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PHILIPPINES PHARMACEUTICAL INDUSTRY DEVELOPMENT

DP/PHI/87/019

PRE-FEASIBILITY STUDY ON PROCESSING CINCHONA FOR QUININE IN THE PHILIPPINES

Contract No. 89/42

Amendment No. 1

Report on Cinchona bark analysis methods and results

Prepared for the Government of the Philippines
on behalf of the United Nations Industrial Development
Organization acting as executing agency for
the United Nations Development Programme.

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SYNOPSIS

This study completes the analysis of fourteen samples of Cinchona bark, covering 8 and 11 year old trees of each of the seven species of Cinchona growing in the Phillipines, and compares two different methods of analysis.

Results lead to the following conclusions:-

(1) a method developed for the analysis of Cinchona bark by High Performance Liquid Chromatography (HPLC) is most useful and suitable for use in analysing large numbers of samples.

(2) setting up of a small laboratory to be equipped for HPLC analysis, preferably under direction of DENR 10 in Mindanao, is recommended.

(3) that Cinchona trees in the Philippines are still significantly increasing in Quinine content between the eighth and eleventh years and some very good contents have been observed.

(4) it must be recommended that only planting of the Cinchona species Ledgeriana should be persevered with in the future, but the varieties Tjiniroena and Kartamanah should be included.

(5) Study programmes should be set up to extend the analysis of existing trees and for monitoring, as an essential feature, any future forestry programmes.

CONTENTS

Introduction.

As a recommendation resulting from the Philippines Pharmaceutical Industry Development Study (DP/PHI/87/019) of 1988 a pre-feasibility Study on Processing of Cinchona for Quinine in the Philippines, under the same funding, was completed in August, 1989.

Information on the composition and level of alkaloids in the bark of the Cinchona trees was sparse and some fourteen samples were collected from growing trees covering the seven species growing at Kaatoan, Bukidnon, Mindanao (the Centre for Cinchona Reforestration Project in the Philippines) for analysis. Samples were taken from trees of eight and eleven years old of each of the seven species.

It was proposed to analyse these samples by two different methods, namely (1) the classical Bruxelles method which is universally accepted by Quinine producers and users, but which is rather tedious and (2) a method to be developed on the use of High Performance Liquid Chromatography (HPLC) which would give more general information on alkaloid distribution and be a more rapid method.

For the purpose of the Pre-feasibility study it was only possible to analyse three samples by the classical Bruxelles method and to develop the HPLC method by which seven samples were analysed. Choice of samples was such that only in the case of one sample was a direct comparison between the methods possible.

It was considered most important for the possible development of the Cinchona plantations in the Philippines, with a view to commercial exploitation, that the analyses of all fourteen samples of Cinchona bark collected (by both the Bruxelles and HPLC methods) be completed. The recommendation of such has led to this extension study under DP/PHI/87/019.

Important aspects of completion of the analyses include:-

(1) confirming the reliability and use of the developed HPLC analysis method,

(2) provision of some initial information on alkaloid development with age,

(3) confirm, and possibly extend, recommendations given in the Pre-feasibilitiy study as to the future development of the Cinchona p antations.

Report.

Fourteen samples of Cinchona bark were collected in July 1989 and transported to the U.K. All samples were dried on receipt in the U.K. and ground to preserve the quality. Storage for extended periods of wet bark, with moisture content above 12%, can possibly leaad to deterioration. All analyses were performed on the dried samples and, although samples analysed to satisfy this report were delayed in execution, can be considered reliable.

Seven species of Cinchona tree are growing at Kaatoan, Bukidnon and one sample from a tree of each species at eight years old together with one sample from a tree of each species at eleven years old was harvested making fourteen samples in total.

The species of Cinchona trees which are growing are listed below together with the abbreviations which will be used later in reporting results.

Cinchona Ledgeriana	= C.Ledg.
Cinchona Ledgeriana, variety Tjiniroena	= C.LvT.
Cinchona Ledgeriana, variety Kartamanah	= C.LvK.
Cinchona Officinalis	= C.Off.
Cinchona Calisaya	= C.Cal.
Cinchona Succirubra	= C.Suc.
Cinchona Hybrid	= C.Hy.

Methods of analysis.

An object of this study was to analyse the fourteen samples of Cinchona bark by two different methods of analysis, the classical Bruxelles method and a method developed while preparing the Pre-feasibility study referred to in the introduction utilising HPLC, and determine the general suitability and usefulness of the latter.

Bruxelles method.

This is the classical method used consistently by the Quinine manufacturers and Cinchona growers throughout the world for commercial application.

The method is based on the extraction of total alkaloids of Cinchona and the precipitation of quinine and cinchonidine tartrates. By measurement of the optical rotation of the dried tartrates and the application of published calibration tables (COMMELIN tables) the relative contents of quinine and cinchonidine may be determined. The method does not give precise levels of quinine or cinchonine as the respective dihydro-derivatives are also precipitated. However, from experience the method gives a good guide as to the amount of quinine which can be commercially isolated.

Results reported on analysis by this method are generally given on an 'as received basis' as this is most important to a buyer or seller who will contract for the sale/purchase of some finite weight of bark. For the purpose of this assessment of bark and tree quality, as well as comparison of methods, it is more convenient and useful to present results on the basis of dried bark and such are reported.

The precise method and details of execution, together with the calibration tables of CONNELIN are given in Appendix 1.

HPLC method.

HPLC analysis of Cinchona alkaloids had only previously been applied to the analysis of samples derived from tissue culture samples and authentic mixtures (ref.1)

Work was initiated within the Pre-feasibility study on the utilisation of Cinchona on the development of a method utilising HPLC for the analysis of bark samples. Some initial problems were experienced and overcome. The main problem was related to the method of extraction. A paper was followed which advocated the destruction of cells by pre-treatment with trichloroacetic acid (ref.2.). It was shown that this produced artifacts which unforunately especially enhanced the response peaks of quinine leading to spurious results. To overcome this the standard method of extraction used in the Bruxelles method was applied on reduced scale and led to reliable results. This method was used for the 7 samples previously analysed and for the subsequent 7 samples analysed under the current study.

It is thought that direct extraction with methanol, rather than alkalising and extraction with toluene, might be equally acceptable but this must remain as a subject for future confirmation. Use of methanol extraction would slightly reduce the work involved in preparing samples.

The HPLC methods which are applicable are also given, for ease of abstracting, in Appendix 2.

Results.

The following abbreviations, in addition to those previously indicated for Cinchona tree species, are used in reporting results.

TAA	= Total alkaloids anhydrous
SQ_2	= Quinine sulphate dihydrate
Qn	= Quinine
HQn	= Dihydroquinine
Qd	= Quinidine
HQd	= Dihydroquinidine
Cd	= Cinchonidine
HCd	= Dihyrocinchonidine
Cn	= Cinchonine
HCn	= Hydrocinchonine

Note: SQ_2 = 1.2067 x Qn

All results are reported as % based on dried bark.

Bark alkaloid analysis - Bruxelles method.

Sample No.	Specie	Age years	TA%	SO_2%	Cd%
2604	C.Ledg.	8	8.9	6.8	2.3
2605	C.Ledg.	11	12.2	9.9	2.2
2606	C.LvK.	8	9.0	6.9	1.4
2607	C.LvK.	11	7.35	2.8	1.6
2608	C.LvT.	8	6.9	5.7	0.6
2609	C.LvT.	11	12.6	11.6	1.5
2610	C.Off.	8	7.8	3.1	1.9
2611	C.Off.	11	10.1	7.75	3.8
2612	C.Cal.	8	4.5	1.1	1.3
2613	C.Cal.	11	9.8	3.9	2.6
2614	C.Hy.	8	8.95	3.5	1.5
2615	C.Hy.	11	9.1	2.2	2.9
2616	C.Suc.	8	9.5	3.3	4.1
2617	C.Suc.	11	8.9	1.75	3.4

Bark alkaloid analysis - HPLC method.

Sample No.	Specie (Age yrs)	Cn%	Cd%	HCn%	HCd%	Qd%	Qn%	HQd%	HQn%
2604	C.Ledg. (8)	0.22	1.13	-	-	0.16	6.40	-	0.31
2605	C.Ledg. (11)	0.34	0.25	-	-	0.51	9.37	-	-
2606	C.LvK. (8)	0.35	0.79	-	-	-	6.41	-	0.38
2607	C.LvK. (11)	1.85	1.16	-	-	0.16	3.04	-	-
2608	C.LvT. (8)	0.08	-	-	-	0.19	5.27	-	0.21
2609	C.LvT. (11)	0.09	0.06	-	-	0.16	11.55	-	-
2610	C.Off. (8)	0.96	1.27	0.16	-	0.16	3.58	-	0.35
2611	C.Off. (11)	1.51	1.00	-	-	0.49	7.27	-	-
2612	C.Cal. (8)	0.25	1.09	-	-	-	1.12	-	-
2613	C.Cal. (11)	1.62	1.20	-	-	0.18	4.93	-	-
2614	C.Hy. (8)	1.61	0.75	0.25	-	0.56	3.55	0.06	0.06
2615	C.Hy. (11)	1.55	0.41	-	-	0.12	2.85	-	-
2616	C.Suc. (8)	0.52	2.30	0.08	0.07	-	4.00	-	0.21
2617	C.Suc. (11)	1.34	2.20	-	-	0.10	2.36	-	-

Note: All even numbered samples were analysed at an earlier date using a Lichrosorb column, whereas all odd numbered samples have been recently analysed using a Bondapak column. See Appendix 2 for details.

Calibration traces for both columns, together with some typical traces can be found in Appendix 3.

Comparison of results.

For easy comparison purposes the results from the Bruxelles method of analysis and the HPLC method are re-tabulated below listing only the relevant elements which can be compared. Quinine is being reported both in terms of anhydrous alkaloid and also SQ_2 (representing quinine sulphate dihydrate) since this is the term most usually used to indicate quality of Cinchona bark and can be more easily related to the projections given in the earlier pre-feasibility study.

Sample No.	Specie (Age yrs)	SQ_2		Qn.		Cd.	
		HPLC	Brux.	HPLC	Brux.	HPLC	Brux.
2604	C.Ledg. (8)	7.72	6.80	6.40	5.64	1.13	2.30
2605	C.Ledg.(11)	11.31	9.90	9.37	8.20	0.25	2.20
2606	C.LvK. (8)	7.73	6.90	6.41	5.72	0.79	1.40
2607	C.LvK. (11)	3.66	2.80	3.04	2.32	1.16	1.60
2608	C.LvT. (8)	6.36	5.70	5.27	4.72	-	0.60
2609	C.LvT. (11)	13.93	11.60	11.55	9.61	0.06	1.50
2610	C.Off. (8)	4.32	3.10	3.58	2.57	1.27	1.90
2611	C.Off. (11)	8.77	7.75	7.27	6.42	1.00	3.80
2612	C.Cal. (8)	1.35	1.10	1.12	0.91	1.09	1.30
2613	C.Cal. (11)	5.95	3.90	4.93	3.23	1.20	2.60
2614	C.Hy. (8)	4.28	3.50	3.55	2.90	0.75	1.50
2615	C.Hy. (11)	3.44	2.20	2.85	1.82	0.41	2.90
2616	C.Suc. (8)	4.83	3.30	4.00	2.73	2.30	4.10
2617	C.Suc. (11)	2.84	1.75	2.36	1.45	2.20	3.40

Interpretation and discussion of results.

HPLC comments.

In the earlier runs made using the Lichrosorb column all the components were evaluated on a single run (always performed in duplicate). This is possible when the appropriate calibration of the unit is made and it can be ensured that all levels of peaks will be fully integrated. Care must be taken to see this is the case particularly in the case of high quinine content barks. Typical traces are reproduced in Appendix 3.

In the recent runs on the Micro-Bondipak column it was, in fact, established that the quinine peaks during Run 1 were not being fully integrated especially in the cases of 2605 and 2609 where very high quinine contents were expected. Run 1 gave a good analysis for the other alkaloids present at lower levels. A second calibration of the equipment was made especially to determine the levels of quinine and these are reproduced also, together with most traces of Run 1 also in Appendix 3.

Under regular running conditions it would be proposed to set parameters such that a single run is all that is necessary.

Results.

The results of the Bruxelles analysis method and the HPLC analysis method are considered to be in agreement especially with respect to the most important information relating to the quinine content of barks.

Only the contents of quinine and cinchonidine can be compared by the two methods and the precise levels reported do differ in magnitude. On average the HPLC method indicates a quinine alkaloid content approximately 1.2 times greater than the Bruxelles method. Consequently the Qn. level reported by HPLC approximates to the SQ_2 level reported by the Bruxelles method.

In the case of cinchonidine lower figures are indicated by the HPLC method than the Bruxelles method. In this case there is less consistency of variation.

It could be possible to calculate a total alkaloids figure (TAA) from the HPLC integrations, but this information is less useful than the general picture of the HPLC trace indicating distribution of constituents.

Some explanations of the differences can be proposed:-

(1) HPLC indicates the actual levels of all cinchona alkaloids present in the bark (subject to very small extraction losses) There are no chemical processing losses.

(2) Bruxelles method essentially indicates the isolatable level of quinine in the bark. Significant chemical processing losses must be anticipated due to solubility of Qn/Cd tartrates in the process liquors (such is also known to be somewhat enhanced by co-solubility effects due to other alkaloids)

(3) It is possible that the COMMELIN tables, originally published in 1912, are slightly biassed towards cinchonidine rather than quinine.

These suggestions are merely speculative.

It may be concluded that the HPLC method is considered to be suitable for use in analysis of Cinchona bark for any assessment programme or monitoring of forestry programmes. It may also be used to indicate the likely commercial value of bark if the Qn. analysis figure by HPLC is interpreted as the estimated SO_4 content for evaluation purposes. At this stage of development, however, it would be unlikely to be acceptable as an official international assay method.

Selection of analysis method.

The HPLC method is relatively simple and easy to reproduce. Some time has to be expended on the preparation of samples but these can be done on a battery basis and provided the simple extraction equipment needed is available a large number of samples can be readily processed. With a little further development work into the use of methanol as extraction solvent there is indication that the method could be further simplified.

The Bruxelles method is still accepted as the international method for commercial evaluation of Cinchona bark, but the lengthy operations are not suitable for assessment of large numbers of samples at one time.

Fluorimetry: It was mentioned in the Pre-feasibility study on utilisation of Cinchona that a fluorimetric method might be most useful for the analysis of a large number of samples, having been used for this purpose in Kenya. The method was applied to only one species of C.Ledgeriana and considerable calibration work was necessary before it could be usefully applied. One reason for this is that the method relies on the measurement of fluorimetric absorption. Both quinine and quinidine actively absorp whilst cinchonine and cinchonidine do not. It is necesssary to establish a factor for the enhancement effect of the appropriate level of quinidine in the species to calculate the quinine content.

No other information is provided by the method other than the quality in terms of quinine and it is considered that this method would be much less useful than the HPLC method.

Cinchona bark qualities.

Analysis results indicate that only the Cinchona ledgeriana species can be considered of good ouality for commercial exploitation. It is now clear though that almost certainly all three species of bark, C.ledgeriana, C.ledgeriana v. Tjiniroena and C.ledgeriana v. Kartamanah may now be considered as suitable planting stock and not solely the C.ledgeriana provisionally proposed in the Pre-feasibility study. This improves the situation with respect to commercial exploitation in the future.

There is one anomalous result seen in the sample of the eleven year old C.ledgeriana v. Kartamanah which analyses badly, but the eight year old sample is satisfactory. Both Bruxelles and HPLC analyses agree indicating a correct analysis. It is

suggested that this sample could not be representative and the result should be ignored at this stage and until further samples can be analysed.

Although only a very small sample has been taken it is felt that the suspected problem of reducing the quality of trees by cross pollination has not occurred, particularly in the case of the Ledgeriana species. This may be a result of relatively good segregation. This is encouraging.

It also appears that especially in the Ledgeriana species development of the level of alkaloids, and particularly quinine, is considerable between eight and eleven years growth. In recent years there has been a tendency to harvest trees at a younger age, down to seven years. Such trees have mostly been grown essentially in the open and not under a canopy. In the Philippines Cinchona trees are grown under a natural canopy and this may explain later development. It would appear that harvesting of trees from Kaatoan would be beneficial at an age not less than eleven years. Apart from the increase in alkaloid quinine content a considerable weight growth will also have developed. Commercial projections should be rather better than those indicated latterly in the Pre-feasibilty study although some delay in commencement could be necessary to accomodate older trees.

The final encouraging aspect is the level of quinine indicated in the eleven year old C.Ledgeriana and C.Ledgeriana var. Tjiniroena at respectively 9.9% and 11.6% SO_2. These are very good quality trees.

Future progress.

In addition to the programme for planting a wider analysis of existing tree samples should be considered. Also any programmes of re-forestration or of experimental nature should be monitored by chemical analysis at appropriate times using HPLC.

With regards existing trees emphasis should be given to checking different areas and ages of trees of the Ledgeriana species. Useful information could also be gained by extention of analysis of more mature C.Officinalis species as these could possibly provide supplementary material suitable for commercial extraction although recovery yields would be depressed if the cinchonidine levels are as high as initial samples indicate.

The value of C.Hybrid, C.Calisaya and C.Succirubra for any commercial exploitation is low and any further analysis of these trees should be very limited.

To pursue this work a small laboratory should be set up, preferably under the direction of DENR 10 and probably at the premises of this organisation in Cagayan de Oro City.

Basic requirements would be:-

A room, no larger than 4 x 3 m. fitted with two benches, desk, electrical power, water supply and ventilation.

For basic equipment :-

1 x Coffee mill (grinder)
1 x modern balance
1 x Air drying oven
6 x small mortar & pestels

12 x soxhlet extraction units with flasks & condensers and sufficient electrical battery heaters or sand trays. Adequate supply thimbles.

Various graduated flasks, 5ml, 50ml & 1.000ml. Miscellaneous measuring cylinders, conical flasks, pipettes, spatulas and other glassware. Glassware drier.

1 x HPLC unit complete with pumps, integral 10µl loop injection, UV (220 nm) detector and computing integrator. Syringes and columns (2 x Lichrosorb RP8 Select B and/or 2 x Micro-Bondipak C_{18}.)

It would be valuable occassionally to make checks against the Bruxelles method and equipment necessary to do so as far as obtaining dry tartrates can be included in the above glassware but including two larger soxhlet extractors. The number of samples being limited, the inclusion of an automatic polarimeter would not be justified, but arrangements could be made for the optical rotations to be performed at an analytical laboratory (probably in Manila) where such equipment is available.

The principle element of cost lies in the HPLC unit and the most expensive or sophisticated is not essential. An overall setting-up cost is estimated at between USD 25,000 & USD 40,000.

Conclusions and recommendations.

1. Conclusions.

This report concludes that the object of the study has been acheived most satisfactorally for the following reasons:-

a) use of a HPLC method for relatively rapid analysis of a large number of samples and for screening purposes is considered to have been proved satisfactory.

b) preliminary indication, as a result of the small screening exercise, indicates that all C.Ledgeriana species appear to be of good quality.

c) fears of possible problems of adverse cross pollination are probably seen to be groundless, particularly in the Ledger species.

d) considerable increase in alkaloid content, especially quinine, is observed between eight year old and eleven year old trees and suggests that any harvesting should be delayed until this older age.

e) some particularly high content trees have been observed and if confirmed in a wider survey suggests a very interesting commercial venture could ensue.

2. Recommendations.

a) For future planting programmes only C.Ledgeriana should be propagated, but the species varieties Tjiniroena and Kartamanah may now also be included.

b) A wider screening programme should be undertaken, and for this purpose a small testing laboratory should be set up. This should be preferably under the direction of DENR 10 and probably situated in the premises at Cagayan de Oro City. An estimated cost of between USD 25,000 and USD 40,000 is envisuaged.

c) The above analytical facility should also be used for the monitoring of any re-forestration programmes.

d) When qualities of a broader spectrum of representative samples has been recorded further projections, as in the Pre-feasibility study, should be made for assessing an up-dated commercial feasibility.

APPENDIX 1.

CINCHONA BARK ANALYSIS

Based on Bruxelles 1949 Standard Method for Analysis Of Cinchona Bark

1. Grind the representative sample until all passes through No.30 BS Sieve.

2. Moisture Content

   Dry 5 g. at 110°C for 2 hours

3. (i) Liberation of Alkaloids

   Mix thoroughly in a mortar:-

   20 g. of ground sieved bark
   6 g. $Ca(OH)_2$
   20 ml. 5% NaOH

   LEAVE FOR HALF HOUR

   (ii) Extraction

   Transfer to extraction thimble (33 x 118 mm), cover with cotton wool and extract in soxhlet with 200 ml. Toluene in a 250 ml. flask on a heating unit for six hours at 8 to 10 syphonings per hour. Solvent to be kept as warm as possible.

   (iii) Formation of Hydrochloride of Alkaloids

   Distil off the Toluene on a hot plate until about 20 ml. is left in the flask. Introduce 20 ml. of N/2 HCl and 20 ml. water. Boil off the remainder of the Toluene removing Toluene vapours in a current of air. Allow to cool and filter through a plug of cotton wool, wash with boiling water until the Hydrochloride has been removed. Volume of filtrate made up to 100 ml.

   (iv) Total Alkaloid

   Titrate the solution as hot as possible with N/1 NaOH to pH 6.5 (6.2 - 6.8 permissable). Methyl red spot test yellow; or bromcresol purple - blue. Filter titrated solution through a No. 1 fluted paper. Rinse beaker and wash through filter paper. Evaporate to 50 ml. again.

(v) Formation of Tartrates of Quinine and Cinchonidine

Add 0.25 ml. 1.0 HCl, (4 drops) then with stirring 10 ml. of 40% Sodium D-Tartrate. Continue to stir only until crystals start to separate, leave over night. Note temperature of the solution before filtering.

Filter the tartrates into a tared sintered crucible, (previously dried at 110°C) measure volume of filtrate and use a portion of recovered mother liquor to effect transfer. Wash the crystals five times with 2 ml. portions of a solution saturated at room temp. with quinine and cinchonidine tartrates (ratio 9.1 formed from pure Hydrochlorides). Dry at 110°C for three hours; cool in dessicator and re-weigh.

Under these conditions Quinine tartrate contains 1 mol of water and cinchonidine tartrate is anhydrous.

(vi) Determination of Quinine Content of Combined Tartrates

Weigh out, accurately, 0.4g. add from burette 3.00ml. N.HCl make up to 20 ml. (If too coloured add 15-25 mg. of decolourising charcoal). Filter, through a No. 50 paper and reject the first few mls. Fill 2 dm. polarimeter tube and determine optical rotation (note temp. of solution) (14-21°C). Temperature as near as possible to 17°C.

4. (i) Calculation of Results

Total Alkaloid: 1 ml. HCl - 0.31 g. alkaloid.

(ii) Wt. of Tartrates

Add on corrections for solubility and temperature (J.W. Commelin).

Solubility correction (for prescribed volume) (50 + 10)ml. is 25 mg. Temperature correction = 25 x .02 (t-17)mg.

(iii) Quinine Content

Correction for temperature of optical rotation .0137° (t-17) to be added to observed rotation at t°C only applicable to 2% solution
i.e. negative coefficient.

Quinine content of combined tartrates obtained directly from table of Dr. J.W. Commelin. Or use formula, or prepare calibration from pure samples.

Quinine content of bark calculated from total weight of tartrates (corrected value) and weight of original sample.

Tables for the determination of quinine & cinchonidine contents in tartrates by polarimetry - COMMELIN tables.

Optical rotation	Wt.anhydrous base per gm. tartrate dried at 110°C.		Optical rotation	Wt.anhydrous base per gm. tartrate dried at 110°C.	
deg.°min.'	Quinine	Cinchonidine	deg.°min.'	Quinine	Cinchonidine
8°51'	0.7941	0.0000	8°04'	0.6092	0.1854
8°50'	0.7902	0.003944	8°03'	0.6053	0.1892
8°49'	0.7861	0.007878	8°02'	0.6015	0.1832
8°48'	0.7823	0.01183	8°01'	0.5974	0.1972
8°47'	0.7784	0.01578	8°00'	0.5936	0.2011
8°46'	0.7745	0.01972	7°59'	0.5896	0.2050
8°45'	0.7705	0.02367	7°58'	0.5857	0.2090
8°44'	0.7665	0.02761	7°57'	0.5819	0.2129
8°43'	0.7626	0.03155	7°56'	0.5779	0.2169
8°42'	0.7588	0.03549	7°55'	0.5740	0.2209
8°41'	0.7548	0.03944	7°54'	0.5701	0.2248
8°40'	0.7508	0.04338	7°53'	0.5661	0.2288
8°39'	0.7469	0.04733	7°52'	0.5620	0.2327
8°38'	0.7430	0.05126	7°51'	0.5582	0.2367
8°37'	0.7391	0.05521	7°50'	0.5543	0.2405
8°36'	0.7350	0.05916	7°49'	0.5503	0.2445
8°35'	0.7311	0.06310	7°48'	0.5464	0.2484
8°34'	0.7273	0.06704	7°47'	0.5425	0.2524
8°33'	0.7233	0.07099	7°46'	0.5386	0.2564
8°32'	0.7194	0.07494	7°45'	0.5346	0.2602
8°31'	0.7155	0.07886	7°44'	0.5307	0.2642
8°30'	0.7115	0.08281	7°43'	0.5267	0.2681
8°29'	0.7076	0.08676	7°42'	0.5229	0.2720
8°28'	0.7037	0.09069	7°41'	0.5188	0.2761
8°27'	0.6997	0.09464	7°40'	0.5150	0.2800
8°26'	0.6958	0.09858	7°39'	0.5110	0.2839
8°25'	0.6918	0.1025	7°38'	0.5071	0.2878
8°24'	0.6879	0.1065	7°37'	0.5031	0.2918
8°23'	0.6839	0.1104	7°36'	0.4992	0.2958
8°22'	0.6800	0.1144	7°35'	0.4953	0.2997
8°21'	0.6761	0.1183	7°34'	0.4914	0.3037
8°20'	0.6722	0.1223	7°33'	0.4874	0.3076
8°19'	0.6682	0.1262	7°32'	0.4835	0.3116
8°18'	0.6643	0.1301	7°31'	0.4796	0.3155
8°17'	0.6604	0.1341	7°30'	0.4756	0.3195
8°16'	0.6564	0.1380	7°29'	0.4718	0.3233
8°15'	0.6525	0.1420	7°28'	0.4677	0.3273
8°14'	0.6486	0.1459	7°27'	0.4638	0.3313
8°13'	0.6446	0.1498	7°26'	0.4599	0.3352
8°12'	0.6407	0.1538	7°25'	0.4560	0.3391
8°11'	0.6368	0.1578	7°24'	0.4521	0.3431
8°10'	0.6328	0.1617	7°23'	0.4481	0.3470
8°09'	0.6290	0.1656	7°22'	0.4442	0.3510
8°08'	0.6250	0.1696	7°21'	0.4402	0.3549
8°07'	0.6212	0.1736	7°20'	0.4363	0.3588
8°06'	0.6172	0.1774	7°19'	0.4324	0.3628
8°05'	0.6132	0.1814	7°18'	0.4285	0.3667

COMMELIN tables continued.

deg. ° min. '	Quinine	Cinchonidine	deg. ° min. '	Quinine	Cinchonidine
7°17'	0.4245	0.3707	6°22'	0.2084	0.5876
7°16'	0.4207	0.3746	6°21'	0.2044	0.5916
7°15'	0.4167	0.3786	6°20'	0.2004	0.5955
7°14'	0.4128	0.3825	6°19'	0.1965	0.5994
7°13'	0.4088	0.3865	6°18'	0.1926	0.6034
7°12'	0.4049	0.3904	6°17'	0.1887	0.6073
7°11'	0.4010	0.3944	6°16'	0.1848	0.6112
7°10'	0.3970	0.3983	6°15'	0.1808	0.6151
7°09'	0.3931	0.4023	6°14'	0.1769	0.6191
7°08'	0.3891	0.4061	6°13'	0.1730	0.6232
7°07'	0.3852	0.4102	6°12'	0.1690	0.6270
7°06'	0.3814	0.4141	6°11'	0.1651	0.6310
7°05'	0.3774	0.4180	6°10'	0.1612	0.6349
7°04'	0.3735	0.4220	6°09'	0.1572	0.6389
7°03'	0.3695	0.4259	6°08'	0.1533	0.6428
7°02'	0.3656	0.4298	6°07'	0.1494	0.6468
7°01'	0.3616	0.4338	6°06'	0.1454	0.6507
7°00'	0.3577	0.4377	6°05'	0.1416	0.6546
6°59'	0.3538	0.4417	6°04'	0.1376	0.6586
6°58'	0.3498	0.4457	6°03'	0.1337	0.6625
6°57'	0.3459	0.4495	6°02'	0.1297	0.6665
6°56'	0.3420	0.4535	6°01'	0.1258	0.6704
6°55'	0.3381	0.4575	6°00'	0.1219	0.6744
6°54'	0.3341	0.4614	5°59'	0.1180	0.6782
6°53'	0.3302	0.4654	5°58'	0.1140	0.6822
6°52'	0.3263	0.4692	5°57'	0.1101	0.6861
6°51'	0.3223	0.4733	5°56'	0.1061	0.6901
6°50'	0.3184	0.4772	5°55'	0.1022	0.6940
6°49'	0.3145	0.4811	5°54'	0.09836	0.6981
6°48'	0.3106	0.4851	5°53'	0.09434	0.7020
6°47'	0.3066	0.4890	5°52'	0.09040	0.7060
6°46'	0.3027	0.4929	5°51'	0.08648	0.7099
6°45'	0.2987	0.4969	5°50'	0.08254	0.7139
6°44'	0.2948	0.5008	5°49'	0.07861	0.7178
6°43'	0.2909	0.5048	5°48'	0.07469	0.7218
6°42'	0.2869	0.5088	5°47'	0.07076	0.7256
6°41'	0.2830	0.5126	5°46'	0.06682	0.7297
6°40'	0.2791	0.5166	5°45'	0.06290	0.7335
6°39'	0.2752	0.5206	5°44'	0.05896	0.7374
6°38'	0.2712	0.5245	5°43'	0.05503	0.7415
6°37'	0.2673	0.5284	5°42'	0.05109	0.7454
6°36'	0.2634	0.5323	5°41'	0.04718	0.7494
6°35'	0.2594	0.5363	5°40'	0.04324	0.7532
6°34'	0.2555	0.5403	5°39'	0.03931	0.7571
6°33'	0.2516	0.5443	5°38'	0.03538	0.7612
6°32'	0.2477	0.5481	5°37'	0.03145	0.7650
6°31'	0.2437	0.5521	5°36'	0.02752	0.7690
6°30'	0.2397	0.5560	5°35'	0.02359	0.7731
6°29'	0.2359	0.5601	5°34'	0.01965	0.7769
6°28'	0.2319	0.5639	5°33'	0.01572	0.7809
6°27'	0.2280	0.5679	5°32'	0.01180	0.7848
6°26'	0.2241	0.5719	5°31'	0.007861	0.7886
6°25'	0.2202	0.5758	5°30'	0.003931	0.7927
6°24'	0.2163	0.5797	5°29'	-	0.7968
6°23'	0.2122	0.5837			

APPENDIX 2.

CINCHONA BARK ANALYSIS

Based on High Performance Liquid Chromatography (HPLC) method of analysis.

This method was developed on the basis of extraction by the Bruxelles method and use of published procedures of D.McCalley, (ref.1).

Method.

1. Grind a representative sample until all passes through a No. 30 BS sieve.
2. Moisture content & drying.

   Dry 5.0 g. of ground bark at 110°C for 2 hours or to constant weight. Record moisture content
3. Preparation of samples for HPLC.

   (i) Mix thoroughly in a mortar,
   1.0 g. dried bark
   0.3 g. calcium hydroxide
   1.0 ml. 5% sodium hydroxide solution.

   After mixing leave to stand for 30 minutes.

   (ii) Transfer all the alkalised sample to a soxhlet extraction thimble and extract with hot toluene for 6 hours. (use about 50 ml. toluene).
   Drain thoroughly and wash cake with a little toluene. Make up volume to 50 ml. and mix well.

   Remove 1.0 ml. extract and evaporate dry at room temperature with a stream of nitrogen. Dissolve the residue in mobile phase (acetontrile-0.1M potassium dihydrogen phosphate (3:17) adjusted to pH 3 with ortho-phosphoric acid) and make up with same solution to 5.0 ml.

   Use this solution for injection.
4. HPLC analysis.

   The following equipments have been used to perform the analyses reported upon here.

   (i) Used for samples 2604, 2606, 2608, 2610, 2612, 2614, and 2616.

   Equipment: Altex 110A double reciprocating pump, Philipps LC3 ultra-violet detector (set at 220 nm), a Rheodyne valve fitted with a 10µl loop.

   Column: Lichrosorb RP-8 Select B, 25cm. length, 0.4cm. i.d. (Merck, Darmstadt, FRG), 5um. particle size.

Measurement: Retention times and peak areas measured with Trivector 2000 computerised data station.

Peak assignments based on comparison of retention times with those of authentic standards (Fluka, Sigma).

Standards: These have to be assayed by HPLC prior to use since some contain large quantities of corresponding di-hydro compounds (see reference traces (Appendix 3)

(ii) Used for samples 2605, 2607, 2609, 2611, 2613, 2615, and 2617.

Equipment: Varion 5000 HPLC. Ultra-vilot detector (set at 220 nm), 10 µl injection loop.

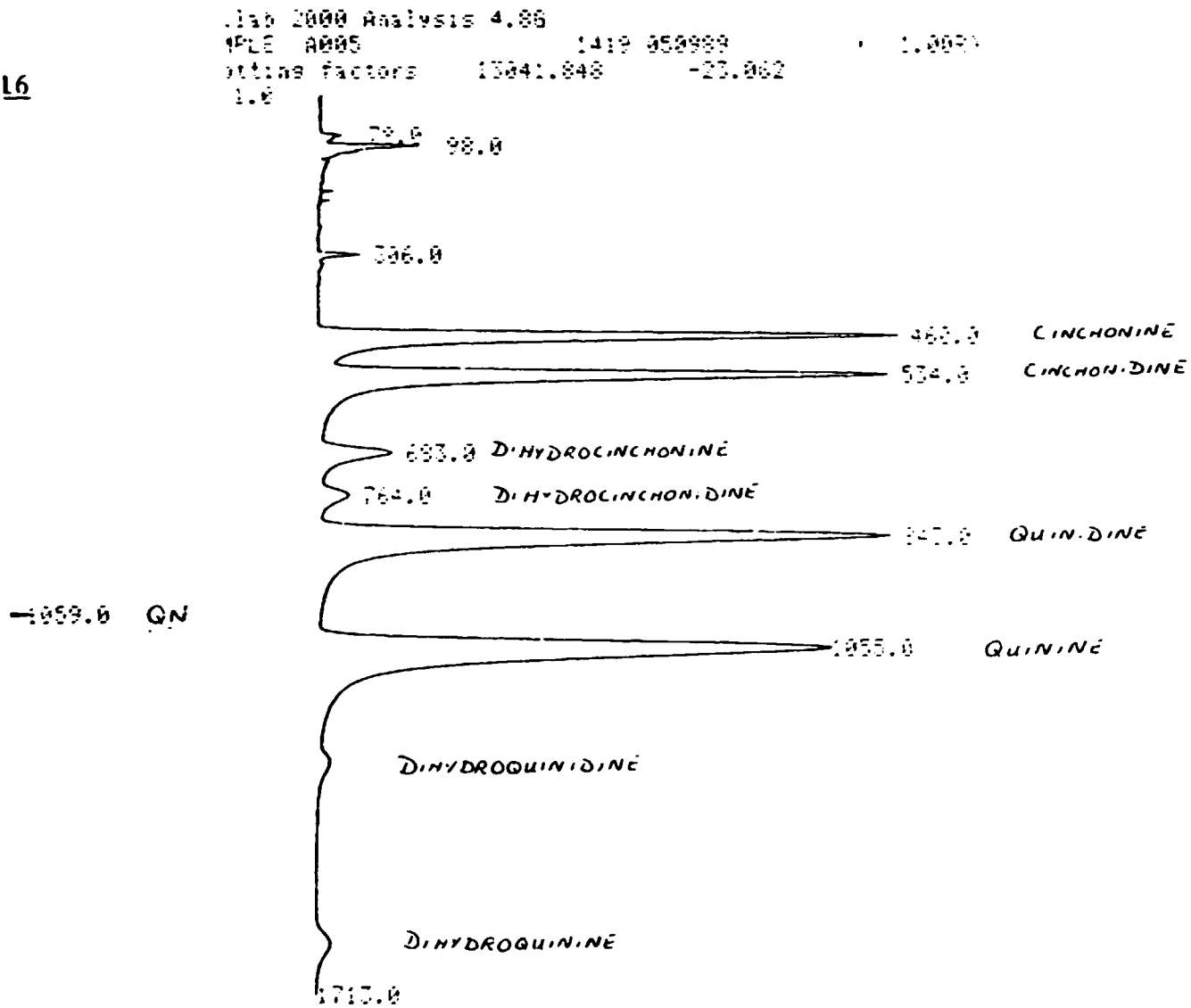
Column: µBondapak C_{18} column. (Waters)

Measurement: Philipps PU 4811 Computing integrator.

Reference traces for both columns, together with some typical sample traces are to be found in Appendix 3.

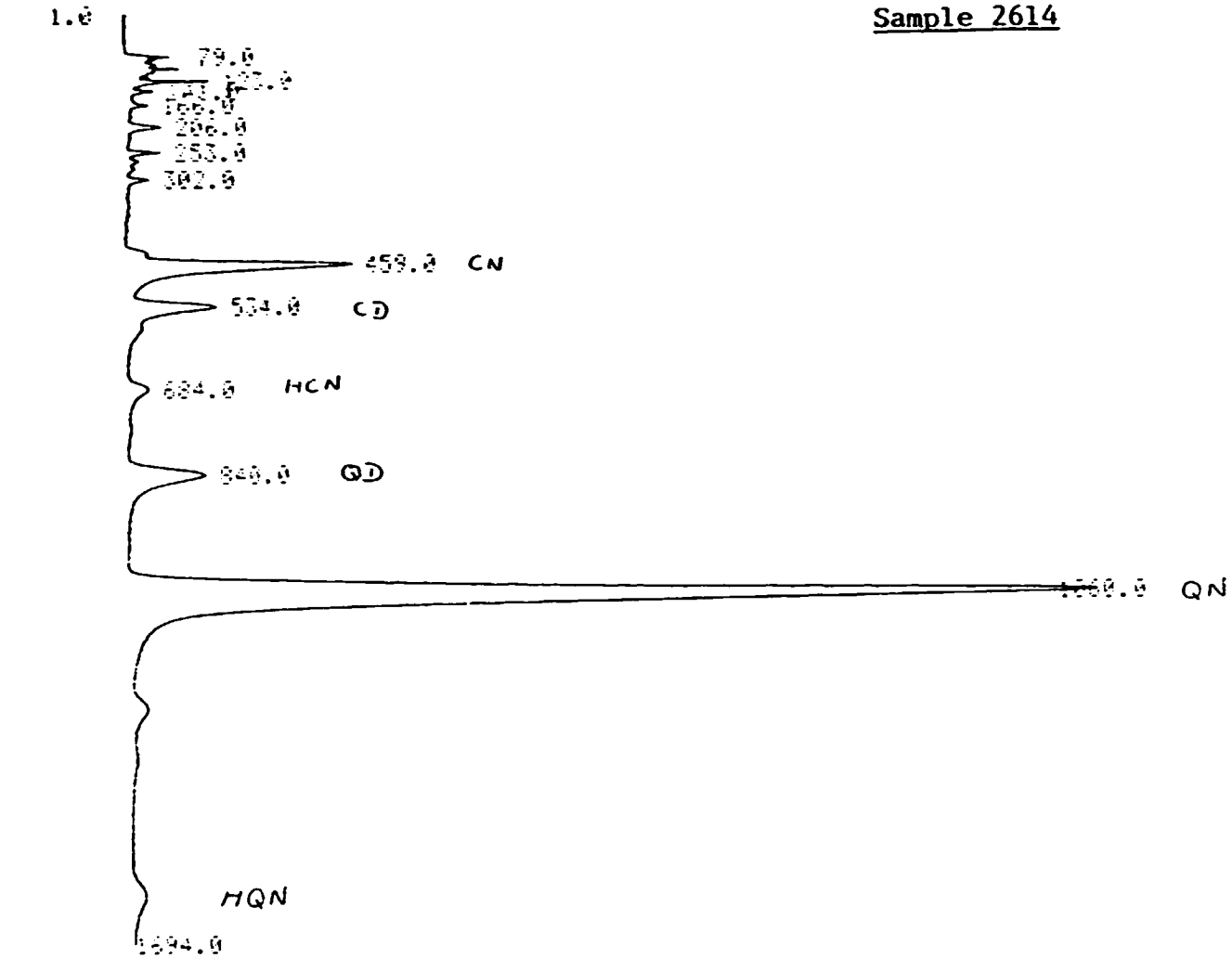
CALIBRATION.

Standard trace - Lichrosorb RP8 Select B column

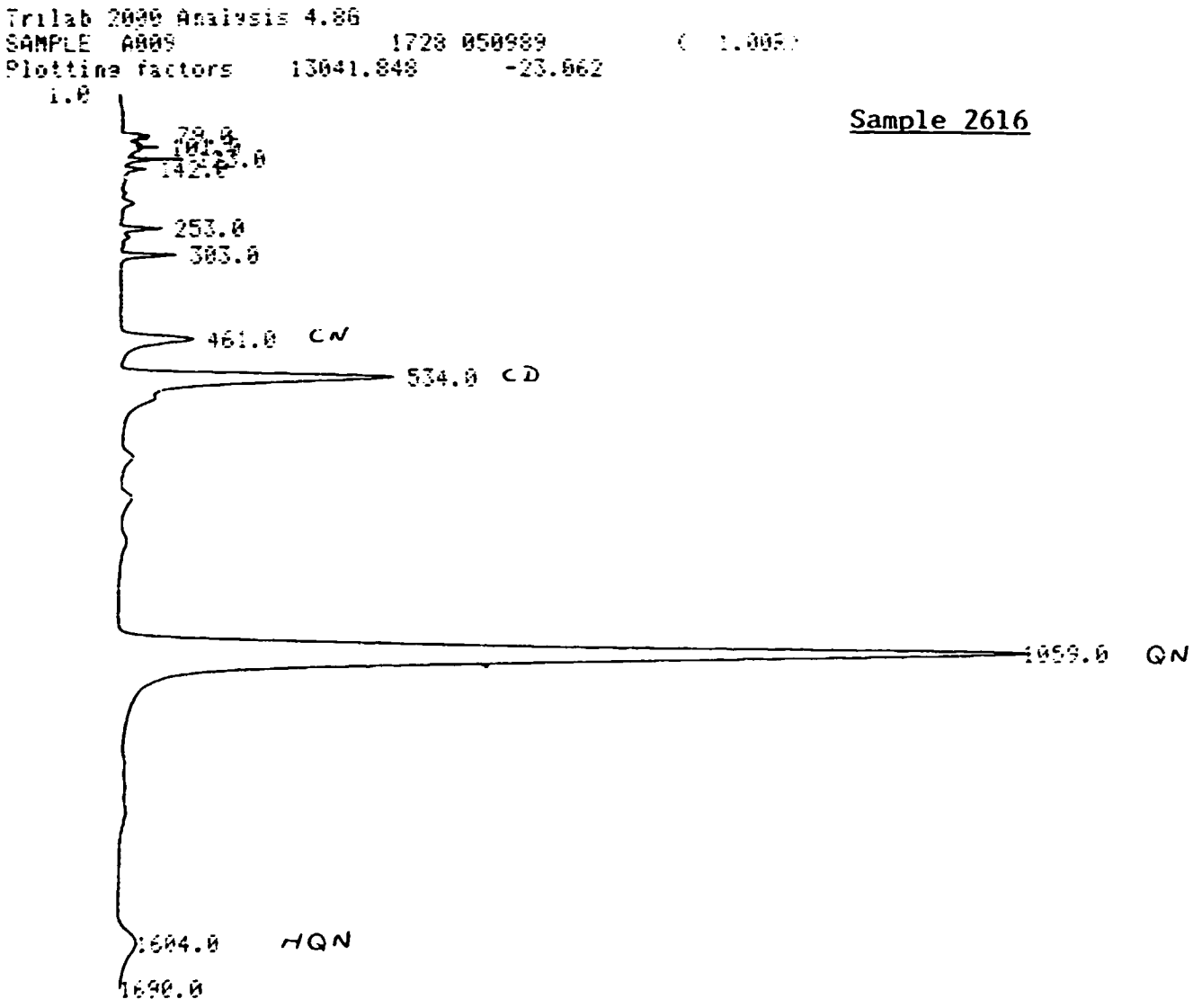


16

Trilab 2000 Analysis 4.05
SAMPLE A008 1651 050989 (1.00R)
Plotting factors 13041.848 -23.062

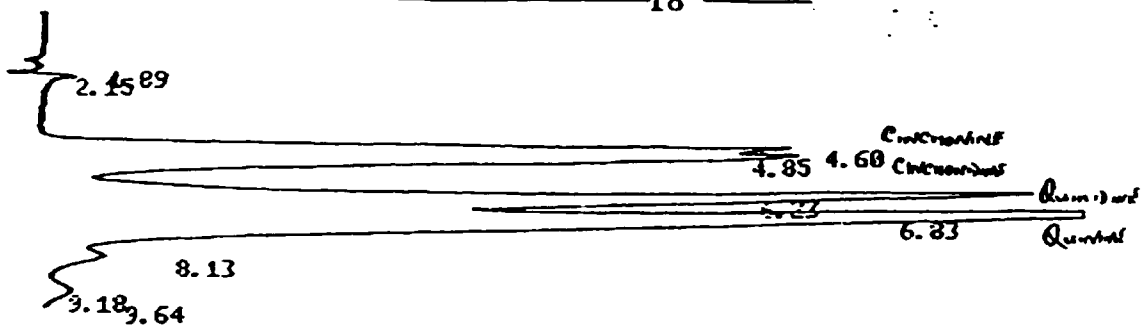


Sample 2614


Trilab 2000 Analysis 4.86
SAMPLE A009 1728 050989 (1.005)
Plotting factors 13041.848 -23.862
1.0
Sample 2616
253.0
303.0
461.0 CN
534.0 CD
1059.0 QN
1604.0 HQN
1690.0

CALIBRATION.

Standard trace - Micro-Bondipak C_{18} column Used on Run 1



05.06.90 15:30:44 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 11 INDEX 11

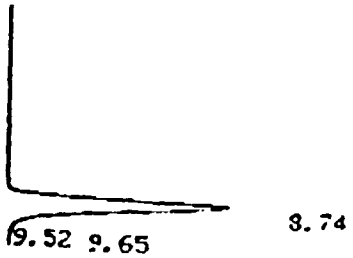
PEAK#	AREA%	RT	AREA BC
1	0.569	1.89	2316 02
2	2.306	2.15	9378 03
3	11.296	4.6	52296 02
4	16.685	4.85	65828 02
5	26.857	6.23	105635 02
6	37.939	6.83	149226 02
7	1.707	8.13	6716 02
8	0.698	9.18	2747 02
9	0.022	9.64	87 03
TOTAL	100.		393329

Samples:

Cn	14.75 mg%
Cd	12.70 mg%
Od	13.90 mg%
On	19.40 mg%

CALIBRATION.

Standard trace - Micro-Bondipak C_{18} column Used on Run 2
Quinine only



07.06.90 15:39:57 CH= "A" PS= 1.

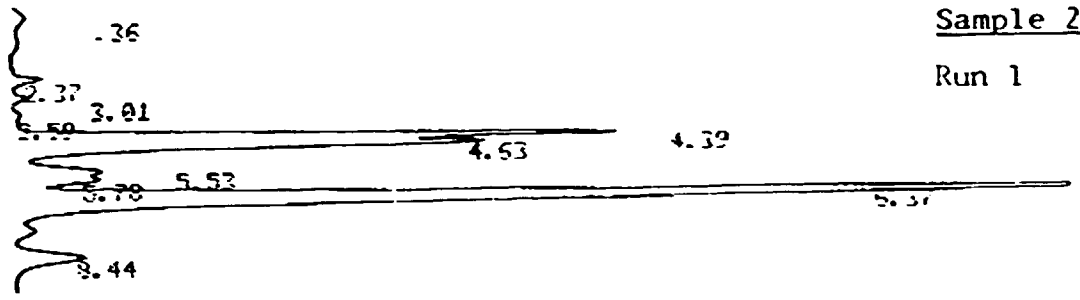
FILE 1. METHOD 0. RUN 19 INDEX 19

PEAK#	AREA%	RT	AREA BC
1	98.855	8.74	46641 02
2	0.638	9.52	301 02
3	0.507	9.65	239 03

CHANNEL A INJECT 05.06.90 18:11:51

Sample 2607

Run 1



05.06.90 18:11:51 CH= "A" PS= 1.

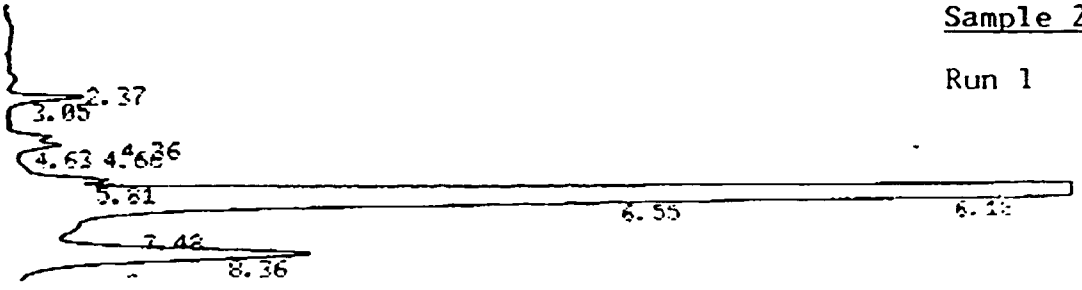
FILE 1. METHOD 0. RUN 24 INDEX 24

PEAK#	AREA%	RT	AREA	BC
1	0.491	0.36	592	01
2	0.889	2.37	1533	02
3	0.427	3.01	736	02
4	0.151	3.59	261	03
5	18.2	4.39	31396	02
6	15.154	4.63	27367	02
7	3.282	5.53	5662	02
8	3.302	5.78	5696	02
9	53.246	6.37	91852	03
10	3.948	8.44	6811	01
TOTAL	100.		172506	

CHANNEL A INJECT 05.06.90 17:30:26

Sample 2609

Run 1

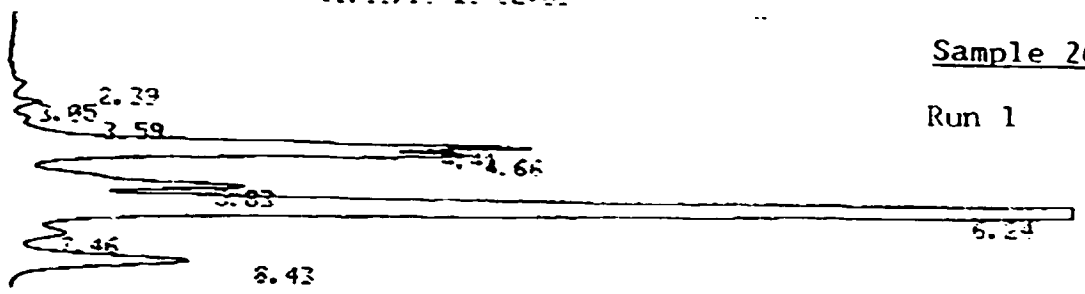


05.06.90 17:30:26 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 20 INDEX 20

PEAK#	AREA%	RT	AREA	BC
1	0.234	2.37	495	02
2	1.806	3.05	3816	03
3	0.929	4.36	1964	02
4	0.654	4.63	1383	02
5	0.606	4.86	1281	03
6	2.488	5.81	5253	02
7	48.236	6.12	102032	02
8	27.874	6.55	58900	02
9	1.535	7.48	3244	02
10	15.587	8.36	32936	03
TOTAL	100.		211393	

CHANNEL A INJECT 05.06.30 16:02:03



Sample 2611

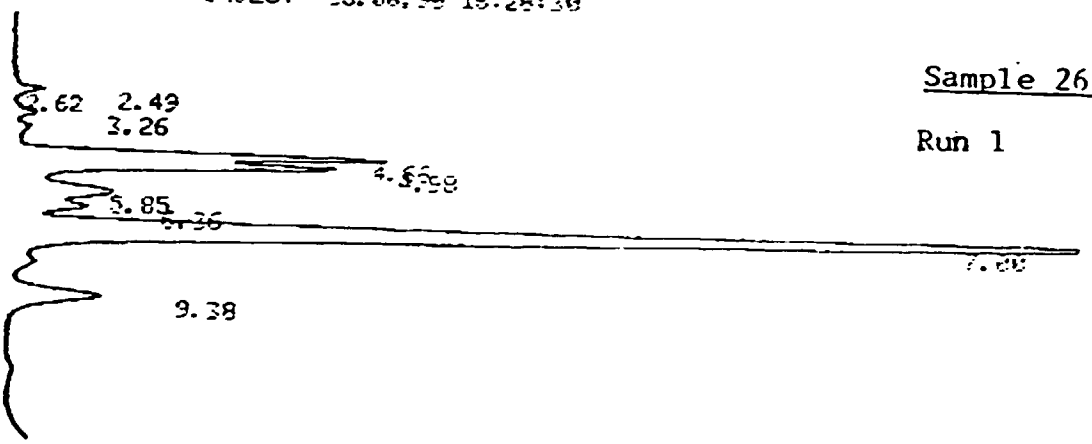
Run 1

05.06.30 16:02:03 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 23 INDEX 23

PEAK#	AREA%	RT	AREA BC
1	0.148	2.39	360 01
2	0.427	3.85	1037 01
3	0.401	3.59	974 02
4	12.802	4.41	31126 02
5	11.922	4.66	28987 02
6	8.722	5.83	21207 02
7	55.945	5.24	136024 02
8	2.208	7.46	5369 02
9	7.425	8.43	18056 03
TOTAL	100.		243140

CHANNEL A INJECT 05.06.90 16:28:30



Sample 2613

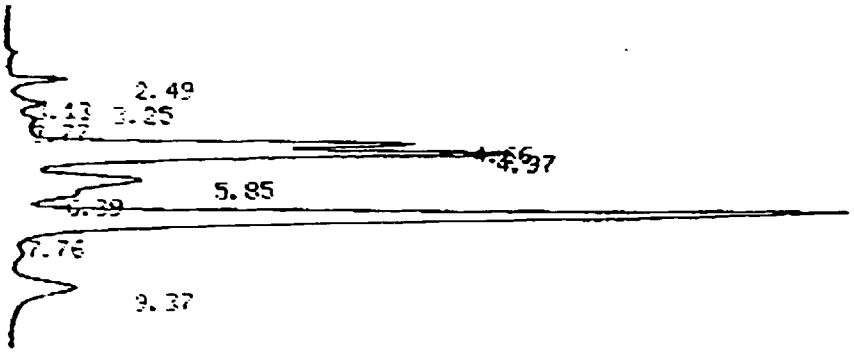
Run 1

05.06.90 16:28:30 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 15 INDEX 15

PEAK#	AREA%	RT	AREA BC
1	0.473	2.49	818 02
2	0.031	2.62	53 03
3	0.312	3.26	539 01
4	11.555	4.66	19991 02
5	12.359	4.90	21382 02
6	5.768	5.85	9980 02
7	2.992	6.36	5177 02
8	61.284	7.	106028 03
9	5.227	9.38	9044 01
TOTAL	100.		173012

CHANNEL A INJECT 05.06.90 16:14:56



Sample 2615

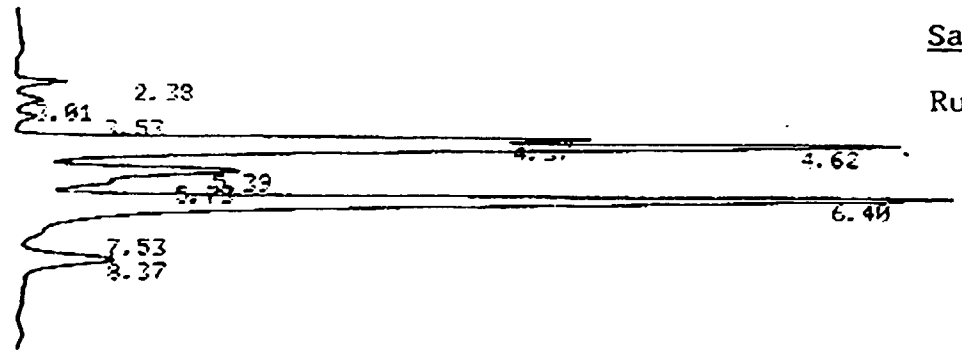
Run 1

05.06.90 16:14:56 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 14 INDEX 14

PEAK#	AREA%	RT	AREA	BC
1	1.532	2.49	2333	01
2	0.094	3.13	143	02
3	0.853	3.25	1300	02
4	0.315	3.77	480	03
5	13.27	4.66	20215	02
6	22.031	4.97	33560	02
7	7.609	5.85	11591	02
8	2.005	6.39	3054	02
9	47.23	7.07	71948	02
10	0.033	7.76	51	03
11	5.029	9.37	7659	01
TOTAL	100.		152334	

CHANNEL A INJECT 05.06.90 17:50:07



Sample 2617

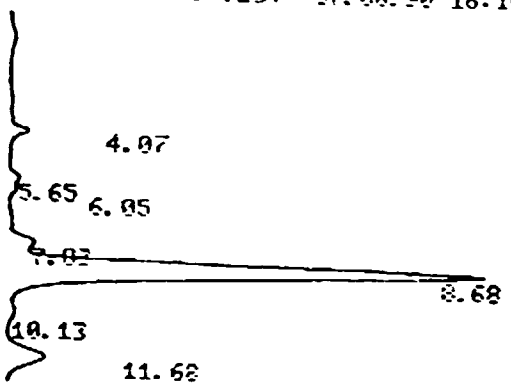
Run 1

05.06.90 17:50:07 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 22 INDEX 22

PEAK#	AREA%	RT	AREA	BC
1	0.925	2.38	1913	01
2	0.691	3.01	1429	02
3	0.493	3.53	1020	03
4	12.464	4.37	25776	02
5	27.723	4.62	57455	02
6	9.046	5.39	18706	02
7	2.679	5.73	5541	02
8	41.525	6.4	85873	02
9	0.012	7.53	24	05
10	4.281	8.37	3859	05
TOTAL	100.		206796	

CHANNEL A INJECT 07.06.90 16:10:48



Sample 2605

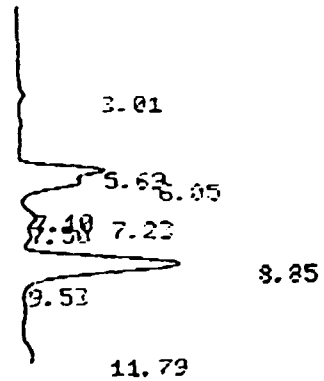
Run 2

07.06.90 16:10:48 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 20 INDEX 20

PEAK#	AREA%	RT	AREA BC
1	1.447	4.07	1925 01
2	1.195	5.65	1590 02
3	0.836	6.95	1113 02
4	5.052	7.83	6722 02
5	83.428	8.68	111008 02
6	0.511	10.13	680 05
7	7.531	11.68	10020 01
TOTAL	100.		133058

CHANNEL A INJECT 07.06.90 17:09:25



Sample 2607

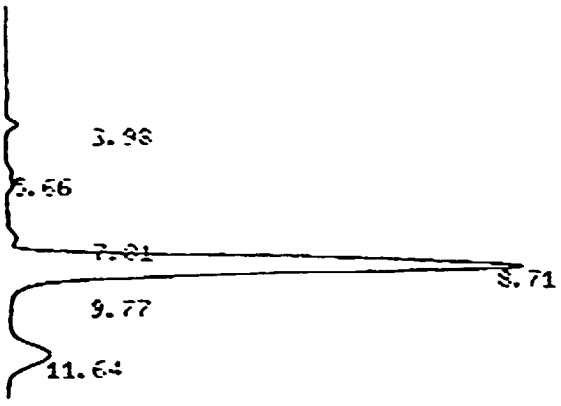
Run 2

07.06.90 17:09:25 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 24 INDEX 24

PEAK#	AREA%	RT	AREA BC
1	0.533	3.01	384 01
2	21.483	5.69	15480 02
3	13.882	6.05	10003 02
4	1.089	7.1	785 02
5	3.093	7.23	2229 02
6	3.196	7.5	2303 02
7	55.614	8.85	40074 02
8	0.366	9.53	264 03
9	0.742	11.79	535 01
TOTAL	100.		72057

CHANNEL A INJECT 07.06.90 16:55:47



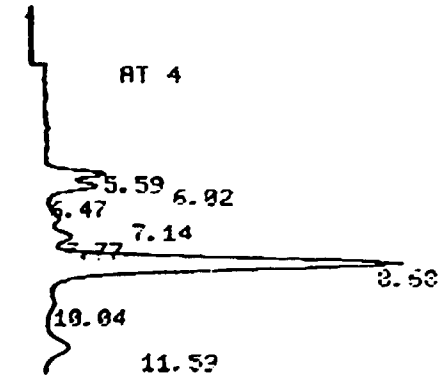
Sample 2609
Run 2

07.06.90 16:55:47 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 23 INDEX 23

PEAK#	AREA%	RT	AREA BC
1	0.968	3.98	1532 01
2	1.373	5.66	2173 01
3	0.9	7.81	1424 02
4	87.573	8.71	138583 02
5	0.091	9.77	144 03
6	9.095	11.64	14392 01
TOTAL	100		158248

CHANNEL A INJECT 07.06.90 15:46:49



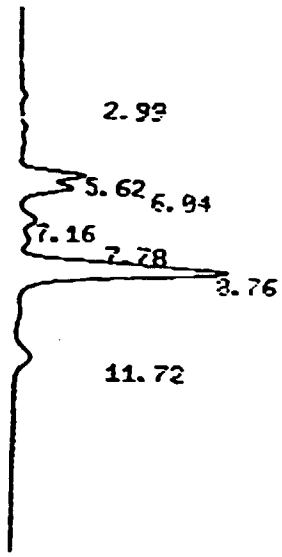
Sample 2611
Run 2

07.06.90 15:46:49 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 18 INDEX 18

PEAK#	AREA%	RT	AREA BC
1	7.188	5.59	7732 02
2	7.337	6.02	7893 02
3	0.059	6.47	63 03
4	1.872	7.14	2014 02
5	4.337	7.77	4665 02
6	71.525	8.68	76942 02
7	1.723	10.04	1854 02
8	5.96	11.59	6411 02
TOTAL	100.		107574

CHANNEL A INJECT 07.06.99 16:23:49



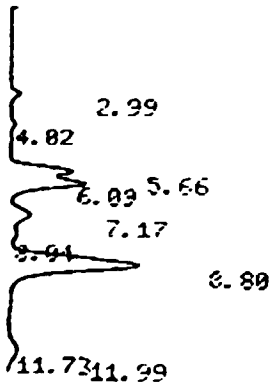
Sample 2613
Run 2

07.06.99 16:23:49 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 21 INDEX 21

PEAK#	AREA%	RT	AREA BC
1	0.669	2.99	486 01
2	12.425	5.62	9027 02
3	11.561	6.04	8399 03
4	3.715	7.16	2699 02
5	2.109	7.78	1532 02
6	64.078	8.76	46554 03
7	5.444	11.72	3955 01
TOTAL	100.		72652

CHANNEL A INJECT 07.06.90 16:42:38



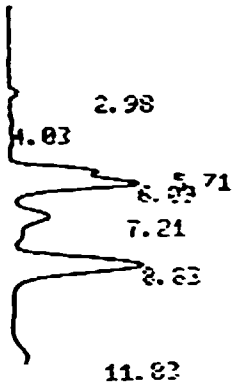
Sample 2615
Run 2

07.06.90 16:42:38 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 22 INDEX 22

PEAK#	AREA%	RT	AREA BC
1	1.553	2.99	954 01
2	0.638	4.02	392 01
3	15.007	5.66	9220 02
4	21.389	6.09	13141 02
5	8.552	7.17	5254 02
6	0.433	8.94	266 02
7	49.106	8.8	30170 03
8	2.508	11.73	1541 02
9	0.815	11.99	501 02
TOTAL	100.		61439

CHANNEL A INJECT 07.06.90 17:21:50



Sample 2617
Run 2

07.06.90 17:21:50 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 25 INDEX 25

PEAK#	AREA%	RT	AREA BC
1	1.173	2.98	870 01
2	0.255	4.03	189 01
3	15.027	5.71	11143 02
4	33.368	6.09	24744 02
5	8.075	7.21	5988 03
6	41.252	8.83	30590 01
7	0.25	11.83	630 01
TOTAL	100.		74154

TOTAL P.05

Bibliography.

Ref.		
1.	Analysis of the cinchona alkaloids by high-performance liouid chromatography.	D.V.McCalley, Journal of Chromatography, 357 (1986) 221-226
2.	Beitrag zur Bestimmung des Gesamptalkaloid-gehaltes von Cortex Chinae.	A.Haznagy, Pharmazie 31 H. 10 (1976)