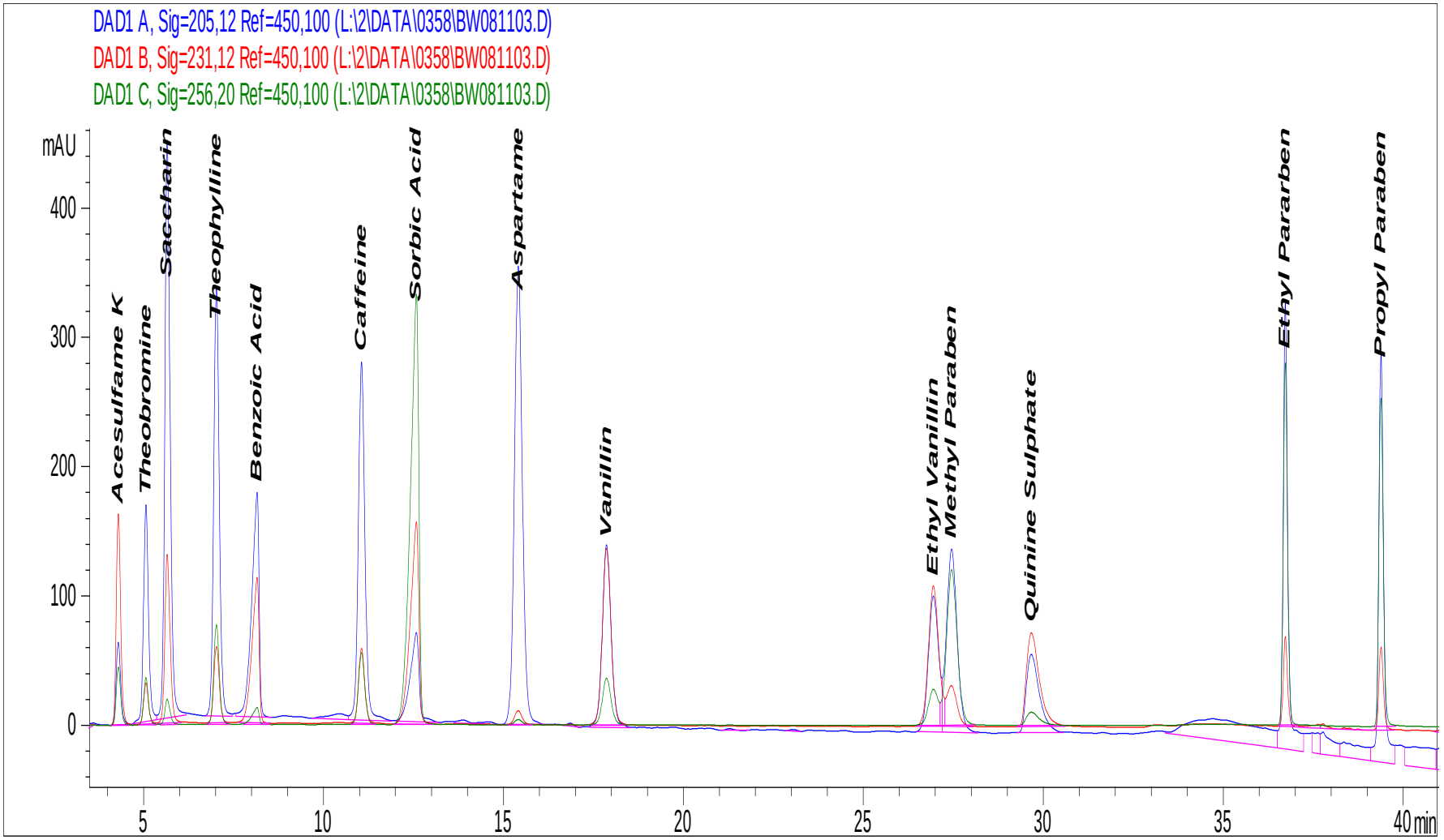
	Absolute difference Standard Deviation	
	Foods (mg/kg)	Drinks (mg/L)
Theobromine	31.6 (10 samples in the range 823 to 3117)	-
Caffeine	-	1.2 (14 samples in the range 12 to 91)

15. Internal Quality Control

15.1.	Instrument Calibration	:Refer to instrument manual.
15.2.	Blank determination	:Take an appropriate blank through the procedure.
15.3.	Standard Control	:Each batch of samples should include analysis of an in-house standard control material.

Repeatability Check:	At least every tenth sample should be analysed in duplicate and the difference between the results should conform to the performance characteristics in the laboratory.

SOP Appendix 1



APPENDIX 8

Detection and Quantification of Cocoa Butter Equivalents in Milk Chocolate

Milk chocolate is permitted to contain small amounts of fat other than cocoa butter. A method has been validated and published by Dr Manuela Buchgraber[31], manuela.buchgraber@ec.europa.eu in 2007 working at Institute of Reference Materials and Measurements in Geel, Belgium. The SOP was published and presented by Manuela at meeting at LGC workshop.

The basis of the method is a simple solvent extraction to remove triglycerides which are analysed by capillary gas chromatography. The results are entered into a custom spreadsheet and the presence of non-cocoa triglycerides are identified and quantified.

Manuela was happy to supply enforcement laboratories with copies of the SOP and spreadsheet.

APPENDIX 9
Table 6
Total Nitrogen measurement in Cocoa Liquor

Sample Lab Ref	Total N (%)	Theobromine from tot N (%)	Theobromine found (%)
20090631	2.164	6.962	1.2147
20090638	2.251	7.242	1.3132
20090780	2.081	6.694	0.8374
20090800	2.343	7.540	1.1669
20091327	2.067	6.651	1.0359
20091327	2.049	6.592	
20091328	2.199	7.077	1.1149
20100079	2.211	7.113	1.1246
20100080	2.291	7.371	1.2706
20100081	2.305	7.417	1.1498
20100085	2.163	6.961	0.9507
20100093	2.333	7.508	1.0581
20100252	2.347	7.551	1.3697
20100254	2.160	6.949	1.0779
20100255	2.074	6.673	0.9416
20100255	2.064	6.642	
20100256	2.187	7.037	0.9002
20100265	2.301	7.404	1.1759
20100959	2.281	7.338	1.0599
20100972	2.154	6.931	1.1917
NIST2384	2.119	6.818	
20101818	2.322	7.470	1.0892
20101822	2.246	7.226	1.1575
20101834	2.151	6.920	0.9664
20101842	2.324	7.476	1.1998
20101844	2.186	7.032	1.0963
20101845	2.174	6.994	1.1071

Analysis was carried out on samples of cocoa liquor for total nitrogen using the Kjeldahl method, modified to use with a hot block digestion unit. The shaded results are duplicate analyses.