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# The fruit of *Annona squamosa* L. as a source of environmental neurotoxins: From quantification of squamocin to annotation of Annonaceous acetogenins by LC–MS/MS analysis

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ABSTRACT

Annonaceous acetogenins (AAGs) are neurotoxins possibly responsible for atypical Parkinsonism/dementia clusters, *via* the consumption of edible Annonaceae fruits. Their presence was investigated in fruit pulps of *Annona squamosa* from different locations. Qualitative analysis of other AAGs was performed. We here report the identification of squamocin in batches from Asia, the Caribbean Basin and the Indian Ocean. This molecule was quantified by HPLC-UV, evidencing a content of 13.5–36.4 mg/fruit. HPLC-ESI-Q-TOF allowed the detection of 25 different raw formulas matching with AAGs. LC–MS/MS methodological development was performed using 4 representative standards. The main AAGs could be annotated, including bullatacin (rolliniastatin-2) and annonacin. This study evidences a remarkable homogeneity for the main AAGs within the species, and discrepancies for minor compounds. These findings indicate that *A. squamosa* should be considered a risk factor for neurodegenerative disorders.



## 1. Introduction

The consumption of *Annona* fruits and herbal teas has been linked to atypical forms of Parkinsonism/dementia in the French West Indies, as well as in other tropical areas (Caparros-Lefèbvre & Elbaz, 1999; Caparros-Lefèbvre & Steele, 2005; Caparros-Lefèbvre et al., 2002; Lannuzel et al., 2007). Annonaceous acetogenins (AAGs) are lipophilic molecules of the Annonaceae family, with suspected involvement in the occurrence of these neurodegenerative diseases (Champy et al., 2008). To date, more than 450 AAGs have been described, all displaying homogeneous structures (Bermejo et al., 2005). These molecules are generally constituted by 35 or 37 carbon atoms, with an oxygenated aliphatic chain bearing a terminal butyrolactone. Their classification, as established by Cavé and co-workers (Cavé, Cortes, Figadère, Laurens, & Pettit, 1997), relies on their main structural features, with types depending on the number of THF rings on the aliphatic chain, and sub-types defined by the structure of the lactonic ring. They most commonly belong to the A-type (mono-THF AAGs) and to the B-type (adjacent bis-THF AAGs) structures. The main sub-types are the sub-type 1a for an α,β-unsaturated γ-methyl-γ-lactone, and the sub-type 1b in which this lactone is associated with a hydroxyl-group on the C-4 position. Since AAGs are potent inhibitors of mitochondrial complex I (NADH ubiquinone oxydo-reductase) in the respiratory chain, structure-activity relationships have been thoroughly explored for this target, showing a tendency towards higher potency for B-type and 1b sub-type structures (Bermejo et al., 2005). Interestingly, additional protein targets have been proposed using fluorescent analogues of the AAG squamocin (B-type, sub-type 1a), which displayed targeting towards the mitochondrial membrane (Derbré, Roué, Poupon, Susin, & Hocquemiller, 2005; Derbré et al., 2008). Several AAGs, including squamocin, have been found to decrease ATP levels, to induce neuronal cell death and to cause tau protein redistribution in neuronal primary cultures (Höllerhage et al., 2009). Moreover, annonacin (A-type, sub-type

*Abbreviations:* AAG, Annonaceous acetogenin; amu, atomic mass unit; CID, collision induced dissociation; EIC, extracted ion chromatogram; HPLC-ESI-Q-TOF, high performance liquid chromatograhy-electrospray-quadrupole-time of flight; HPLC–MS/MS, high-performance liquid chromatography–tandem mass spectrometry; HPLC-UV, high performance liquid chromatograhy-ultra violet detection; LC–MS/MS, liquid chromatograhy–tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; Rt, retention time; THF, tetrahydrofuran; TIC, total ion current.

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1b), which has been identified as the main AAG in *Annona muricata* L. (soursop), proved to be neurotoxic in rodents and to trigger tau-pathology upon subchronic exposure (Champy et al., 2004; Yamada et al., 2014). Similar findings were observed in mice through dietary exposure to the fruit juice of *A. muricata* over a one-year period (Rottscholl et al., 2016). Since evidence of possible neurotoxic long-term effects in humans was brought to light, estimation of exposure to AAGs raised the attention of several research groups. Already largely described in leaves and other parts of *Annona* trees, AAGs have also been reported in the edible fruit pulps of *A. muricata* and *Asimina triloba* (L.) Dunal, along with quantitative estimations (Champy et al., 2005; Levine et al., 2015; Potts et al., 2012). AAG compositions of some *Annona*-derived food products have been described (Champy, Guérineau, & Laprévote, 2009; Le Ven et al., 2012; Le Ven et al., 2014), evidencing mainly $C_{35}$ AAGs for the species *A. muricata* and *A. cherimolia*. Squamocin is one of the main AAGs in *Annona squamosa* L. (Bermejo et al., 2005) and was reported in high amounts in the seeds of this species (Champy, 2011; Yang et al., 2009). The presence of AAGs in *A. squamosa* fruit pulp yielded scarce and limited reports (Bonneau et al., 2012; Champy et al., 2008; Hollerhage et al., 2015). Precise identification, unambiguous quantitative data and insight on possible variabilities are still acutely needed. In the present study the fruit pulp of *A. squamosa* was examined, using four batches from different cultivation areas, in order to confirm the presence of squamocin in *A. squamosa* pulp, and to describe the structural features of the other major AAGs, in a dereplicative approach. The identification of squamocin and its quantification using a robust method with the requisite validation are presented, as well as a methodological development by HPLC–MS/MS with post-column infusion of lithium (Le Ven et al., 2012), allowing an overview of the variety of AAGs in the batches studied, and details of the main representatives of this molecular series to be described.

## 2. Material and methods

### 2.1. Chemical and reagents

Standards (see Fig. 2) were isolated in-house (purity > 97%), under previously described conditions (Bermejo et al., 2005), from the seeds of *Annona muricata* L. for annonacin (**1**) and annonacinone (**2**), seeds of *Annona bullata* A. Rich. for bullatacin (**3**), and seeds of *Annona squamosa* L. for squamocin (**4**). Methylene chloride, methanol and acetonitrile (HPLC-grade) were supplied by CarloErba SDS (Val de Reuil, France). Lithium iodide (LiI) was provided by Sigma-Aldrich (St. Quentin Fallavier, France). Ultra-pure water was prepared in-house with a Millipore Milli-Q purification system (Darmstadt, Germany).

### 2.2. Plant material

Four distinct batches (A-D) of *Annona squamosa* L. ripe fruits were directly obtained from food retailers. Places of origin (country, region), of purchase and months of harvest, respectively, were: batch A: Thailand (4 fruits; Asian food store, France; January; 1062 g); batch B: Mauritius (Indian Ocean; 1 fruit; local fruit market; March; 217 g); batch C: Martinique (French West Indies; 1 fruit; local fruit market; March; 151 g); batch D: Vietnam (4 fruits; Rungis market, France; February; 1003 g). Fruit pulps (batch A: 741 g; batch B: 123 g; batch C: 80 g; batch D: 601 g; fresh pulp) were carefully separated from the seeds and pericarps, and then air-dried before powdering. Their water content comprised between 79 and 81%.

### 2.3. Crude extracts

Dried pulp powders were subjected to 3 successive macerations of 3 h in distilled $CH_2Cl_2$ (10% m/v). Extractions were performed

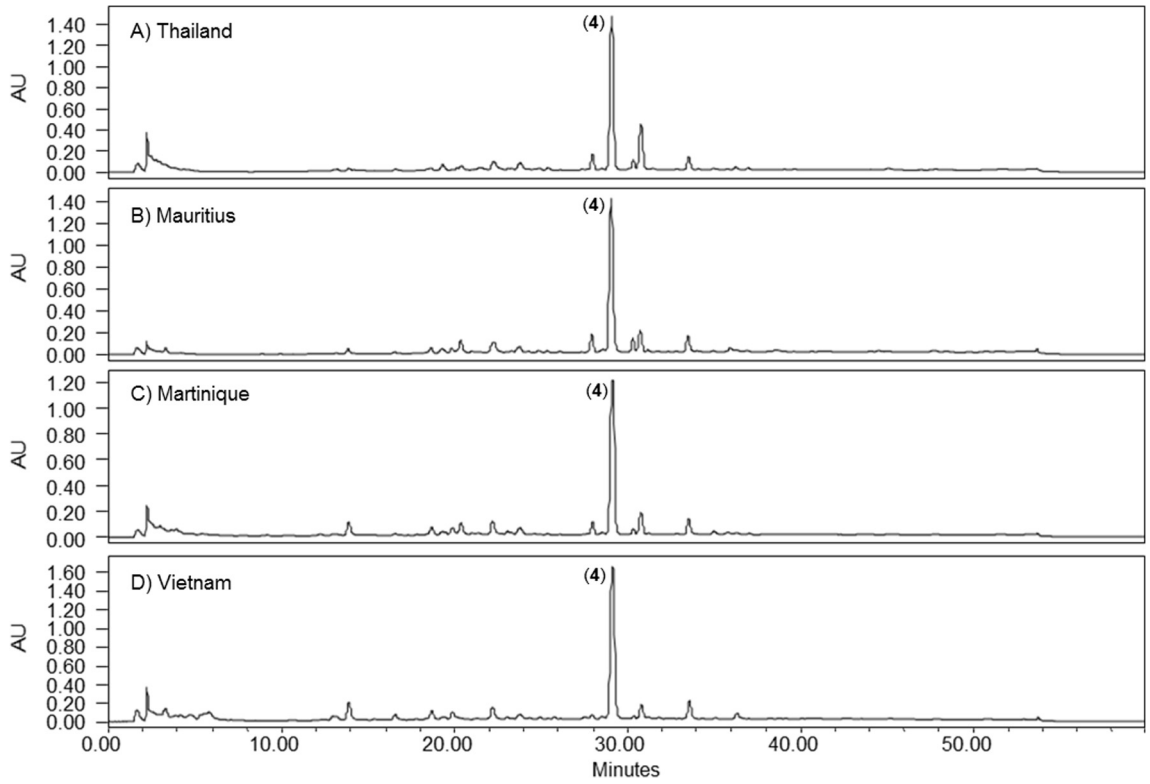


**Fig. 1.** HPLC-UV chromatograms recorded at λ = 210 nm, showing the crude extracts of *Annona squamosa* fruit pulp from the 4 batches (10 mg/ml in MeOH). The most intense peak (Rt = 29.0 min) was identified as squamocin (4). Quantitative data are presented in Supplementary Table S1.