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Application of a new optimization strategy for the separation of tertiary alkaloids extracted from *Strychnos usambarensis* leaves☆

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ABSTRACT

The HPLC separation of six alkaloids extracted from *Strychnos usambarensis* leaves has been developed and optimized by means of a powerful methodology for modelling chromatographic responses, based on three steps, i.e. design of experiments (DoE), independent component analysis (ICA) and design space (DS). This study was the first application of a new optimization strategy to a complex natural matrix. The compounds separated are the isomers isostrychnopentamine and strychnopentamine, 10-hydroxyusambarine and 11-hydroxyusambarine, also strychnophylline and strychnofoline. Three LC parameters have been optimized using a multifactorial design comprising 29 experiments that includes 2 center point replicates. The parameters were the percentage of organic modifiers used at the beginning of a gradient profile which consisted in different proportions of methanol (MeOH) and acetonitrile (MeCN), the gradient time to reach 70% of organic modifiers starting from the initial percentage and the percentage of MeCN found in the mobile phase. Subsequent to the experimental design application, predictive multilinear models were developed and used in order to provide optimal analytical conditions. The optimum assay conditions were: methanol/acetonitrile-sodium pentane sulfonate (pH 2.2; 7.5 mM) (33.4:66.6, v/v) at a mobile phase flow rate of 1 ml/min during a 40.6 min gradient time. The initial organic phase contained 3.7% MeCN and 96.3% MeOH. The method showed good agreement between the experimental data and predictive value throughout the studied parameters space. Improvement of the analysis time and optimized separation for the compounds of interest was possible due to the original and powerful tools applied. Finally, this study permitted the acquisition of isomers profiles allowing the identification of the optimal collecting period of *S. usambarensis*.

1. Introduction

Malaria still remains the major parasitic infection, affecting hundreds of countries around the world, especially tropical regions in Africa, Asia and Latin America. The impact of the disease in terms of morbidity and mortality, as well as the ever increasing emergence of resistance to currently used treatments enhances the importance of developing new drugs. The plant world represents a huge, varied, promising and a countless source of new potential therapeutic substances.

1.1. Alkaloids from Strychnos usambarensis leaves

Our study concerns *S. usambarensis* Gilg (Loganiaceae). This small tree growing widely in Eastern Africa, particularly in Rwanda, is used in traditional pharmacopoeias. It is well known not only for its use as arrow poison due to quaternary curarizing alkaloids contained in the roots [1,2] but also for its tertiary alkaloids isolated from leaves because some of them possess antiplasmodial [3] and cytotoxic properties [4–7]. These activities could be related to their original structure: they are all asymmetrical monoterpenoid bisindolic usambarane-type alkaloids. The major compounds are isostrychnopentamine (Fig. 1A) and its epimer strychnopentamine (Fig. 1B), 10-hydroxyusambarine (Fig. 1C) and its position isomer 11-hydroxyusambrine (Fig. 1D), strychnophylline (Fig. 1E),

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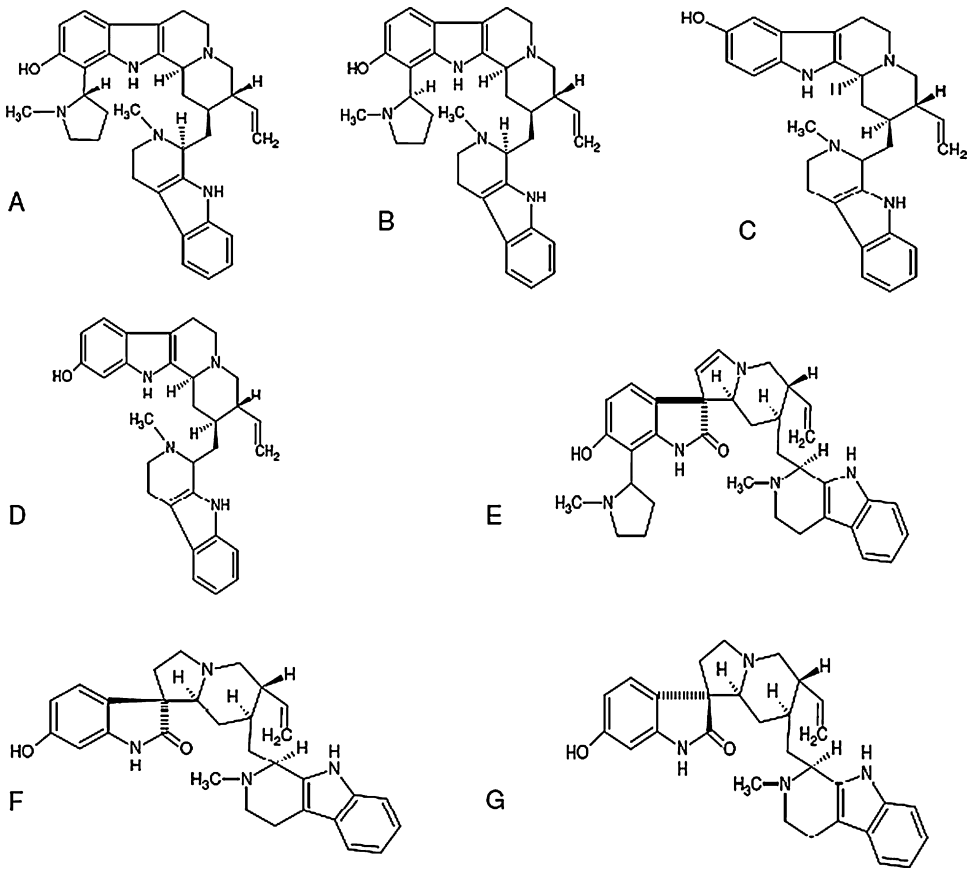

Fig. 1. Chemical structures of (A) isostrychnopentamine, (B) strychnopentamine, (C) 10-hydroxyusambarine, (D) 11-hydroxyusambarine, (E) strychnophylline, (F) strychnofoline and (G) isostrychnofoline.

strychnofoline (Fig. 1F) and its epimer isostrychnofoline (Fig. 1G). From a therapeutically point of view, isostrychnopentamine (ISP), and to a lesser extent, its isomer strychnopentamine (SP) seem to be the most promising substances [8]. ISP proved to be active *in vitro* and *in vivo* against *Plasmodium falciparum* with under μM concentrations [9]. In the recent years, ISP also showed a potential anti-tumor activity against apoptosis-resistant cancer cells [10,11].

1.2. Analytical and biostatistical methods

To our knowledge, this work is one of the first studies dedicated to the chromatographic separation of these tertiary alkaloids extracted from *S. usambarensis* leaves, representing also the first application of a new optimization strategy to a complex natural matrix. Considering the complexity of the plant material, the difficulty lies in finding a method that could separate and identify all the structurally similar compounds (epimers and isomers). Two LC procedures have been described in the 1990s about alkaloids separation of *Strychnos* species. The first one exposed the isolation of monomeric indole alkaloids in *Strychnos nux-vomica* and *Strychnos ignatii* seeds [12], and the second one, the separation of bisindolic alkaloids in *S. usambarensis* roots [13]. Both were assessed by reversed-phase chromatography using acetonitrile (MeCN), an ion-pairing reagent (sodium acetate and sodium salt of heptanesulfonic acid, respectively) and phosphate buffer (pH ~3.00).

The goals of the present study were to develop and optimize HPLC conditions for the separation of six target alkaloids from *S. usambarensis* leaves that are strychnopentamine, isostrychnopentamine, 10-hydroxyusambarine, 11-hydroxyusambarine, strychnophylline and strychnofoline, respectively. To achieve this, three methodologies were combined. Firstly, design of experiments (DoE) was implemented to gather experimental data in order to achieve statistical modelling. The major advantage of using design of experiments to develop this method is that it allows all potential factors to be evaluated concurrently, systematically and quickly [14–16]. Secondly, a design space was built over the design of experiment domain to simultaneously optimize the chromatographic method separation and assess the robustness of its future use [17–19]. Finally, independent component analysis methodology was used to facilitate peaks detection and identification even for co-eluted peaks [20]. Another aim was to reduce analysis cost using methanol (MeOH) as organic modifier rather than MeCN because at the time of the study, the market presented itself with a paucity regarding the last one.

Ion-pairing chromatography was used in this study as it is an efficient strategy to control the retention of protonised bases by reversed phase liquid chromatography (RP-LC) [21–24]. Additionally this new method showed to be precise and suitable to qualitatively identify different samples of *S. usambarensis* leaves collected over a period of three years in various seasons.

2. Materials and methods

2.1. Chemicals

Orthophosphoric acid 85% (pro analysis), sodium carbonate, dichloromethane, methanol and acetonitrile of HPLC-grade were obtained from Merck (Darmstadt, Germany). The 1-pentanesulfonic acid (sodium salt monohydrate 99%) was purchased from Acros Organics (Geel, Belgium). The ultra pure water