Estimation of Fat-Free Cocoa Solids in Chocolate and Cocoa Products – Global Survey of Typical Concentrations of Theobromine and Caffeine Determined by HPLC

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Summary

A method for the measurement of the alkaloids theobromine and caffeine in cocoa and chocolate products is described. The method was subjected to a ring trial test with 5 laboratories from across Europe. The method involved minimal sample preparation and analysis of the alkaloids was by HPLC-DAD. The method was used to determine the dry fat free cocoa solids in 191 cocoa liquor samples from around the world. The mean content of theobromine and caffeine in the dry fat free cocoa liquor samples analysed by this method were 24,572 mg/kg and 3,165 mg/kg respectively. Full details of the project can be obtained from the UK Defra website, project code FA 0202 "Measurement of theobromine content in cocoa for determining cocoa solids content in chocolate and chocolate products".

Keywords

Theobromine, caffeine, cocoa, chocolate, HPLC.

Introduction

Cocoa and chocolate products comprise a multi-million pound industry in the UK and throughout Europe. More chocolate is consumed in Europe than any other continent. The amount of cocoa present in chocolate products is generally regarded as a guide to quality and its accurate quantification has been attempted for over 100 years.

The labelling and composition of chocolate products are controlled by the Cocoa and Chocolate Products (England) Regulations 2003[1] which implement the EC Directive 2000/36/EC[2]. Identical provisions for labelling and composition are included in parallel regulations across the devolved areas of the U.K. The regulations specify a range of reserved descriptions for chocolate products which have minimum requirements for, *inter alia*, levels of cocoa solids to be present. The alkaloids theobromine and caffeine are naturally present in cocoa. Hence the dry fat free cocoa content of food has been estimated in manufacturers' and enforcement laboratories[3] for many years by determination of these alkaloids and application of a conversion factor derived from analysis of genuine cocoa samples.

Over time, the methods used have changed and been improved. Direct measurement of alkaloid, theobromine and/or caffeine content enables the determination of total non-fat cocoa solids. In the past 50 years or so little systematic work has been done to establish whether the level of alkaloids has changed as a result of botanical and agricultural changes, different methods of production or environmental factors although it is recognised that changes in production methods and climate over the last 50 years has probably resulted in significant changes in the alkaloid levels in cocoa. To investigate what changes of significance had taken place a project was commissioned by the UK Food Standards Agency (FSA project Q01122)[4] and the main findings reported here. In essence, an updated conversion factor was calculated from 191 samples of cocoa liquor samples analysed as part of that project using the trialled method described later.

All of the in-house quality control checks carried out during the lifetime of the project were satisfactory.

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 Nib – That part of the cocoa bean remaining after raw cocoa beans have been roasted, cracked and winnowed

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)

Method Development and Ring Trial

The first objective was to optimise the liquid chromatographic (LC) method (Appendix 1) for determining theobromine and caffeine in cocoa and chocolate products including drinking chocolate, milk chocolate, plain chocolate, and chocolate. The method consists of a simple and rapid procedure for extraction of alkaloids and analysis by HPLC with diode array detection.

Six samples of cocoa liquor were analysed for theobromine and caffeine, or total alkaloids by 5 different laboratories. All participants were asked to analyse each sample in duplicate and record the identity of the analytes reported and the concentrations, as mg/kg. The results are summarised in Appendix 2.

Survey of Theobromine and Caffeine Concentrations

The optimised method was then applied to the determination of the theobromine and caffeine concentrations, over a two year period, of 191 samples of cocoa liquor, prepared from beans grown around the world. The extended time period of sampling and analysis was to take account of seasonal variations in growing conditions and possible natural changes in alkaloid levels. From the data obtained it is possible to calculate a conversion factor from the average theobromine and caffeine levels in cocoa, and compare them with previously established factors.

The results showed that the levels of theobromine present were generally lower than those found in prior survey work. This could be due to a combination of factors such as changes in the cocoa varieties being grown, e.g.

increasing use of hybrid varieties of cocoa that are hardy and high yielding, as well as changes in climate and cultivation methods.

Whilst in the past analysts were probably measuring total alkaloids accurately the types of samples under test were not described clearly i.e. samples were often described as "cocoa" or "cocoa powder". This means that the alkaloid contents being reported could not be correlated with the current data. The current results indicate that the conversion factor, calculated from theobromine content in the 191 samples of cocoa liquor tested, is higher than the factor currently used by enforcement laboratories to calculate dry fat free cocoa solids.

The mean theobromine content of the cocoa liquor samples tested was 27,737 mg/kg leading to 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 in question. The standard deviation of the results from 191 samples was 3,470 mg/kg and the range was 19803 – 39,168 mg/kg. This wide variation indicates that considerable caution must be taken when reporting on cocoa content of foods when the composition of the cocoa liquor used in their preparation is unknown. Enforcement organisations will generally take the statistical variation of the results into account when reporting on composition obtained from analysis of a sample. Indication of adverse results on samples usually leads to further investigation of further samples and/or the source of raw materials used.

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 newly-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

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

Full details of the method optimisation, ring trial, sample acquisition and analytical data obtained are given in the FSA report.[3]

Calculation

The amount of fat free dry cocoa in chocolate products may be calculated from the theobromine content as follows:

$$\text{Fat free dry cocoa (g/100g)} = \text{theobromine (g/100g)} \times 40.7$$

Where total alkaloids are measured the dry fat free cocoa content may be calculated from:

$$\text{Fat free dry cocoa (g/100g)} = \text{total alkaloids (g/100g)} \times 36.1$$

The major producers of cocoa are currently the countries of Africa. The sampling plan was biased towards cocoa samples obtained from this source. The alkaloid content of cocoa liquors were found to vary in different continents so that where the country of origin of the sample is known, then alternative, location-specific, factors may be more appropriate.

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References

1 The Cocoa and Chocolate Products (England) Regulations 2003, SI 1659/2003 http://www.legislation.gov.uk/uksi/2003/1659/contents/made

2 Directive 2000/36/EC of the European Parliament and of the Council of 23 June 2000 relating to cocoa and chocolate products intended for human consumption http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:197:0019:0025:EN:PDF

3 Validated Enforcement Method VEMS 0358 – Determination of additives and flavourings in food by HPLC, APA Publication for internal use

4 FSA project Q01122 Measurement of theobromine content in cocoa for determining cocoa solids content in chocolate and chocolate products. This project is published on the UK Defra Website, Project code: FA0202

Appendix 1

Measurement of the Alkaloids Theobromine and Caffeine in Cocoa and Chocolate Products

1. Scope of Method

1.1. 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. A range of permitted additives permitted in cocoa and chocolate products, which may interfere with the analysis of theobromine and caffeine, are also extracted including saccharin, benzoic acid, aspartame, acesulfame K, sorbic acid, vanillin, ethyl vanillin and 3 parabens. The chromatography has been optimised to separate each of these compounds.

2. Health and Safety

2.1. EYE PROTECTION SHOULD NORMALLY BE WORN AT ALL TIMES.

2.2. 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.

2.3. 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.

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

3. Reagents

Analytical Reagent grade reagents are suitable unless otherwise stated.
Water should be deionised, distilled or of similar quality.

3.1. Glacial Acetic Acid

3.2. Mobile Phase A – Acetonitrile, HPLC grade. Degas and filter through a 0.45 µm membrane filter (4.3)

3.3. Caffeine

3.4. Hydrochloric acid, 11mol/L

3.5. Methanol, HPLC grade

3.6. Potassium Hexacyanoferrate(II) Trihydrate

3.7. Sodium Dihydrogen Orthophosphate

3.8. Sodium Hydroxide

3.9. Theobromine

3.10. Zinc acetate dihydrate

3.11. Sodium hydroxide – 0.1mol/L – Weigh 4.0g of sodium hydroxide (3.8) and dissolve in water, allow to cool, dilute to 1 litre

3.12. Mobile phase B – phosphate buffer – Accurately weigh 3.12g of sodium dihydrogen orthophosphate (3.7) and make up to 1 litre with water in a volumetric flask. Adjust to pH 5.0 with 0.1mol/L sodium hydroxide. Filter through a 0.45 µm filter (4.3)

3.13. Hydrochloric acid – 0.5mol/L – Add 44.5mL of hydrochloric acid (3.4) to water and dilute to 1 litre in a volumetric flask

3.14. Stock standard caffeine 1000mg/L – Weigh 0.5g, accurate to 0.001g of caffeine (3.3). Transfer quantitatively into a 500mL volumetric flask with water. Dissolve in water, dilute to volume with water and mix by inversion 6 times.

3.15. Stock standard theobromine 500mg/L – Weigh 0.25g, accurate to 0.001g, of theobromine (3.9) 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.

3.16. 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 100mL volumetric flasks, dilute to volume with water and mix by inversion 6 times. This gives working standard solutions of concentrations 10, 25, 50 and 100mg/L of each alkaloid. Working standard solutions should be prepared fresh on the day of use.

3.17. 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.

3.17.1. 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.

3.17.2. 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.

3.18. 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.

3.19. Clearing reagents 1 and 2

3.19.1. Dissolve 21.9g, accurate to 0.1g, zinc acetate dihydrate (3.10) in water containing 3g of acetic acid (3.1) and make up to 100mL with water.

3.19.2. Dissolve 10.6g, accurate to 0.1g, potassium hexacyanoferrate(II) trihydrate (3.6) in water and make up to 100mL with water.

4. Apparatus

Normal laboratory glassware and apparatus

4.1. Analytical balance of appropriate accuracy as specified

4.2. 0.45µm disposable syringe filters or 0.45µm sample membrane filter kit (Millipore or equivalent)

4.3. Solvent filter system with 0.45µm membrane filters

4.4. Ultrasonic bath

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

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

4.7. HPLC Chromatographic column – 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

4.7.1. The following HPLC conditions were found to be suitable. The conditions can be modified if necessary to achieve suitable resolution of any additives and flavourings of interest.

Gradient Time (min)	Mobile Phase A (3.2) (%)	Mobile Phase B (3.12) (%)
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

To help maintain column efficiency, flush it 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.

5. Procedure

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

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

5.1.2. Add 25mL of water and 2mL of 0.5mol/L hydrochloric acid (3.13) and mix by inversion 6 times.

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

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

5.1.5. Add 1mL of each clearing reagent (3.19), make to volume with water and mix by inversion 6 times.

5.1.6. Allow the solution to stand for about 30 minutes and then filter through a filter paper (4.6), rejecting the first 5mL and then through a syringe filter (4.3) for HPLC analysis. The filtration rate depends upon the amount of precipitated solids present. Solutions are stable for at least 24 hours.

5.2. Chromatography

Set up the HPLC system (4.5) according to the manufacturer's instructions. The instrument must be fitted with a suitable column (4.7). 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 3.

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

5.4. Determine the peak areas (by electronic integration) at the appropriate wavelengths (see 4.7.1). If a diode array detector is not available then separate runs at each wavelength may be required.

5.5. Preparation of calibration curve.

5.5.1. Successively analyse each working standard solution (3.16) according to steps 5.3-5.4.

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

5.5.3. When fresh stock standards are prepared a new calibration curve must be prepared also.

5.6. Calibration check.

5.6.1. 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.

5.6.2. Analyse working standard 50mg/L according to steps 5.3 – 5.4. Carry out a duplicate injection of the working standard solution.

5.6.3. 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.

5.6.4. 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.

6. Calculation

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

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

6.3. 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

7. Performance Characteristics

7.1. Limit of Detection

	Liquids (direct)	**Solid Foods***
Theobromine	0.5 mg/L	25 mg/Kg
Caffeine	0.5 mg/L	10 mg/Kg

*Assuming 1g diluted to 50mL for theobromine

7.2. 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

7.3. Precision

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

Appendix 2

Ring Trial Data

Origin		Theobromine (g/100g)					Caffeine (g/100g)					Total Alkaloid (g/100g)					
		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 1	Lab 2	Lab 3[1]	Lab 4	Lab 5	Lab 5[2]
Venezuela	A	1.18	1.17	-	1.22	1.20	0.26	0.27	-	0.24	0.26	1.45	1.44	1.54	1.46	1.46	1.527
	B	1.18	1.26	-	1.26	1.20	0.26	0.29	-	0.25	0.26	1.44	1.55	1.57	1.51	1.46	1.536
Papua New Guinea	A	1.04	1.07	-	1.10	1.03	0.15	0.16	-	0.14	0.15	1.19	1.23	1.30	1.24	1.18	1.241
	B	1.02	1.11	-	1.05	1.04	0.15	0.16	-	0.13	0.15	1.16	1.27	1.31	1.18	1.18	1.282
Ecuador	A	1.15	1.18	-	1.20	1.17	0.19	0.21	-	0.19	0.19	1.35	1.39	1.44	1.39	1.36	1.442
	B	1.15	1.27	-	1.21	1.18	0.19	0.22	-	0.18	0.19	1.34	1.49	1.43	1.39	1.37	1.443
San Tome	A	1.19	1.23	-	1.25	1.18	0.10	0.11	-	0.10	0.10	1.30	1.34	1.40	1.35	1.28	1.419
	B	1.17	1.39	-	1.25	1.19	0.10	0.12	-	0.10	0.11	1.27	1.51	1.41	1.35	1.29	1.425
Madagascar	A	1.12	1.15	-	1.18	1.12	0.16	0.18	-	0.15	0.16	1.28	1.33	1.41	1.33	1.28	1.381
	B	1.11	1.23	-	1.15	1.13	0.16	0.18	-	0.15	0.15	1.27	1.41	1.41	1.30	1.28	1.385
Tanzania	A	1.20	1.29	-	1.20	1.21	0.16	0.17	-	0.13	0.14	1.36	1.46	1.48	1.33	1.36	1.442
	B	1.16	1.26	-	1.22	1.21	0.15	0.17	-	0.13	0.15	1.32	1.43	1.49	1.35	1.36	1.443
NIST 2384	A	1.11					0.11					1.22					
	B	1.10					0.11					1.21					

Reference values for NIST CRM 2384 – Theobromine 1.16% ± 0.11%, Caffeine 0.11% ± 0.05%

Notes:

1. Lab 3 only measured total alkaloids using a spectrophotometric method
2. Lab 5 used HPLC for theobromine and caffeine determination and measured total alkaloids using a spectrophotometric method

Statistical Analysis of Ring Trial Data

Origin	Theobromine (g/100g)			Caffeine (g/100g)			Total Alkaloid (g/100g)		
	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
Papua New Guinea	1.11	1.02	1.06	0.16	0.13	0.15	1.31	1.16	1.23
Ecuador	1.27	1.15	1.19	0.22	0.18	0.20	1.49	1.34	1.40
San Tome	1.39	1.17	1.25	0.12	0.10	0.11	1.51	1.27	1.36
Madagascar	1.23	1.11	1.16	0.18	0.15	0.16	1.41	1.27	1.34
Tanzania	1.29	1.16	1.22	0.17	0.13	0.15	1.49	1.32	1.39

Appendix 3

Typical Separation of Alkaloids and Food Additives using HPLC-DAD