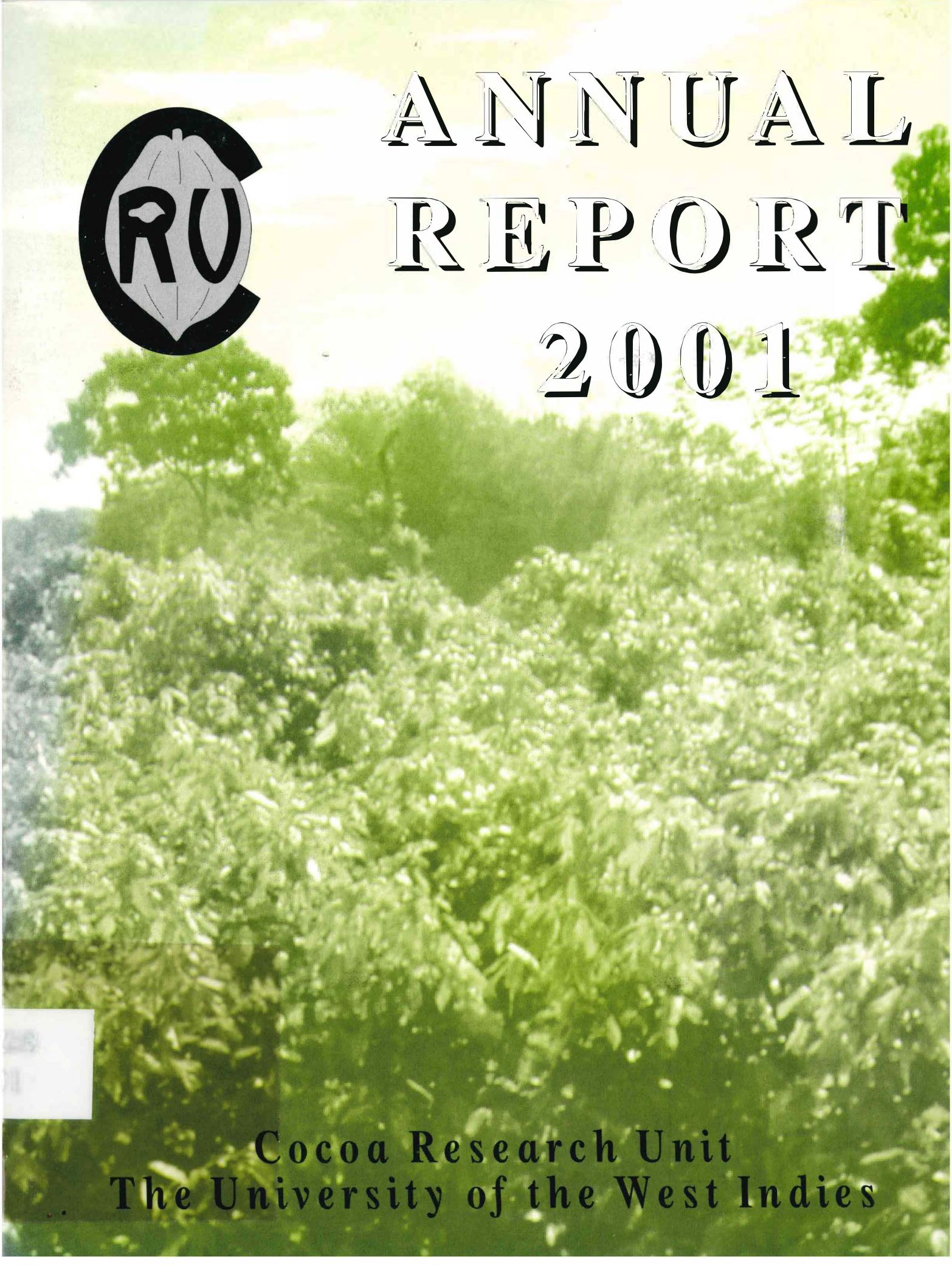

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Cover photograph. Canopy of closely planted trees on a commerical cocoa estate in east Trinidad.

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Preliminary results from chemical analyses of selected cacao (*Theobroma cacao* L.) accessions – training experiences at CIRAD

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Introduction

In response to an invitation from CIRAD, Darin Sukha went to Montpellier, France from 6 September to 5 November 2001 for training in High Performance Liquid Chromatography (HPLC) techniques on different cacao varieties. He was supervised by Dr. Emile Cros, who has over 26 years of experience working on the flavour chemistry of cocoa.

Aims and Objectives

The use of HPLC for chemical analyses is a new area of research for CRU. For the purpose of training, an experiment was designed to exploit the diversity of cacao germplasm at the ICG, T, and also include some accessions which were selected as common clones for the CFC/ICCO/INIAP Flavour Project.

The experiment comprised 23 cacao accessions representing seven groups within the *T. cacao* species *viz.* AMELONADO, CATONGO[2], Criollo, Forastero, Hybrid, Refractario[3] and Trinitario. The preliminary investigation was to determine the possibility of differentiating between these groups by extracting Purine, Flavanol and Procyanidin fractions from each accession and analysing the extractions by HPLC to identify and quantify phenolic compounds.

Phenolic Compounds and Separation Techniques

Phenolic compounds are important components of many fruits, vegetables and beverages, since they contribute to flavour, colour and sensory properties such as bitterness and astringency. Recent interest in functional foods and the medicinal use of phenolic compounds, specifically their anti-oxidant properties, have also stimulated interest in their chromatographic separation (Lee, 2000).

Cocoa is rich in phytochemicals and, in unfermented cocoa beans, pigment cells make up about 11-13% of the tissue. The pigment cells contain approximately 65% – 70% polyphenols and 3% anthocyanins by weight. During fermentation the polyphenols undergo a variety of reactions, including self-condensation and reactions with proteins and peptides. Approximately 20% (by weight) of the polyphenols remain at the end of the fermentation process. Roasting and other cocoa processing activities cause further changes. It is generally agreed that the level of polyphenols varies with the variety of cocoa bean and with the degree of fermentation (Zumbe, 1998).

According to the International Union of Pure and Applied Chemistry, "chromatography" is a physical method of separation, in which the components to be separated are distributed between two

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[2] CATONGO is a mutant with white cotyledons within the Forastero group.

[3] Refractario is viewed as a sub-classification within the Forastero group.

phases, one of which is stationery (the stationery phase), and the other of which (the mobile phase) moves in a definite direction. Different types of chromatography depend on the nature of the mobile phase (liquid, gas or supercritical fluid chromatography). High Performance Liquid Chromatography (HPLC) is a method of separating a mixture of compounds based on their differing physical properties, such as polarity, charge and size. The compounds are partitioned between the liquid mobile phase and a bonded or stationary phase packed within a column. The retention time refers to the time that elapses between injection and elution of a solvent and the retention factor is a measure of the time the sample component resides in the stationery phase relative to the time it resides in the mobile phase (Siouffi, 2000).

Materials and Methods

Purine Determination

Duplicate samples of freeze-dried cocoa beans from the selected accessions were peeled and ground into a powder. Approximately 0.3 g of cocoa powder was extracted with distilled water under reflux for 30 min. After cooling and filtration via vacuum through celite, the extracts were diluted to 100 mL, filtered through a 0.45µm micro pore syringe filter and analysed using a Shimadzu LC-5A HPLC with a Shimadzu SPD-6A UV spectrophotometric detector. Separations were effected at ambient temperature on a 5 µm reverse phase C18 LiChromosphere 100RP-18 column with pre column using mobile phase A = 30% Methanol and B = 70% Water at a flow rate of 1 mL/min and a loop injection of 10 µL. Quantitative levels of caffeine and theobromine were determined at 278 nm relative to a reference standard solution. A column wash of 100% methanol for 30 min. was used between injections. Integration was achieved with a Hewlett Packard 3390A integrator.

Flavanol and Procyanidin Determination

Approximately 1 g of defatted cocoa powder (soxhlet extraction with petroleum ether) was extracted with 100 mL of 75% acetone in water with stirring for 1 h. The extracts were filtered through sodium chloride and allowed to settle, separating the lower aqueous hydroxycinnamic phase from the polar acetone phase containing procyanidin. One mL of an internal standard of gallic acid was added to the acetone phase which was successively dehydrated and re-extracted with chloroform and then with ethyl acetate. The final extract was filtered through anhydrous sodium sulphate, dehydrated and finally re-hydrated with 25 mL of methanol. Analysis was carried out using an Agilent Series 1100 HPLC with a Diode Array Detector (DAD) in the Ultra Violet (UV) visible range.

Separations were effected at ambient temperature on a 5 µm reverse phase C18 LiChromosphere 100RP-18 column with pre-column. The flavanols and procyanidins were eluted with a linear gradient of A = 5% Methanol and B = 95% 2 mM Phosphoric Acid (time, %B): (0, 95), (48, 100) at a flow rate of 1 mL/min and a loop injection of 10 µL. Components were detected in the UV visible range and the software for data acquisition and processing was HPCHEM station Version A06.03. Concentrations of (-) epicatechin were determined relative to the internal gallic acid reference and procyanidin levels were estimated using the response factor for (-) epicatechin. A column wash of 100% methanol for 30 min. was used between injections.

Table 1. Percentage butterfat, purine, flavanol [(-) epicatechin)] and procyanidin isomers B-2, B-5 and C-1 in 14 cacao accessions from different cacao groups.

Accession	Group	Butter Fat (%)	Theobromine (%)	Caffeine (%)	Epicatechin (%)	B2 (%)	B5 (%)	C1 (%)
AMELONADO	AMELONADO	40.0	1.33	0.04	6.25	2.02	0.48	3.06
AMELONADO	AMELONADO	na	1.12	0.04	na	na	na	na
MATINA 1/7	AMELONADO	53.4	1.04	0.20	18.63	6.87	1.61	8.97
CATONGO	CATONGO	48.8	1.25	0.10	7.91	3.20	0.50	4.32
SP1	CRIOLLO	53.1	1.05	0.41	5.02	2.01	0.52	2.74
IMC 67	FORASTERO	51.3	1.08	0.12	16.94	6.37	1.82	8.36
MOCO 1/12	FORASTERO	48.5	1.57	0.15	25.48	11.52	4.88	10.11
LCT EEN 271/S-3	FORASTERO	50.6	1.66	0.16	na	na	na	na
PA 34	FORASTERO	53.1	1.18	0.11	na	na	na	na
NA 33	FORASTERO	55.3	1.02	0.17	30.17	9.58	2.51	12.58
DE 52.B	HYBRID	52.9	1.24	0.14	na	na	na	na
DOPOL 2/1-5	HYBRID	59.5	1.38	0.14	26.67	9.41	2.55	10.80
DOS HERMANOS	HYBRID	53.5	0.93	0.14	11.30	6.82	1.61	14.96
JA 5/39	REFRACTARIO	52.9	1.65	0.28	15.17	4.07	1.75	11.44
B 9/10-23	REFRACTARIO	52.5	1.59	0.25	14.48	7.20	1.13	4.85
AM 2/83	REFRACTARIO	49.6	1.47	0.21	na	na	na	na
LV 20	REFRACTARIO	48.8	1.18	0.16	12.07	6.86	1.35	1.09
NG 3	TRINITARIO	51.5	1.46	0.34	na	na	na	na
TRD 1	TRINITARIO	45.7	1.25	0.30	13.53	26.02	6.61	34.01
COMMERCIAL TRINITARIO	TRINITARIO	55.0	1.17	0.23	16.81	6.04	1.79	8.81
ICS 1 (UWI)	TRINITARIO	43.8	0.73	0.26	2.23	4.63	1.89	11.85
ICS 1 (CENTENO)	TRINITARIO	51.2	1.01	0.21	18.30	0.84	1.29	6.04
VEN A/58	TRINITARIO	52.2	1.50	0.21	na	na	na	na
GS 50	TRINITARIO	47.6	1.04	0.18	71.54	6.9	0.18	0.51

"na" designates missing values. No extraction and analysis was done for this compound.

Integration and Calculation of Results

Chromatograms contain retention times that are used to identify specific compounds. Under specific conditions the same compound will have a constant retention time. This retention time is established and checked via the use of pure standards of the compounds of interest. The retention time, the area under the chromatogram peak and the amount/area ratio are all used to quantify the amount of the compound under investigation relative to the calibration for that particular compound. Calibration standards may be internal or external and the percentage recovery of the standard is used to indicate the efficiency of the extraction process. Once the quantity of the compound in the injected sample is calculated via an integrator or specialised computer software this is then extrapolated to the actual sample from which the initial extraction was done. A final percentage representing the quantity of compound present is then calculated.

Results

The results in Table 1 show the various accessions selected and the group to which they belong. The percentage butterfat, purine (theobromine and caffeine), flavanols ((-) epicatechin) and the procyanidin isomers B-2, B-5 and C-1 for the accessions selected are also presented.

All accessions sampled were analysed for purine content. However, due to time and logistical constraints, a sub-set of accessions sampled were extracted and analysed for flavanol and procyanidin content. However, at least two accessions from each population were analysed for all compounds. Missing values are designated "na" (not available).

Data Analysis

Discriminant data analysis and analysis of variance (ANOVA) were carried out using Minitab Release 12.21 (Minitab Inc.). Due to unavailability of pods, only one accession within the Criollo group and one CATONGO were represented, so these could not be included in the discriminant data analysis.

Table 2 shows the squared distance between the groups represented as a measure of differences between groups based on purine, flavanol and procyanidin contents.

Table 3 shows the mean percentage and standard deviation of the purine, flavanol and procyanidin contents for all groups except Criollo and CATONGO.

Table 2. Squared distance between groups represented.

	Amelonado	Forastero	Hybrid	Refractario	Trinitario
Amelonado	0.00	8.14	3.90	15.95	8.20
Forastero	8.14	0.00	3.91	33.90	6.51
Hybrid	3.90	3.91	0.00	31.32	8.31
Refractario	15.95	33.90	31.32	0.00	18.75
Trinitario	8.20	6.51	8.31	18.75	0.00

Table 3. Variable pooled means and standard deviation for groups based on purine, flavanol and procyanidin contents.

Compound	Amelonado	Forastero	Hybrid	Refractario	Trinitario
% Theobromine	1.18 (0.20)	1.22 (0.30)	1.16 (0.32)	1.47 (0.26)	1.04 (0.20)
% Caffeine	0.12 (0.12)	0.15 (0.03)	0.14 (0.00)	0.23 (0.07)	0.23 (0.05)
% Epicatechin	12.43 (8.76)	24.20 (6.71)	19.00 (10.90)	13.91 (1.63)	24.50 (27.05)
% B2	4.44 (3.44)	9.16 (2.60)	8.11 (1.83)	6.04 (1.71)	8.89 (9.86)
% B5	1.04 (0.80)	3.07 (1.61)	2.08 (0.67)	1.14 (0.31)	2.35 (2.47)
% C1	6.01 (4.18)	10.35 (2.12)	12.88 (2.94)	5.80 (5.24)	12.24 (12.86)

Discussion

These preliminary results are based on a very small sample size from each group, so any conclusions are tentative and serve only as a basis for further research in the new area of collaboration for CRU.

Epicatechin represents a class of flavanols present in cocoa. Due to its high concentration, ease of analysis and availability of authentic standards much research has been done on its quantification in various cocoa accessions. Clapperton *et al.* (1992) revealed a strong correlation between epicatechin levels and astringent and bitter flavour attributes. From the results presented in Table 1, epicatechin levels did not generally vary significantly between groups, however, a notable exception was GS 50 in the Trinitario group, which contained the highest epicatechin concentration (71.54%). Accession SP 1 from the Criollo population had the lowest epicatechin concentration (5.02%). These epicatechin values correlate with the findings of Clapperton (1992) in that Criollo varieties are generally not bitter or astringent and require little fermentation (as little as 24 hours). Trinitario varieties on the other hand are much more bitter and astringent and require up to 168 hours (7 days) fermentation.

Theobromine and caffeine represent a class of compounds known as xanthine alkaloids. They too are responsible for bitter and astringent flavours. From the ANOVA of the data, average percentage caffeine was the only compound that varied significantly ($P<0.05$) between groups. Theobromine content ranged from 0.73% - 1.66% across the cacao groups, although differences were not statistically significant.

The average percentage butterfat was assessed as a part of the procyanidin extraction process. Butterfat content did not vary significantly between groups but ranged from 40.0% in AMELONADO to 59.5% in DOPOL 2/1-5 (a Hybrid).

The procyanidin isomers B-2, B-5 and C-1 can also be correlated to bitter and astringent flavours as well as increasing cocoa flavour as they are reduced during the course of fermentation (Clapperton *et al.*, 1992). A corollary to this observation was noted in NA 33, an accession that is noted for its intense cocoa flavour when properly fermented (Sukha *et al.*, 2002). This flavour attribute corresponds to reductions in polyphenol composition and subsequent bitter and astringent taste. Clapperton *et al.* (1992) quantified the purine, flavanol and procyanidin contents of this accession at 0-, 3- and 5-day fermentation times. We also performed the same extractions on fresh beans (0-day fermentation) from this accession using a different procedure. The results for the epicatechin levels and the procyanidin isomers at day 0 are almost the same to those presented by

Clapperton *et al.* (1992), highlighting the robustness of the methodology used in this study.

ICS 1 was sampled from two different growing locations; UWI Campus cocoa fields, St. Augustine and also from the La Reunion Estate, Centeno. Both locations have different soil types and microclimatic rainfall patterns. The percentage butterfat, theobromine and caffeine contents in Table 1 for these two samples (ICS 1 UWI and ICS 1 Centeno) did not vary widely. However, the percentage epicatechin content was higher for ICS 1 from Centeno whilst the percentage procyanidin isomer content was higher for ICS 1 from UWI. These observations, though very preliminary, allude to possible environmental effects on epicatechin and procyanidin contents and ultimately flavour. Further research in this area is needed and is currently being undertaken at CRU.

The results from discriminant analyses in Tables 2 and 3 allow tentative conclusions to be made about the groups based on the compounds analysed, excluding CATONGO and Criollo. The Refractario group was separated from the other groups by the largest squared distance. Large squared distances imply large differences between groups, and vice versa. The distances are squared to account for negative values.

The relative difference of Refractario from the other groups must be viewed against the limited sampling of accessions. However, if the result was subsequently confirmed, it would be interesting since Refractarios are viewed by many as an "artificial" group selected from within the Forastero group.

The pooled means and standard deviations in Table 3 indicate the variability of each compound relative to the different populations. Percentage caffeine and theobromine varied little between groups whilst percentage epicatechin was more variable, especially within the Forastero group. The procyanidin isomers also varied widely between groups.

Analyses for AMELONADO were repeated to check the consistency of the extraction and quantification process for theobromine and caffeine. Both instances showed good consistency between repetitions since there was no variation in caffeine values whilst theobromine content varied by 0.21%.

Conclusions

Even though these results are very preliminary, they afford an interesting insight into the potential value of the association between identification and quantification of key chemical compounds and their link to flavour attributes. This work has added a new depth of understanding to some fundamental principles behind flavour chemistry and created the potential for new areas of collaboration between CIRAD and CRU. Future collaboration is planned and will build on the existing strong links between CIRAD and CRU in plant biochemistry and pathology. This collaboration is appropriate since the diversity of cacao germplasm at the ICG, T has not been examined in this manner up to now and the genebank makes sampling for this type of exercise very easy. A vacuum in knowledge exists in this area in spite of such a diverse and relatively easily accessible selection of germplasm.

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