Research Paper

EXPERIMENTAL DESIGN OPTIMIZATION FOR SCREENING RELEVANT FREE PHENOLIC ACIDS FROM VARIOUS PREPARATIONS USED IN BURKINA FASO FOLK MEDICINE

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Abstract

Experimental design methodology was used to optimize the HPLC separation of various relevant phenolic acids from an artificial mixture. The effect of four characteristic factors of the HPLC procedure on the Chromatographic Response Function was investigated by a Central Composite Face-Centred Design and Multi Linear Regression fitting. The eight phenolic acids were successfully separated. The optimal HPLC setting condition was applied to rapid screening of free phenolic acids from *Striga hermonthica* (Del.) Benth. (Scrophulariaceae), *Guiera senegalensis* J. F. Gmel (Combretaceae) and unifloral honey from *Acacia* sp. (Mimosaceae). None of reference phenolic acid was identified in *S. hermonthica* extract when some of them were detected in *G. senegalensis* and honey extracts. This work is considered as a first step for a largest phenolic acids screening from various preparations used in Burkina Faso folk medicine to justify some of their properties or potentialities.

Key words: Central Composite Face-Centred Design, Chromatographic Response Function, Phenolic acids

Introduction

Phenolic acids are plant secondary metabolites widely spread throughout the plant kingdom (Bruneton, 1993; Herrmann, 1989). Recent interest in phenolic acids stems from

their potential as protective factors against cancer and heart diseases in part because of their potent antioxidative properties (Breinholt, 1999). They are also known to exhibit antibacterial effects (Weston et al., 1999) antimutagenic and anti-inflammatory activities in bacteria and mammalian (Loarca-Pina et al., 1998; Ihantola-Vormisto et al., 1997; Kaur et al. 1997). Besides, some phenolic acids were reported to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase (Middleton et al., 2000). Therapeutic uses of medicinal plants in Burkina Faso folk medicine could partly be assigned to their free phenolic acids (Nacoulma, 1996). A screening program on free phenolic acids in medicinal plants and foods from Burkina Faso is then undergoing in our laboratory to justify some their biological properties and potentialities.

The potential healthful of phenolic acids have prompted the interest in developing analytical methodologies for their detection and measurement from plant sources. A number of analytical techniques have been presented for the analysis of phenolic acids, including thin-layer chromatography (Schmidtlein and Herrmann, 1975), gas-liquid chromatography (Schulz and Herrmann, 1980), gas chromatography-mass spectrometry (Wu et al., 1999), or capillary electrophoretic methods (Fernandez et al, 1996). However, high-performance liquid chromatography is presently the most widely used method (Amakura et al., 2000; Escarpa and Gonzales, 2000; Hahn et al., 1983). Despite the numerous HPLC methods for phenolic acids determination described in the literature, an optimized method, convenient for all phenolic acids from complex mixture like plant extracts is not available to our knowledge. Hence, this study aimed to develop an optimized HPLC method for the separation of caffeic, ferulic, vanillic, ellagic, gallic, benzoic, cinnamic and hydrocinnamic acids. Their choice was focused either on their relatively close structure and their well-known antimutagenic, antibacterial, antioxidant and anti-inflammatory activities (Rukkumani et al., 2004; Friedman et al., 2003; Op de Beck et al., 2003; Privadiarsin et al., 2002; Fernandez et al., 1998; Kaur et al., 1997; Fernandez et al., 1996; Garcia and Marhuenda, 1985; Wood et al., 1982).

The selection of optimal HPLC operating conditions is rather difficult and complex for multi-component mixtures due to the high degree of process variable interaction. Several process variables must be investigated. HPLC methods development was traditionally accomplished through a trial-and-error process. This procedure is overly expensive due to both the time involved and the potential product wasted in the many experiments that must be performed to find acceptable, though not necessarily optimal, operating conditions. Experimental design methodologies, more practical and efficient, have been reported as a suitable tool to optimize HPLC separations (Karnka et al., 2002; Onjia et al., 2002; Klein and Rivera, 2000). It involves a statistical design of experiments, which uniformly samples the experimental domain to generate an experimental test matrix. Response or criteria functions are employed to quantify results numerically and rank chromatograms in order of their desirability. These criteria functions are mapped to response surface models, which are then optimized within the constraints of the experimental domain with respect to the process variables.
In this paper, we propose an experimental design with HPLC for a suitable separation of caffeic, ferulic, vanillic, ellagic, gallic, benzoic, cinnamic and hydrocinnamic acids

mixture. The optimization process was carried out on an artificial mixture and the optimal HPLC setting applied to biological samples.

Material and Methods

Whole plant *Striga hermonthica* (DEL.) Benth (Scrophulariaceae) and galls from *Guiera senegalensis* J. F. Gmel (Combretaceae) were collected at Gampela (25 km, east of Ouagadougou, Burkina Faso). After being identified by Prof. Millogo, voucher specimens were deposited at the herbarium of the University of Ouagadougou as Lamien 01 (galls from *Guiera senegalensis*) and Kiendrebeogo 01 (*Striga hermonthica*). Honey sample (n°3) was collected from the east of Burkina Faso. Pollen morphologies were compared with reference slide from the Laboratory of Biology and Ecology, University of Ouagadougou. Sample was identified as *Acacia* sp. unifloral honey (Meda et al., 2005).

Test samples preparation

Plant extracts were obtained by refluxing 10 g of powdered material for 30 min with 100 ml of aqueous acetone 80 %. Acetone was removed under reduced pressure and the aqueous residue freeze-dried. A 10 mg/ml solution was made from a (1:1) methanol/water solution for HPLC analysis. Honey sample (25 g) was mixed with 125 ml acidic water pH 2 and filtered through an amberlite XAD2 column (25 x 2 cm). The column was eluted with 100 ml of acidic water pH 2, 300 ml of water and then 300 ml of methanol. Methanolic fraction was concentrated under reduced pressure and partitioned between water and diethyl ether. The organic phase was dissolved in 1 ml methanol for HPLC analysis. HPLC Chromasolv® methanol and G Chromasolv® water (Flucka chemie GmbH, Switzerland) were used to prepare samples solutions for HPLC analysis.

Artificial Phenolic acids mix

Phenolic acids (caffeic, ferulic, cinnamic, hydroxycinnamic, gallic, vanillic, benzoic, ellagic acid) were purchased from Sigma. Their structures and CAS numbers are given in Fig. 1. Standard mixture solution made in methanol (100 µg/ml of each of all phenolic acids) was used for the optimization procedure.

HPLC system and chromatographic separation efficiency estimation

The liquid chromatographic system consisted of a Varian 9012 pump module, a Varian 9100 automatic sampler and a Varian 9065 UV detector. Star Chromatography Workstation software version 5.51 was used to acquire and analyse chromatographic data. All the experiments were performed on Kromasil C_{18} column (250 mm × 4.6 mm internal diameter, 10 µm particle size, 100 Å pore size). Ultraviolet detection was achieved at 210 nm and 273 nm. Samples (20 µl) were injected in triplicate. Gradient elution regimes were employed using HPLC Chromasolv® acetonitrile and 0.1 % (v/v) orthophosphoric acid in

G Chromasolv® water (Fluka chemie GmbH, Switzerland). Orthophosphoric acid (85 %) was HPLC grade (Fischer scientific). The quality of chromatographic separation has been evaluated by the Chromatographic Response Function (CRF) (Karnka et al., 2002; Klein and Rivera, 2000). The CRF (Eq. 1) is a global quality criterion that allows specifying time and resolution parameters.

Eq. 1. $\quad CRF = \sum R_i + L^a - b \, | \, T_M - T_L \, | - C(T_0 - T_1)$

Where R_i is the resolution between adjacent pairs of peaks, L the total number of peaks, T_M the maximal acceptable duration of analysis, T_L the retention time of the last eluted peak, T_0 the specified minimum retention time and T_1 the retention time of the first eluted peak. a, b and c are the arbitrary weighting factors. To maximize L parameter, the weighting factors were fixed as following: a = 2, b = 1 and c = 1. All R_i values exceeding 2.0 were systematically discarded from CRF calculation to avoid overestimated CRF.

Experimental design

Experimental design was processed and analyzed using Modde 5.0 software. Central Composite Face-Centred Design (CCFD) was used because it requires only three levels for each factor and lead to a quadratic model (Eq. 2) which is efficient to describe a potentially complex function:

Eq. 2. $\quad \eta = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{\substack{i,j=1 \\ i \neq j}}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2$

Where η is computed response of CRF, β_0 the constant, β_i the linear coefficients, β_{ij} the cross product coefficients, β_{ii} the quadratic coefficients, k the number of independent factors. Normality of the experimental data was examined using the Box-Cox transformation procedure (Box and Cox, 1964). Model coefficients were estimated with the well known Multi Linear Regression (MLR), which allows fitting of the observed response with the analytical model (Box et al., 1978). Initial model including all the coefficients was refined using stepwise technique (Yin and Carter, 1996). This procedure involves removing step by step each eligible coefficient in regard to subsequent statistic quality of the model. The model efficiency was estimated by the coefficient of correlation R^2, the coefficient of determination R^2_{Adj}, the coefficient of prediction Q^2 (Eq. 3) and the analysis of variance (ANOVA) based on the Fischer F-test.

Eq. 3 $\quad Q^2 = \dfrac{\left[\sum_{1}^{n}\left(y_i - \overline{Y}\right)^2 - \sum_{1}^{n}\left(\eta_i' - y_i\right)^2 \right]}{\sum_{1}^{n}\left(y_i - \overline{Y}\right)^2}$

In this equation η_i' represent a prediction of the ith observed response made from a model regressed on all of the available data except the ith observation (Eriksson et al., 2000). P-values criteria estimated by Student t-test was employed to control the coefficient to be removed. In this iterative approach, coefficient with the minus P-value was removed from the sub-model of the step. Stepping was stopped when statistic criteria corresponding to the best sub-model were obtained. Optimisation of the experimental conditions was extrapolated using Nelder Mead simplex method module, as implemented in Modde 5.0 software (Nelder and Mead, 1965). Then, predicted response efficiency (Eq. 4) was evaluated by "distance to target" coefficient (D) applied in only one response case (Eriksson et al., 2000):

Eq. 3. $\quad D = \log\left(\dfrac{\eta - T}{T - L}\right)^2$

Where η is the computed response of CRF, T the desired response target and L the worst acceptable response value.

Results and Discussion

The CRF optimization study was carried out in two steps. First, the eight commercial phenolic compounds of interest were mixed together to obtain a complex test medium as a mimic of plant extract. The above artificial mix of the commercial phenolic acids was used to optimize the HPLC identification by surface method procedure. In a second step, the optimal HPLC procedure was applied to the screening of natural extract from *Striga hermonthica*, galls of *Guiera senegalensis* and honey sample.

The HPLC procedure was carried out in 3 steps:
(i)   An initial isocratic elution during 15 min for column equilibration
(ii)   A gradient elution during X_3 min (to be determined through the optimization procedure) for phenolic acids separation
(iii)   A second gradient elution for 10 min followed by a 5 min isocratic elution to extract potentially by-products residuals from the HPLC column.

Elution solvent was composed of acetonitrile and orthophosphoric acid 0.1 %. Optimization of the chromatographic separation was investigated on four factors implicated in the 2nd step (step ii): initial acetonitrile percentage (X_1), acetonitrile percentage at the end of the first gradient elution (X_2), duration of the first gradient elution (X_3) and pump flow rate during the first isocratic phase and the first gradient elution (X_4). Acetonitrile percentage as well as solvent flow rate at the end of the second gradient and during the last isocratic elution was settled at 75 % and 1 ml/min. The different factors levels values producing the experimental domain were chosen after brainstorming and some preliminary studies. Then X_1 interval values were chosen between 1 and 5 % acetonitrile and X_2 values between 40 and 70 % acetonitrile. X_3 interval was limited by 40

and 50 min and X_4 by 1 and 2 ml/min. Artefacts caused by the different magnitudes and units of the factors were avoided using variables explained in a centred coded levels. For each factor, (+1) constitute the high level value, (-1) the low level value and (0) the centred value.

Due to the potential complex interactions suspected between the factors, the experimental design was chosen to support a quadratic model. CCFCD was then investigated in 23 experimental runs including 3 central points. Run sequence of experimental units was completely randomized to minimize the effect of noise and the chromatographic separation quality was evaluated using CRF (Table 1). No deviating experiment (outlier) was detected using Normal probability plot.

Chromatographic method optimization

The Box-Cox transformation of experimental raw data gives $\lambda_{lower} = 0.5539$, $\lambda_{max} = 1.0297$, $\lambda_{upper} = 1.5792$ at 95 % confidence level. No data transformation was then necessary to improve the model (Draper and Smith, 1981; Box and Cox, 1964), assessing the normal distribution of experimental data. The model coefficients were obtained by fitting the worksheet data by MLR. Fig. 2 shows all the regression coefficients and their uncertainty given by 90 % confidence interval. The best sub-model (Eq. 5) adopted by the stepwise technique for the CRF response was obtained as described in material and method section:

Eq. 4.
$$\eta = 49.313 - 11.1548X_1 - 8.46117X_2 + 3.56258X_3 + 2.06971X_4 + 3.03885X_1X_4$$
$$+ 3.3337X_2X_3 - 8.34727X_2X_4 - 13.3077X_1^2 + 6.59799X_2^2 - 8.05465X_3^2 + 7.0351X_4^2$$

The coefficient of correlation $R^2 = 0.959$ and the coefficient of determination $R^2_{Adjusted} = 0.918$ showed that quadratic model is well fitted by MLR. The CRF response is very well predicted as indicated by the coefficient of prediction $Q^2 = 0.773$. Generally speaking, $Q^2 > 0.5$ is considered to be good, $Q^2 > 0.7$ very good, and $Q^2 > 0.9$ excellent (Eriksson et al., 2000). Besides, R^2 and Q^2 difference was favourably inferior to 0.2. The ANOVA analyse shows that probability for the model regression is significant at 95 % (P-value = 0.000 << 0.05) but the probability for lack of fit is significant at 95 % (P-value = 0.001 < 0.05). However, the model validity was not dramatically depreciated by this slight lack of fit because the predictive power evaluated by the coefficient of prediction Q^2 was greater than 0.5 (Eriksson et al., 2000). In the statistically point of view, the quadratic model appears good within the range of variables employed.

The positive sign of the linear coefficients (β_i) refers to an increasing effect on the CRF response, whereas the negative sign indicate a decreasing effect on this response. Direct determination of optimal factor setting was very difficult in regard to the number and the antagonist influence of interaction and quadratic terms also implicated in the model.

Table 1: Central Composite Face-Centred Design matrix and corresponding CRF response

Experimental unit	Run Order	Experimental factors				CRF
		X_1	X_2	X_3	X_4	
1	1	-1	-1	-1	-1	53.7367
2	14	+1	-1	-1	-1	27.2283
3	22	-1	+1	-1	-1	51.3441
4	8	+1	-1	+1	-1	21.6544
5	18	-1	+1	+1	-1	54.0935
6	9	+1	+1	+1	-1	36.1383
7	15	-1	-1	-1	+1	66.9711
8	2	-1	+1	-1	+1	27.8605
9	6	+1	+1	-1	+1	12.3068
10	16	-1	-1	+1	+1	68.5150
11	20	+1	-1	+1	+1	52.1348
12	3	+1	+1	+1	+1	27.3733
13	10	-1	0	0	0	51.2591
14	17	+1	0	0	0	21.638
15	19	0	-1	0	0	68.9428
16	21	0	+1	0	0	43.7657
17	7	0	0	-1	0	32.6095
18	4	0	0	+1	0	50.7937
19	5	0	0	0	-1	54.4539
20	11	0	0	0	+1	59.1288
21	12	0	0	0	0	48.6639
22	13	0	0	0	0	48.5918
23	23	0	0	0	0	48.8198

(+1) high level value, (-1) low level value and (0) the centred value of each factor
(X_1) initial and (X_2) final acetonitrile, (X_3) duration of the first gradient, (X_4) solvent flow rate of the first isocratic and first gradient elutions.

Table 2: Phenolic acids suspected on the base of retention time

Phenolic acid (λ nm)	RT (min) ± SD	S. h	G. s	Honey
Benzoic acid; **273** nm	4.59 ± 0.06	-	-	-
Gallic acid; **210** and 273 nm	5.96 ± 0.08	-	X	-
Caffeic acid; **210** and 273 nm	15.19 ± 0.12	-	-	X
Vanillic acid; **210** and 273 nm	15.89 ± 0.15	-	X	X
Ferulic acid; **210** and 273 nm	21.67 ± 0.13	-	-	X
Ellagic acid; 210 and **273** nm	22.83 ± 0.16	-	-	X
Hydrocinnamic acid; **210** nm	32.12 ± 0.07	-	-	X
Cinnamic acid; 210 and **273** nm	33.46 ± 0.07	-	X	X

Mean value ± Standard deviation of 3 injections
Wavelengths in bold character are preferably used
RT: retention time
X: Detected compounds
-: Non detected compounds
S.h: *Striga hermonthica*
G. s: *Guiera senegalensis*

For example, final acetonitrile proportion have a decreasing effect (β_2 = -8.4611) on CRF response but an increasing effect is observed in interactions with phase duration (β_{23} = +3.3337), in interactions with pump flow rate (β_{24} = +8.34727) and within quadratic effect (β_{22} = +6.59799). Analysis was aided by surface response plot drawn from the model when varying two factors while the other factors were kept constant at the medium level (x_i = 0). For sake of simplicity, only the response 3D surface plot and corresponding 2D contour plot for X_2 and the other significant factors set one by one were reported in Fig. 3. As illustrated, the CRF was maximized for the following setting parameters: the final concentration of acetonitrile (X2) was minimized, the initial concentration of acetonitrile (X_1) to about 2 %, the gradient duration (X_3) to about 45 min and the pump flow rate (X_4) to about 2 ml/min.

Further, to determine more precisely the optimal factors range, a Nelder mead simplex method was investigated. This method has an advantage over the response surface plot and 2D contour plot because the response is optimized using all the factors in the same time. Then, an interesting value for CRF was arbitrary focused to 76. The experimental conditions predicted by the model were: Initial acetonitrile (X_1) = 2 %; Final acetonitrile at the end of gradient I (X_2) = 43 %; Gradient I duration (X_3) = 45 min and Flow rate (X_4) = 2 ml/min.
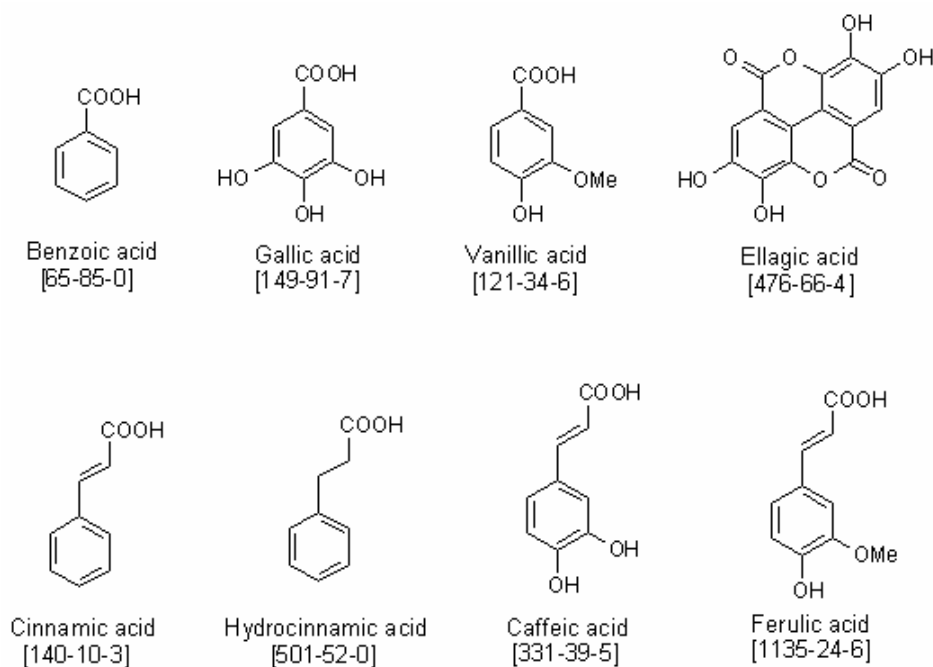
Figure 1: Structure and CAS number of Phenolic acids of study
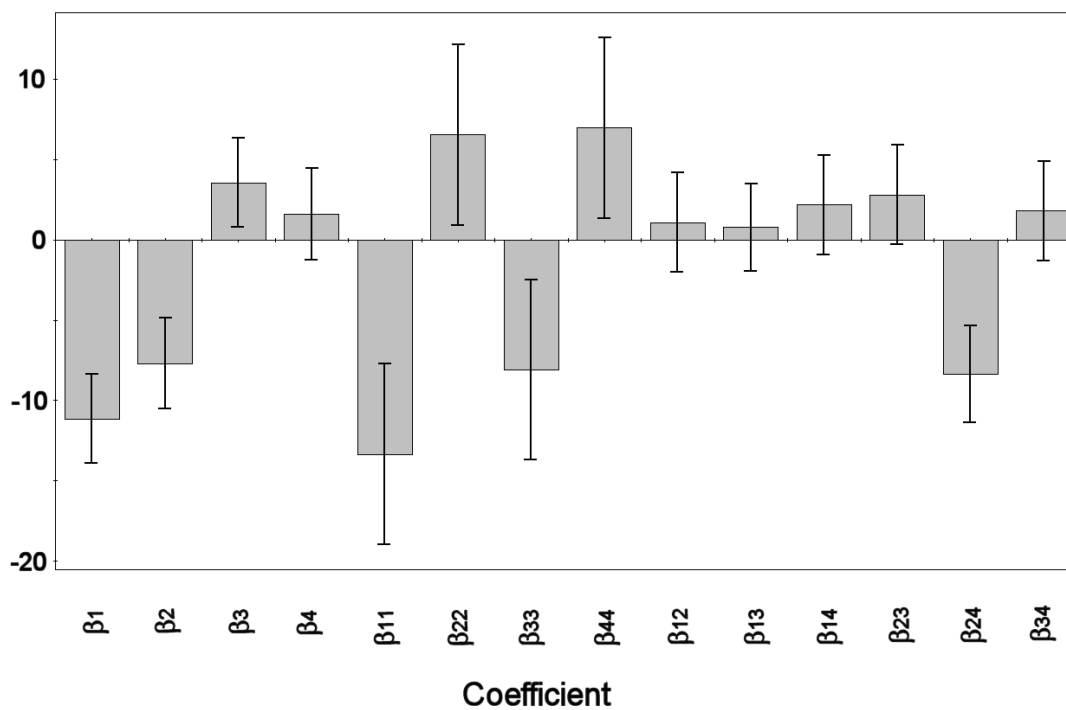
**Figure 2:** Regression coefficient plot obtained by MLR process

Thus, factors settings were very close to the previous tendencies obtained by plot analysis. Experimental investigation was carried out with this experimental setting to check the adequacy of the model. Calculated CRF of 68.6 was in accordance with the predicted CRF value within confidence interval of 99 %. Consequently, the quadratic model was statistically and experimentally found to be good.
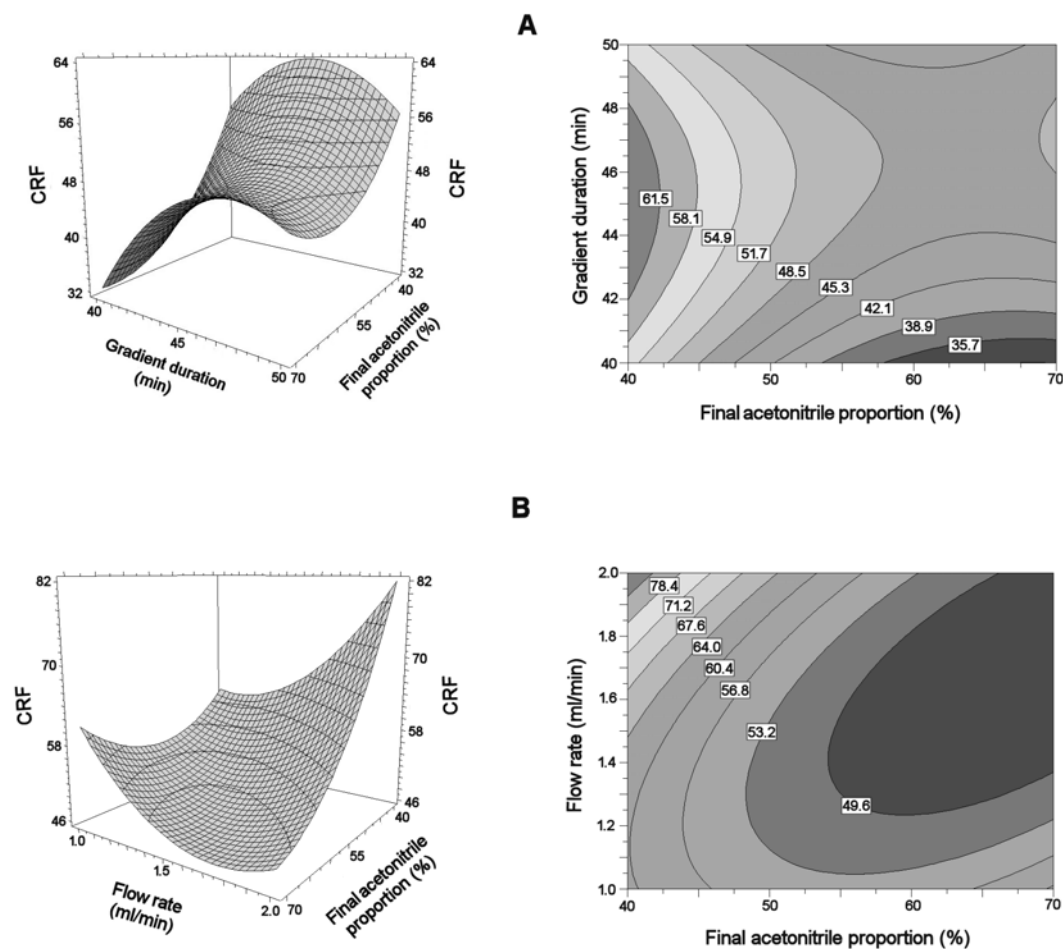
Figure 3:   3D response surface plot and corresponding 2D contour plot showing the variation of CRF values versus final acetonitrile proportion and gradient duration (A) or pump flow rate (B).

The chromatographic profiles at 210 and 273 nm of the artificial phenolic acids mix obtained with the optimized HPLC method are illustrated by Fig 4. In our operating conditions, hydrocinnamic acid was only detected at 210 nm when benzoic acid was

detected at 273 nm. The other phenolic acids were detected at both 210 and 273 nm. However, 210 nm is more suitable for gallic, caffeic, vanillic, ferulic and hydrocinnamic acids when ellagic and cinnamic acids were better detected at 273 nm. The retention time and suitable wavelength corresponding to the different relevant free phenolic acids in the optimized HPLC procedure were summarized in Table 2. The optimized chromatographic method was successfully applied to the screening of free phenolic acids from *Striga hermonthica*, *Guiera senegalensis* and *Acacia sp.* honey. Results are summarized in Table 2. In comparison with the standard mix reference, relevant free phenolic acids may be encountered in galls of *G. senegalenesis* and the unifloral honey of *Acacia sp.*, *S. hermonthica* didn't contain any. Gallic, vanillic and cinnamic acids were identified in *G. senegalenesis* when caffeic, vanillic, ferulic, ellagic hydrocinnamic and cinnamic acids were detected in the honey sample. In previously published work, gallic acid was identified in *G. senegalenesis* (Bouchet et al., 2000) when caffeic, vanillic, ellagic, hydrocinnamic and cinnamic acid were identified (Gheldorf et al., 2002; Thomas-Barberan et al., 2001; Amiot et al., 1989) from the unifloral honey of *Acacia* sp.
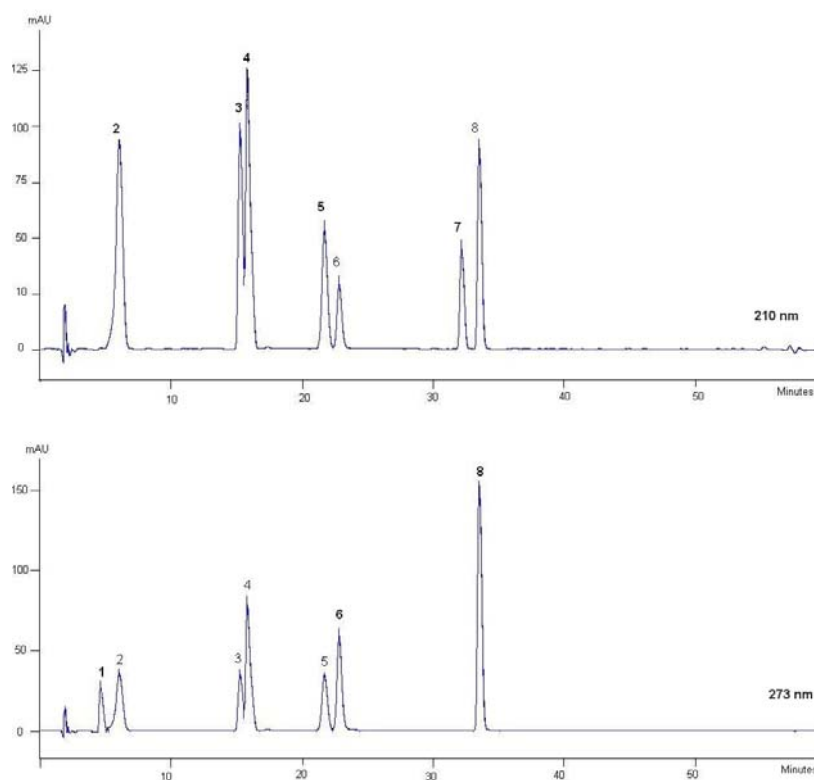
Figure 4: Optimized chromatographic separation of the artificial phenolic acids mix:
1 Benzoic acid, 2 Gallic acid, 3 Caffeic acid, 4 Vanillic acid, 5 Ferulic acid, 6 Ellagic acid, 7 Hydrocinnamic acid, 8 Cinnamic acid at 210 and 273 nm

Conclusion

In conclusion, this experimental design methodology allows us to set HPLC conditions for optimized separation of 8 relevant free phenolic acids. These conditions were successfully applied to the screening of *S. hermonthica*, *G. senegalensis* and honey extracts. This study is a preliminary to a largest screening of phenolic acids from Burkina Faso medicinal plants in order to correlate them with their antioxidant properties and biological benefits.

Acknowledgments

References

1. Amakura, Y., Okada, M., Tsuji, S. and Tonogai, Y. (2000). Determination of phenolic acids in fruit juices by isocratic column liquid chromatography. J. Chromatography A **891**: 183-188.
2. Amiot, M. J., Aubert S., Gonnet M. and Tacchini M. (1989). Les composés phénoliques des miels: étude préliminaire sur l'identification et la quantification par familles. Apidologie **20**: 115-125.
3. Bouchet N., Levesque J. and Pousset J. L. (2000). HPLC isolation, identification and quantification of tanins from *Guiera senegalensis*. Phytochemical analysis **11**: 52-56.
5. Box G. E. P., and Cox, D. R. (1964). An Analysis of Transformations. Journal Royal Statistical Society B **26**: 211-252.
6. Box, G. E. P., Hunter W. G. and Hunter J. S. (1978). Statistics for experiment: an introduction to design, data analysis, and model building. John Wiley & Sons: New York, USA.
7. Breinholt, V. (1999). Desirable versus harmful levels of intake of flavonoids and phenolic Acids: In Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease.(Kumpulainen J., Salonen J.);The Royal Society of Chemistry: Cambridge, UK.
8. Bruneton, J. (1993). Pharmacognosie: Phytochimie-Plantes médicinales (2nd edition). Lavoisier TEC & DOC: Paris, France.
9. Draper, N. R. and Smith, H. (1981). Applied Regression Analysis (2nd edition). John Wiley & Sons: New York, USA.
10. Eriksson, L., Johansson E., Kettaneh-Wold N., Wikström C. and Wold S. (2000). Design of experiments, principles and applications. Umetrics Academy: Stockholm, Sweeden.
11. Escarpa A., Gonzalez M. C. (2000). Evaluation of high-performance liquid chromatography for determination of phenolic compounds in pear horticultural cultivars. Chromatographia **51**: 37-43.

12. Fernandez, M. A., Garcia, M. D. and Saenz, M. T. (1998). Anti-inflammatory activity in rats and mice of phenolic acids isolated from *Scrophularia fructescens.* J. Pharmacy Pharmacology **50(10)**:1183-1186.

13. Fernandez, J. B., Griffiths, D. W., Bain, H. and Fernandes, F. N. (1996). The development and evaluation of capillary electrophoretic methods for the determination of the major phenolic constituents of potato (*Solanum tuberosum*) tubers. Phytochemical Analysis **7:**253-258.

14. Friedman, M., Henika, P. R. and Mandrell, R. E. (2003). Antibacterial activities of phenolic benzaldehydes and benzoic acids against *Campylobacter jejun, Escherichia coli, Listeria monocytogenes and Salmonella enterica*. J.food Protection **66(10)**: 1811-1821.

15. Garcia, M. D. and Marhuenda, E. (1985). Isolement et activité antimicrobienne de l'acide vanillique et de l'acide p-hydroxybenzoique constituant d'*Onis natris*. Fitoterapia **56(6)**: 349-350.

16. Gheldof, N., Wang, X. H. and Engeseth N. J. (2002). Identification and quantification of antioxidant components of honeys from various floral sources. J. Agriculture Food Chemistry **50**: 5870-5877.

17. Hahn, D. H., Faubion, J. M. and Rooney, L. W. (1983). Sorghum phenolic acids, their high performance liquid chromatographic separation and their relation to fungal resistance. Cereal Chemistry **60**: 255-259.

18. Herrmann, K. (1989). Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. Critical Reviews Food Science Nutrition **28**: 315-347.

19. Ihantola-Vormisto, A., Summanen, J., Kankaanranta, H., Vuorela, H., Asmawi, Z. M. and Moilanen, E. (1997). Anti-inflammatory activity of extracts from leaves of *Phylllanthus emblica*. Planta Medica **63**: 518-524.

20. Karnka, R., Rayanakorn, M., Watanesk, S. and Vannesorn, Y. (2002). Optimization of High-Performance Liquid Chromatographic Parameters for the Determination of Capsaicinoid Compounds Using the Simplex Method. Analytical Science **18(6)**: 661-665.

21. Kaur, S., Grover, I. S. and Kumar S. (1997). Antimutagenic potential of ellagic acid isolated from *Terminalia arjuna*. Indian J.Experimental Biol. **35**: 478-482.

22. Klein, E. S. and Rivera, S. L. (2000). A review of criteria functions and response surface methodology for the optimization of analytical scale HPLC separation. J. Liquid Chromatography Related Technology **23(14)**: 2097-2121.

23. Loarca-Pina, G., Kuzmicky, P. A., De Mejia, E. G. and Kadoa, N. Y. (1998). Inhibitory effects of ellagic acid on the direct-acting mutagenicity of aflatoxin B1 in the *Salmonella microsuspension* assay. Mutation Res. **398**: 183-187.

24. Meda, A., Lamien, C. E., Romito, M., Millogo, J. and Nacoulma O. G. (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chemistry **91**: 571-577.

25. Middleton, E., Kandaswami, C. and Theoharides, T. C. (2000). The effect of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. Pharmacology Review **52**: 673-751.

26. Nacoulma, O. G. (1996). Plantes médicinales et pratiques médicales traditionnelles au Burkina-Faso : cas du plateau central ; thèse d'état Université de Ouagadougou, Burkina-Faso, Tome I, 320 pp, Tome II, 261 pp.

27. Nelder, J. A., and Mead, R. (1965). A simplex method for function minimization. Computer Journal 7: 308-313.

28. Onjia, A., Vasilevic, T., Cokesa, D. and Lausevic, M. (2002). Factorial design in isocratic high performance liquid chromatography of phenolic compounds. J. Serbian Chem. Soc. **67(1)**: 745-751.

29. Op de Beck, P., Cartier, G., David, B., Dijoux-Franca, M. G. and Mariotte, A. M. (2003). Antioxydant flavonoids and phenolic acids from leaves of Lea guineese G. Don.(Leeaceae). Phytotherapy Research **17**: 345-347.

30. Privadarsin, K. I., Khopde, S. M., Kumar, S. S. and Mohan H. (2002). Free radical studies of ellagic acid, a natural phenolic antioxidant. J. Agric. Food Chem. **50(7)**: 2200-2206.

31. Rukkumani, R., Aruna, K., Suresh, V. P. and Padmanabhan, M. V. (2004). Hepatoprotective role of ferulic acid: a dose dependant study. J. Medicinal Food **7(4)**: 456-461.

32. Schmidtlein, H. and Herrmann, K. (1975). Quantitative analysis for phenolic acids by thin-layer chromatography. J. Chromatography **115**: 123-128.

33. Schulz, J. and Herrmann, K. (1980). Analysis of hydroxybenxoic and hydroxycinnamic acids in plant material. II. Determination by gas-liquid chromatography. J. Chromatography **195**: 95-104.

34. Thomas-Barberan, F. A., Martos, I., Ferreres, F., Radovic, B. S. and Anklam E. (2001). HPLC flavonoid profiles as markers for the botanical origin of european unifloral honeys. J. Sci. Food Agric. **81**: 485-496.

35. Weston, R. J., Mitchell, K. R. and Allen, K. L. (1999). Antibacterial phenolic components of New Zealand honey. Food chemistry **64**: 295-301.

36. Wood, A. W., Huang, M. T., Chang, R. L., Newmark, H. L., Lehr, R. E., Yagi, H., Sayer, J. M., Jerina, D. M. and Conney A. H. (1982). Inhibition of mutagenecity of bay-region diolepoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. Proceedings of the National Academy of Sciences of the United States of America **79**: 5513-5517.

37. Wu, H., Haig, T., Pratley, J., Lemerle, D. and An M. (1999). Simultaneous determination of phenolic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in wheat (*Triticum aestiVum* L.) by gas chromatography-tandem mass spectrometry. J. Chromatography A **864**: 315-321.

38. Yin, Y. and Carter, C. W. Jr. (1996). Incomplete factorial and response surface methods in experimental design: yield optimization of tRNA[Trp] from in vitro T7 RNA Polymerase transcription. Nucleic Acids Research **24(7)**: 1279-1286.