

# Establishment of Liquid Chromatographic Profile for the Evaluation of Solvents for the Global Extraction of Phytochemical Metabolites from Tradimedicines

Nicaise Francois Bony<sup>1,\*</sup>, Andre Sawa Kpaibe<sup>1</sup>, Timothee Aboua Okpekon<sup>2</sup>,  
Marielle Akassi Assemiam<sup>1</sup>, Christophe N'cho Amin<sup>1</sup>, Michele Ake<sup>1</sup>

<sup>1</sup>Department of Analytical, Mineral and General Chemistry, Food Technology, Training and Research Unit Pharmaceutical and Biological Sciences, Felix Houphouet-Boigny University, Abidjan, Côte d'Ivoire

<sup>2</sup>Laboratory of Organic Chemistry and Natural Substances (LCOSN), Training and Research Unit Sciences of the Structures of Matter and Technology, Felix Houphouet-Boigny University, Abidjan, Côte d'Ivoire

## Email address:

bonynicaise@yahoo.fr (Nicaise Francois Bony), andresawa@yahoo.fr (Andre Sawa Kpaibe),  
okpekon@yahoo.fr (Timothee Aboua Okpekon), christophe.amin@gmail.com (Christophe N'cho Amin),  
assemianakassimarielle@gmail.com (Marielle Akassi Assemiam), dominique\_ake@yahoo.fr (Michele Ake)

\*Corresponding author

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**Abstract:** Assessing the quality of traditional African medicines (tradimedicines) requires the development of methods for the overall analysis of the pharmacologically active and/or chemically characteristic constituents present. The chromatographic profile of phytochemical metabolites is an important tool for the quality control of these drugs. Obtaining a rich chromatographic profile requires a process for the global extraction of phytochemicals. The objective of this work is to propose a profiling protocol by liquid chromatography for the evaluation of different solvents for the global extraction of phytochemical metabolites. The methodology was based on the establishment of an optimized reverse phase liquid chromatographic profile (good selectivity) of a pool of different extracts of a complex plant matrix (mixture of three plant drugs), applied to the determination of better solvents overall extraction of phytochemical metabolites (high number of chromatographic peaks detected). The results showed that 26 solvents (alone or in a binary, ternary and quaternary mixture) were tested; A better chromatographic profile was obtained in binary gradient (water-acetonitrile with added formic acid and ammonia) on C18 stationary phase and the best solvents for the overall extraction of phytochemical metabolites consisted of ternary mixtures of solvent (apolar/alcohol /water). This binary gradient liquid chromatography technique (water-acetonitrile with added formic acid and ammonia) on C18 stationary phase can be proposed for the global profiling of phytochemical metabolites with dichloromethane/ethanol/water or chloroform/ethanol/water mixtures as the overall extraction solvent.

**Keywords:** Liquid Chromatographic Profile, Global Extraction, Phytochemical Metabolite, Tradimedicines

## 1. Introduction

Assessing the quality of tradimedicines (traditional african medicines) is difficult because they are complex mixtures of several herbal medicines or herbal medicine preparations that can be combined with mineral and/or

animal medicines. Also the source, the harvest and the preparation of the raw materials are very variable [1, 2]. The quality of traditional medicines has a direct impact on their safety and effectiveness. Reports have shown side effects associated with adulterations and the presence of contaminants (heavy metals, pesticides and microorganisms) [3].

For the evaluation of the quality of these complex drugs, a multi-technical approach is necessary in order to authenticate the link between the components and the traditional use [4, 5].

The chromatographic profile of polar, apolar or global metabolites, a model of the pharmacologically active and/or chemically characteristic constituents present, constitutes a good tool for the qualitative and quantitative evaluation of traditional medicines [4-6].

Among these techniques, HPLC is a popular and commonly used method for the analysis of traditional herbal medicines, as it is easy to operate and is not limited by the volatility or thermal instability of sample constituents unlike the GC [4-8].

Sample preparation is an important step for chromatographic profiling of herbal medicines. Secondary metabolites belong to different chemical classes and their extraction is usually based on their polarity. Commonly used extraction solvents are water, methanol and ethanol for polar metabolites and chloroform, dichloromethane and hexane for non-polar metabolites [9, 10].

The objective of this work is to propose a profiling protocol by liquid chromatography for the evaluation of various solvents for the global extraction of phytochemical metabolites.

## 2. Materials and Methods

### 2.1. Chemical Compounds

All solvents used were analytical or HPLC grade: acetonitrile (HPLC grade, VWR), formic acid (MERK), ethyl acetate, acetone, chloroform, dichloromethane, ethanol, hexane, 25% ammonium hydroxide, and methanol supplied by CARLO ERBA.

### 2.2. Equipment

A WATERS brand HPLC chain was used. It consists of a 1525 Binary HPLC Pump, an 717 plus autosampler with automatic injector, a column oven and a 2487 Dual  $\lambda$  Absorbance detector. Data acquisition was managed by BREEZE software.

### 2.3. Plant Material

This study was performed on samples of three herbal drugs:

- 1) Samples of powdered leaves of *Combretum micranthum* G. Don. (raw material for the phytomedicine industry provided by the Laboratory of Analytical Chemistry, of the UFR of Pharmaceutical and Biological Sciences, University Félix HOUPOUËT BOIGNY);
- 2) Leaves of *Voacanga africana* (Apocynaceae) and bark of *Enantia polycarpa* (Annonaceae) which were collected in the Yakassé-mé forest, then authenticated at the National Institute of Floristry, Félix HOUPOUËT BOIGNY University and then dried at room temperature and ground evenly.

## 2.4. Preparation of Extracts

### 2.4.1. Choice of Different Solvents

The various solvents are used alone or as a mixture (binary, ternary or quaternary), depending on their polarity (from the most apolar to the most polar). For our study, we selected 26 solvents (Table 1).

Table 1. Different solvents used for the preparation of extracts.

N°	Solvents (proportions)
1	Hexane (100)
2	Dichloromethane (100)
3	Chloroform (100)
4	Ethyl acetate (100)
5	Ethanol (100)
6	Acetone (100)
7	Methanol (100)
8	Water (100)
9	Ethanol/water (50/50)
10	Acetone/water (50/50)
11	Hexane/Ethanol/Water (12/76/12)
12	Hexane/Acetone/water (12/76/12)
13	Hexane/Methanol/Water (7/86/7)
14	Dichloromethane/Ethanol/water (20/60/20)
15	Dichloromethane/Acetone/Water (20/60/20)
16	Dichloromethane/Methanol/Water (20/60/20)
17	Chloroform/Ethanol/Water (20/60/20)
18	Chloroform/Acetone/Water (20/60/20)
19	Chloroform/Methanol/Water (20/60/20)
20	Hexane/Ethyl Acetate/Ethanol/Water (14/14/58/14)
21	Hexane/Ethyl Acetate/Acetone/Water (17/17/49/17)
22	Hexane/Ethyl Acetate/Methanol/Water (9/9/73/9)
23	Chloroform/Ethyl Acetate/Ethanol/Water (17/17/49/17)
24	Chloroform/Ethyl Acetate/Methanol/Water (18/18/46/18)
25	Chloroform/Acetone/Ethanol/Water (20/20/40/20)
26	Chloroform/Acetone/Methanol/Water (20/20/40/20)

### 2.4.2. Preparation of Extracts

The extracts are obtained by refluxing a mixture of 1.7 g of each plant drug powder (5.1 g) with 25 ml of solvent for 15 min, then filtering through Whatman 42 filter paper into a volumetric flask of 25 ml, then make up to 25 ml with the same solvent.

### 2.4.3. Optimization of Chromatographic Separation

The various tests were carried out on a pool of extracts obtained by mixing volume by volume of the 26 extracts. The chromatographic analysis was carried out with 20  $\mu$ l of extract on a BDS Hypersil C18 column (4.6 $\times$ 250 mm, 5  $\mu$ m) with two solvents (water and acetonitrile), at a flow rate of 1 ml/min, in a linear gradient from 5 to 100% acetonitrile over 20 minutes, followed by holding at 100% acetonitrile for 10 minutes and double detection at 210 and 254 nm. The optimization was carried out based on the choice of the operating conditions with or without additive (formic acid and/or ammonia) allowing to obtain a good sensitivity, selectivity (distinction of more peaks) and a better distribution of the chromatographic peaks.

## 3. Results and Discussion

Our study focused on the development of a protocol for

establishing a global liquid chromatographic profile of phytochemicals (polar and apolar). And the application of the protocol developed to the choice of the best solvents for the global extraction of metabolites from a complex plant matrix. The choice of our plant matrix was based on the one hand on the complexity, and on the other hand on the presence of the three major groups of phytochemicals (alkaloids, phenoloids and terpenoids). The matrix consisted of a mixture of three plant drugs (leaves of *Combretum micranthum*, leaves of *Voacanga africana* and bark of *Enantia polycarpa*) in which the presence of the major groups of phytochemicals was identified. [11-17].

### 3.1. Choice of Different Extraction Solvents

The choice of solvents was based on those commonly used for the extraction of phytochemicals [18]: apolar (hexane, dichloromethane, chloroform), intermediate polarity (ethyl acetate and acetone) and polar (water, methanol, ethanol).

We based ourselves on the work of Gullberg et al [14] who obtained better extraction with the ternary mixture chloroform/methanol/water (20/60/20). We have proposed mainly ternary solvent mixtures with an apolar solvent, the most polar solvent (water) and the third solvent of intermediate polarity or the alcohols. Taking miscibility into account, quaternary mixtures have been proposed, especially with ethyl acetate. There are also solvents for the extraction of large groups of phytochemicals [19].

### 3.2. Optimization of Chromatographic Separation

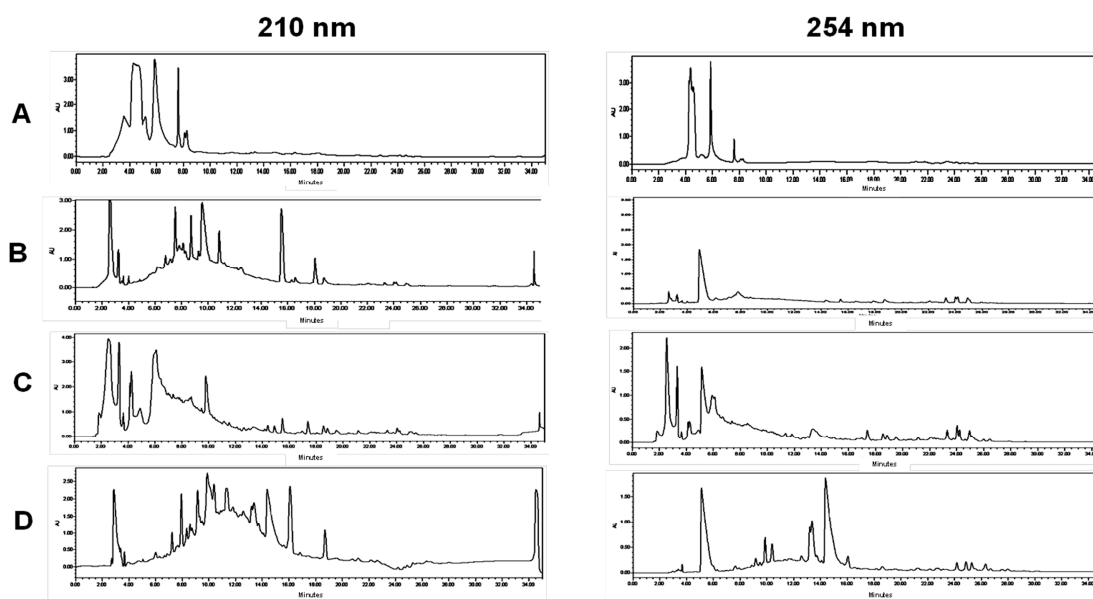
The choice of our chromatographic conditions was based on the work of Cremin and Zeng [20] and Mohn et al. [21], dealing with the global chromatographic analysis of the phytochemical compounds of herbal drugs. The

chromatographic analyzes were carried out in reverse phase with a C18 stationary phase and two mobile phase solvents (water and acetonitrile) with addition of formic acid in gradient mode with at least one detection in the ultraviolet range.

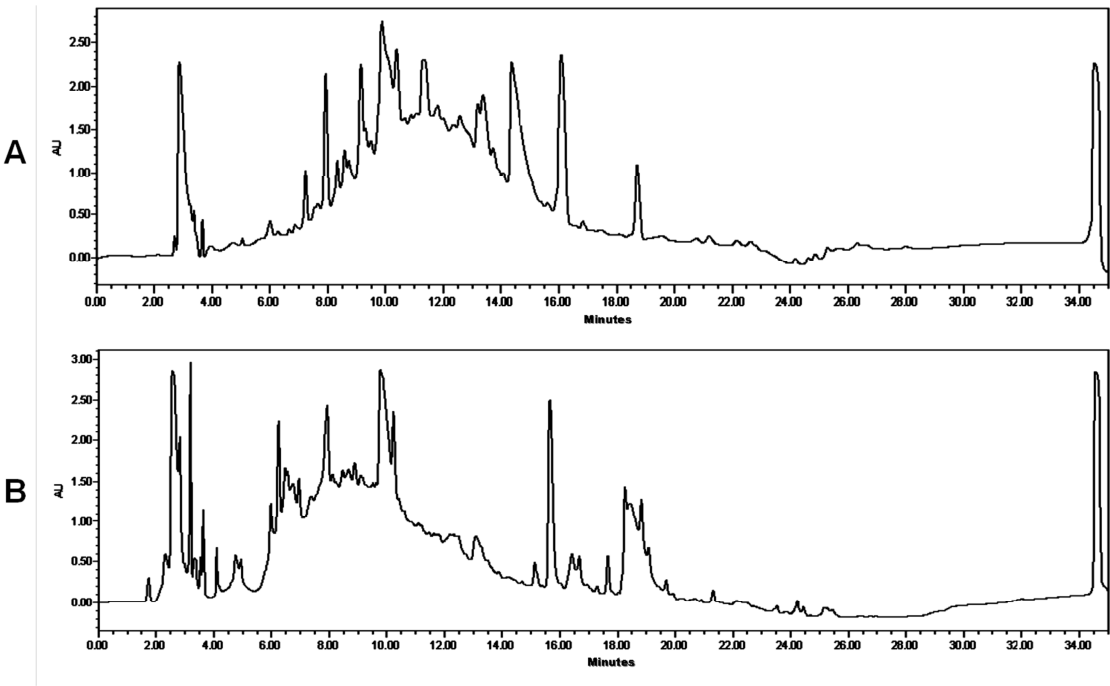
The choice of the different solvents and additives of the mobile phase depended on the physico-chemical characteristics (eluting strength, miscibility and solubility) and on the impact for an analysis by mass spectrometry. The addition or not of a pH modifier (formic acid and/or ammonium hydroxide) was based on one of the HPLC applications of Phenomenex inc. [22] and on the other hand on the work of Deschamps et al. [23], who proposed the addition of a mixture of acid and base in the mobile phases for the chromatographic profiling of different classes of lipids (polar and apolar). The various tests were carried out on a pool of extract obtained by mixing all the extracts in equal volumes in order to be able to ensure the presence of all the compounds extracted with the different solvents.

And the choice of the operating conditions was based on that allowing to obtain a good sensitivity, selectivity (distinction of more peaks) and a better distribution of the chromatographic peaks on the chromatogram with a good analysis time.

The first tests were carried out with 20  $\mu$ l of extract on a BDS Hypersil C18 column (4.6 $\times$ 250 mm, 5  $\mu$ m) using water and acetonitrile with or without the addition of 0.1% formic acid and/or ammonium hydroxide, at a flow rate of 1 ml/min, in a linear gradient from 5 to 100% acetonitrile in 20 minutes, followed by maintenance at 100% acetonitrile for 10 minutes and double detection at 210 and 254 nm, two wavelengths commonly used for the detection of most chemical compounds.



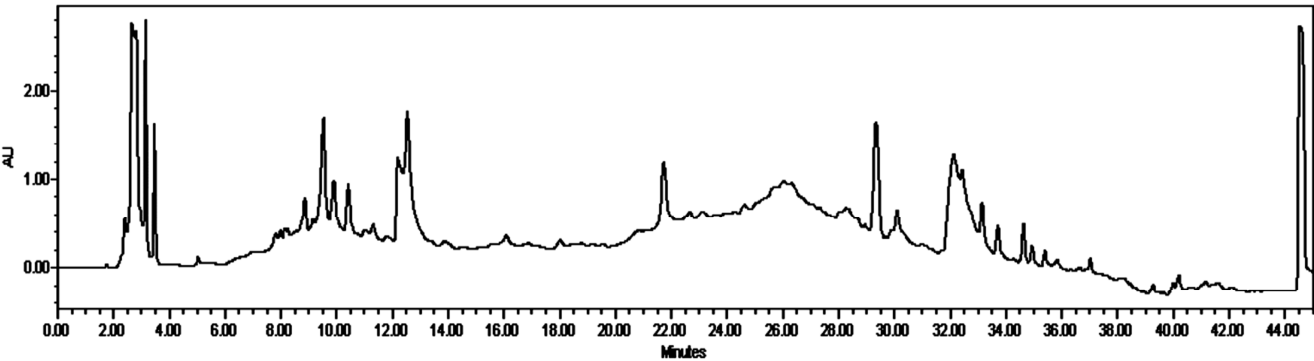
**Figure 1.** Chromatograms of the extract pool on C18 column binary water-acetonitrile gradient at a flow rate of 1 ml/min, linear gradient from 5 to 100% acetonitrile in 20 minutes, followed by holding at 100% acetonitrile for 10 minutes and detection at 210 and 254 nm: -(A) without additive – (B) 0.1% formic acid – (C) 0.1%  $\text{NH}_3$  – (D) 0.1% formic acid + 0.1%  $\text{NH}_3$  + 3% water.



**Figure 2.** Chromatograms of the extract pool on C18 column binary water-acetonitrile gradient at a flow rate of 1 ml/min, linear gradient from 5 to 100% acetonitrile in 20 minutes, followed by holding at 100% acetonitrile for 10 minutes and detection at 210: (A) 0.1% formic acid + 0.1% NH<sub>3</sub> + 3% water – (B) 0.05% formic acid + 0.1% NH<sub>3</sub> + 3% water.

**Table 2.** Binary gradient at a flow rate of 1 ml/min on C18 stationary phase and detection at 210 nm.

Time (minutes)	Water: (0.05% formic acid - 0.1% ammonium hydroxide) (%)	Acetonitrile: (0.05% formic acid - 0.1% ammonium hydroxide - 3% water) (%)
0	97	3
5	85	15
10	83	17
15	80	20
20	70	30
25	50	50
30	30	70
35	0	100
40	0	100
41	97	3
45	97	3



**Figure 3.** Chromatograms of the extract pool with the binary gradient Table 2.

The chromatograms obtained (figure 1) showed that the great majority of the compounds were eluted at the start of the analysis, indicating a low retention of the compounds. And detection at 210 nm provides more chromatographic peaks compared to 254 nm. It is noted that the addition of

formic acid and/or ammonium hydroxide with 3% water for solubilization in acetonitrile improves the chromatographic separation and the sensitivity of various compounds. Better chromatographic separation is obtained with the simultaneous addition of two additives, as

indicated by the work of Deschamps et al. [23]. For the rest of our tests, we retained 210 nm as the detection wavelength and the optimization of the separation by modifying the concentration of the pH modifiers and the elution gradient.

Test results on varying the concentration of pH modifiers showed a greater impact on elution strength and separation with formic acid compared to ammonium hydroxide, as indicated by the results of the application of Phenomenex inc. [22]. We have retained optimal concentrations in an equimolar mixture of the two additives (0.05% formic acid and 0.1% ammonium hydroxide) making it possible to obtain a chromatographic profile (figure 2) with always the elution of the majority of the compounds at the start of the analysis (high eluting force at the start of the gradient).

Tests were then carried out by decreasing the speed of the change of the gradient at the beginning and/or by increasing the duration of the gradient. Good selectivity and distribution of the chromatographic peaks on the chromatogram (figure 3) was obtained with a water-acetonitrile binary gradient for 40 minutes (Table 2) at a flow rate of 1 ml/min on C18 stationary phase and detection at 210 nm.

### 3.3. Application to the Choice of Solvents for the Global Extraction of Phytochemical Compounds

The chromatographic conditions retained were applied to the 26 extracts, to determine the best solvents for the overall extraction of the phytochemical metabolites. The choice was based on the greatest number of chromatographic peaks detected and their good distribution over the entire chromatogram.

The results of the determination of the number of

chromatographic peaks (Table 3) of the various chromatograms showed that the mixture of solvents and particularly the ternary mixtures were relatively the best solvents for the overall extraction of phytochemical metabolites in agreement with the work of Gullberg et al. [14]. There is a greater advantage with ternary mixtures combining water, an alcohol (ethanol or methanol) and an apolar solvent (chloroform or dichloromethane).

And taking into account the physicochemical characteristics, toxicity as indicated by Jones et al [9] propose ternary mixtures (dichloromethane/ethanol/water or chloroform/ethanol/water) as extraction solvent, for the establishment of an overall chromatographic profile of the phytochemicals of traditional medicines.

The method that we propose in this study using liquid chromatography in binary gradient (water-acetonitrile with added formic acid and ammonia) on stationary phase C18 with detection in the ultraviolet makes it possible to establish an overall chromatographic profile (polar and apolar) bioactive or non-bioactive secondary metabolites of herbal drugs.

This liquid chromatographic technique has been applied to the selection of better solvents for the overall extraction of phytochemicals. The best solvents consisted of ternary mixtures combining water, an alcohol (ethanol or methanol) and an apolar solvent (chloroform or dichloromethane).

This binary gradient liquid chromatography technique (water-acetonitrile with added formic acid and ammonia in equimolar quantity) on C18 stationary phase can be proposed for the global profiling of phytochemical metabolites with the dichloromethane/ethanol/water or chloroform/ ethanol/water as extraction solvent.

**Table 3.** Number of chromatographic peaks detected according to the different solvents used for the preparation of the extracts.

Solvents (proportions)	Number of chromatographic peaks detected
Dichloromethane/Ethanol/water (20/60/20)	82
Chloroform/Methanol/Water (20/60/20)	80
Chloroform/Ethanol/Water (20/60/20)	77
Hexane/Acetone/Water (12/76/12)	76
Dichloromethane/Methanol/Water (20/60/20)	66
Dichloromethane/Acetone/Water (20/60/20)	64
Chloroform/Acetone/Methanol/Water (20/20/40/20)	64
Hexane/Ethyl Acetate/Methanol/Water (9/9/73/9)	63
Chloroform/Ethyl Acetate/Ethanol/Water (17/17/49/17)	63
Hexane/Ethyl Acetate/Ethanol/Water (14/14/58/14)	62
Chloroform/Ethyl Acetate/Methanol/Water (18/18/46/18)	59
Hexane/Ethanol/Water (12/76/12)	57
Chloroform/Acetone/Ethanol/Water (20/20/40/20)	57
Hexane/Methanol/Water (7/86/7)	56
Chloroform (100)	53
Hexane/Ethyl Acetate/Acetone/Water (17/17/49/17)	50
Ethanol/water (50/50)	45
Acetone/water (50/50)	44
Ethanol (100)	40
Ethyl acetate (100)	22
Acetone (100)	22
Chloroform/Acetone/Water (20/60/20)	21
Dichloromethane (100)	20
Methanol (100)	11
Hexane (100)	9
Water (100)	5

## 4. Conclusion

The method that we propose in this study using liquid chromatography in binary gradient (water-acetonitrile with added formic acid and ammonia) on stationary phase C18 with detection in the ultraviolet makes it possible to establish an overall chromatographic profile (polar and apolar) bioactive or non-bioactive secondary metabolites of herbal drugs.

This liquid chromatography technique has been applied to the selection of better solvents for the overall extraction of phytochemicals. The best solvents consist of ternary mixtures combining water, an alcohol (ethanol or methanol) and an apolar solvent (chloroform or dichloromethane).

This binary gradient liquid chromatography technique (water-acetonitrile with added formic acid and ammonia in equimolar quantity) on C18 stationary phase can be proposed for the global profiling of phytochemical metabolites with dichloromethane/ethanol/water or chloroform/ethanol /water as extraction solvent.

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