SUPERCRITICAL CO_2 EXTRACTIONS OF ALKALOIDS –*Narcissus pseudonarcissus* ALKALOIDS

Orchidea Rachmaniah[a, b], Rob Verpoorte[c] *, and Geert-Jan Witkamp[b]

[a]Institute Technology of Sepuluh Nopember
Chemical Engineering Department
Surabaya, Indonesia 60111

[b]Delft University of Technology,
Biotechnology Department, Faculty Applied Sciences
Julianalaan 67, 2628 BC, Delft, the Netherlands

[c]Leiden University,
Institute of Biology Leiden, Natural Products Laboratory
PO Box 9502, 2300 RA, Leiden, the Netherlands

Email: verpoort@chem.leidenuniv.nl

Abstract. Supercritical carbon dioxide extraction is an environmentally friendly modern technology and has advantages over the conventional extraction methods for isolation of natural compounds, such as a specific selectivity for compounds and providing higher purity. However, only a limited number of reports on supercritical CO_2 (SC CO_2) extraction of alkaloids was found. Therefore, further studies on SC CO_2 extraction of alkaloids should be are of interest. In this study, SC CO_2 extraction of galanthamine, an alkaloid from *Narcissus pseudonarcissus* bulbs was performed. The extracted alkaloids were identified using GC-MS analysis and quantified by GC-FID. Galanthamine, *O*-methyloduline, haemanthamine, *O*-methyllycorenine, and a haemanthamine derivate were identified in the SC CO_2 extract. High selectivity for galanthamine and *O*-methyllycorenine were found at 690.05 kg/m^3 of CO_2 density (70^{o}C, 220 bar) by using 10%-w of $NaHCO_3$ as modifier. Whereas diethylamine as modifier extracted a broader range of alkaloids: galanthamine, lycoramine, norgalanthamine, narwedine, oduline, haemanthamine, *O*-methyllycorenine, and a haemanthamine derivate.

Keywords: Alkaloids, Carbon dioxide, Galanthamine, Supercritical extraction, *Narcissus pseudonarcissus*.

1. Introduction

Supercritical fluid extraction (SFE) is a promising extraction process. The principle of the process is utilizing a supercritical fluid whose physicochemical properties are between those of a liquid and a gas [1]. Supercritical fluids have better transport properties than liquids because that depends on its density which is tuneable by changing pressure and temperature [2]. SFE is an environmentally benign process compared to conventional industrial solvent extractions i.e. do not require the use of organic solvents. The resulting products are completely free from toxic residues in high purity and selectivity which are important for pharmaceutical industries. Many supercritical fluids can be easily removed from the extract products by depressurization to the atmospheric pressure, leaving the products free of solvent.

One of the most frequently used supercritical fluids is CO_2; it has low critical conditions (T_c = 31.12^{o}C, and P_c = 73.7 Bar) [3,4], is available in high purity, and is safe and cheap. It is good for solubilizing non-polar compounds such as hydrocarbons and can dissolve some medium polar compounds, e.g.. alcohols, esters, aldehydes, and ketones [5]. The solubilizing properties of neat supercritical CO_2 is comparable to hexane and benzene. It can be used at low temperature for extracting thermally labile or easily oxidized compounds. Another advantage of CO_2 is that it is

gaseous at room temperature. Therefore, analyte recovery is simple and provide solvent-free extracts. However, CO_2 has a disadvantage as it has a low polarity and consequently is less effective in extracting middle to highly polar compounds [1,2,4]. With increasing molecular weight of compounds a drop of solubility in supercritical CO_2 (SC CO_2) is observed [1]. This drawback can be minimized by adding other solvents. Such solubility enhancers are called co-solvent or modifier. Several solvents are used as modifiers e.g. methanol (MeOH) [6-11], ethanol (EtOH) [12,13], isopropanol, acetonitrile, dichloromethane, diethylamine (DEA) [6,14,15], and water [6], in SFE of natural products [5]. In some studies, the modifier was added to the sample matrix prior the extraction process [9,12,15,16]. However, the choice of modifier type is limited due to unfavourable properties of many of these solvents considering safety, health, toxicity, and environmental aspect.

Several operating parameters need to be taken in consideration for developing a successful SFE process [1,2]. First, the compound(s) of interest must be sufficiently soluble in the supercritical fluids. The considered operating parameters include the type of sample [2,6], method of sample preparation [6], type of fluid [1,2], choice of modifiers [1,2,6], method of fluid feeding, and condition of extraction including pressure, temperature, flow rate, and extraction time [1,2]. Other factors are water content [2], and particle size of the matrix [12,17,18], and void fraction [18].

Although SFE has been employed for the extraction of many classes of natural products, only few studies involving alkaloids extraction has been published. Therefore the main purpose of this paper is to review and assess the use of SFE for alkaloids as reported in the period 2000-2013. Our recent research on the SFE of alkaloids from *Narcissus pseudonarcissus* cv. Carlton concerning galanthamine extractability by using different chemicals treatment will be briefly mentioned..

2. Experimental

2.1 Materials and chemicals

Powder of *Narcissus pseudonarcissus* cv. Carlton was supplied by Leenen BV (Voorhout, NL). Standard of galanthamine-HBr was donated by Tiofarma BV (Nieuw Beijerland, NL). Pure grade of methanol (MeOH), diethylamine (DEA) were purchased from Sigma-Aldrich (****).

2.2 SC CO_2 extraction

SC CO_2 extraction was conducted in SFX^{TM}-220 (ISCO) extractor system consisting of a 10 mL extractor vessel, a restrictor, an extractor temperature controller (SFX^{TM}-200 controller), and a restrictor temperature controller (ISCO). The SC CO_2 set-up includes a pump for CO_2 (ISCO syringe pump model 260D) and a chiller (cooling unit Hubbler-chiller control). The temperature and pressure of the extraction system were adjusted and controlled at the desired conditions. The CO_2 flow rate was automatically adjusted with the temperature and pressure setting. *Narcissus pseudonarcissus* powder (~5 g) was loaded into the extraction vessel at each experiment for 3 h. The vessel was then placed in the heating chamber to maintain the operating temperature.

2.3 Alkaloid Identification and Quantification

The alkaloids in the obtained extracts were identified by GC-MS. GC-MS was carried out on an Agilent 7890A GC system with a 5975C single quadruple Mass Spectrometric Detector and an Agilent 7693 Auto sampler (Agilent Technologies, Inc.). The GC column used was a 30 m x 0.25 mm i.d., 0.25 μm film thickness of DB-5 column (Agilent JW Scientific, Agilent Technologies, Inc.). A temperature gradient was employed of a 30 min temperature increase from 200-250^{o}C at 2.5^{o}C/min, then 250-270^{o}C at 10^{o}C/min followed by 8 min hold at 270^{o}C. The injector and detector temperatures were 250 and 270^{o}C, respectively. One μL of each sample was injected. The flow rate of the carrier gas (Helium) was 1.5 mL/min and the split ratio was 1:20. The analysis was done in scan mode (m/z 50-350) using electron ionization at 70 eV. Galanthamine identification was accomplished by comparing the measured m/z data with an authentic standard compound while other alkaloids were identified by comparing their mass spectral fragmentation data with literature.

The quantified of alkaloids were done by GC-FID. The sample preparation method was adopted from Gotti et al. (2006). One μL of the resulting solution was injected into the GC-FID. Assuming a similar detector response in GC-

FID, the amount of other identified alkaloids besides galanthamine is expressed as μg galanthamine/g of dry weight of plant material.

3. Results and Discussion

3.1 Overview of Supercritical Extraction of Alkaloids

A literature search was done using the Scopus and Web of Science databases; information about SFE reported during the period 2000-2013 periods. More than 6000 publications were found, including journal articles, conference papers, reviews, editorials, notes, short reviews, conference reviews, letters, errata, books, and abstract reports (Fig.1A) by using of 'supercritical fluid extraction' as keywords with Boolean (logical) operator AND. Thus number proofs that SFE is widely applied in numerous fields. Further searching was done to investigate the most interesting research papers of SFE using the Scopus database with Science direct, Scopus, Springer, MedLine/PubMed as sources. The results are summarized in Fig.1B. Oils, essential oils, fatty acids, bioactive compounds, soil, metals (instead of heavy metal keywords), carotenoids, fragrances, phospholipids, tocopherol, triterpenes and sterols, alkaloids, and natural products were added as the keyword after 'supercritical fluid extraction'. Their truncation was also used for various word endings, singularis and pluralis forms.

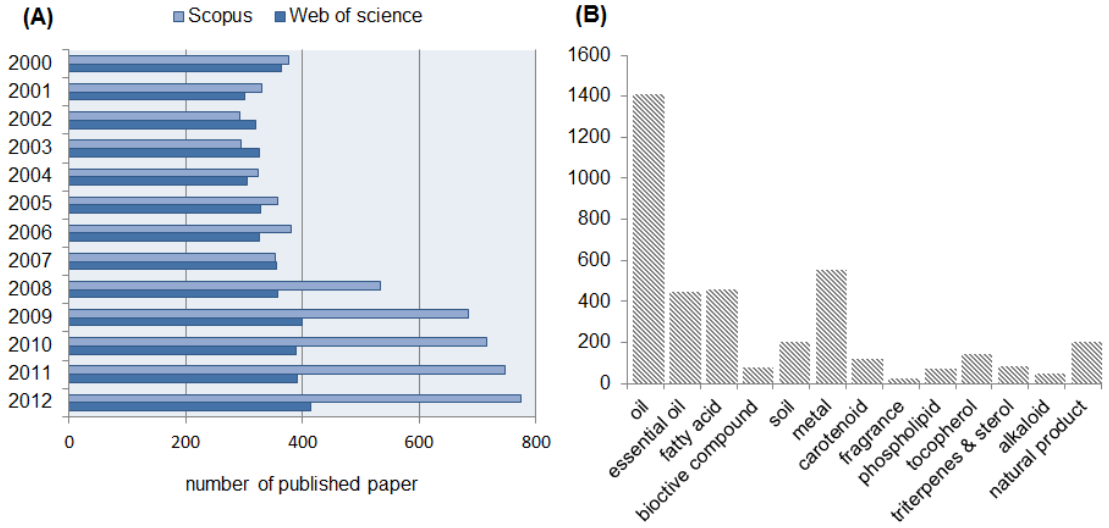

Figure 1. **(A)** SCFE works published per year, and **(B)** Fields of interest of SFE published by Scopus in the period 2000-2013.

This search results do not represent the real mapping of SFE work, as those keywords can be overlapping. However, it describes roughly the most interesting research topics for SFE. We also found that mathematical OR modelling and solubility AND studies were subject of 561 and 920 published titles per year, respectively. It clearly shows that SFE of alkaloids is not much explored, even though about 45 published titles were found both by Scopus and Web of Science (Fig.2) in the period 2000-2013.

SFE of alkaloids has been used quite widely even though only certain classes of alkaloids were extensively investigated, whilst many others are yet to be investigated [5]. Caffeine, a xanthine group alkaloid, is the most studied alkaloid by SFE. Caffeine containing beverages are widely known but caffeine is also contained in some medicines and foods e.g. certain soft and energy drinks, candies and sweets [16]. Fifty published titles were found by Scopus when 'supercritical fluid extraction caffeine' was used as keyword by AND Boolean search strategy. It is as many as were found for 'supercritical fluid extraction alkaloid'. Industrial application of decaffeination of coffee beans and tea were established by applying SFE technology in Italy and Germany, respectively. A summary of some selected papers on SFE of alkaloids is shown in Table 1.

3.2 The Supercritical Extraction of *Narcissus pseudonarcissus* alkaloids

Amaryllidaceae's alkaloids extraction involving SC CO_2 has not yet been reported. In our studies SC CO_2 extraction of dried powder of *Narcissus pseudonarcissus* bulb was conducted with different types of chemical treatment: $NaHCO_3$ solution, DEA, DEA combined with water or Methanol, and NH_4OH (Rachmaniah et al. unpublished results). Four kind of *Narcissus* alkaloids were extracted at 70°C at 150 bar: galanthamine, haemanthamine, *O*-methyllycorenine, and haemanthamine derivate. A strong effect was found of the chemicals used in the plant material treatment on the alkaloids extractability. For example galanthamine, haemanthamine, *O*-methyllycorenine, and a haemanthamine derivate were observed in the extract with $NaHCO_3$ solution as modifier (Fig 3) whereas different alkaloids profiles were observed by using the other modifiers. The extracted alkaloids, in sequence of ascending retention time, were: galanthamine, lycoramine, norgalanthamine, epi-norgalanthamine, narwedine, oduline, haemanthamine, *O*-methyllycorenine, and haemanthamine derivate. The other extraction conditions resulted in different patterns of alkaloids in the extracts.

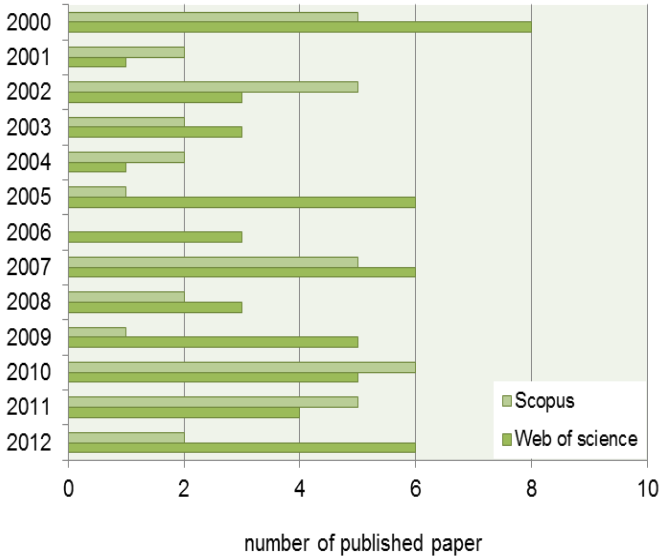

Figure 2. SFE alkaloids published per year in the period 2000-2013.

Benzylamine alkaloids i.e. methylephedrine, norephedrine, ephedrine, and pseudoephedrine from *Ephedra sinica* [7], indole alkaloids i.e. catharantine [10], vindoline, vinblastine, vincristine [14] from *Catharanthus roseus*, coronaridine, and voacangine from *Tabernaemontana catharinensis* [19], purine alkaloids i.e. caffeine [16] and theobromine from *Ilex paraguariensis* [20, 21], quinolizidine alkaloids i.e. matrine, oxysophocarpine, oxymatrine from *Sophora flavescens* Ait. [12], and evodiamine from *Evodia rutaecarpa* [11], isoquinoline alkaloids i.e. dl-tetrahydropalmatine from *Corydalis yanhusuo* [22] and nuciferine from *Nelumbo nucifera* [15], colchichine, colchicoside from *Colchicum autumnale* L. [8], and sinomenine from *Sinomenium acutum* [9] were reported to be extracted by means SFE.

SFE can selectively extract compounds at certain conditions as previously reported [1-3]. Slightly different extraction conditions were applied for the SFE of caffeine or theobromine from leaves of mate, *Ilex paraguariensis* [20,21]: 293 K and 313 K, respectively as the the best extraction temperatures for these two compounds, while 150 bar was the best pressure condition for the extraction. Higher temperature and pressure (373 K and 300 bar) was reported when caffeine was extracted from *Coffea canephora* husks with 32% of moisture content in raw material [16]. Different alkaloid extract profiles were observed when different SFE conditions for *Catharanthus roseus* were applied. Selective production of vinblastine and vincristine was achieved, 35.69 µg/g of vinblastine and trace amount of vincristine, were extracted at 34 MPa, 80°C using methanol-triethylamine as modifiers [14]. A higher yield with lower selectivity was obtained at 280 bar, 62°C when a solely methanol was used as modifier. Catharanthine, vindoline, and vinblastine were obtained at 198.8 µg/g, 208.2 µg/g, and 77.8 µg/g respectively [10] while vincristine

was obtained in trace amounts. It was shown by those two cases of SFE of alkaloids, that no rule of thumb can be used for extracting the alkaloids from their plant matrices.

There are two main factors for compound solubility in supercritical fluids (SFs): the volatility of interested compound and the solvation effect of SFs. Both are a function of temperature and fluid density. Whilst for the SFE from plant matrices the rate of extraction depends on the rate of diffusion of the SFs through the plant matrices, the interaction between targeted compound and its location inside the plant matrix [5], and the influence of compound-matrix desorption effects. Hence, pure solute solubility in SFs, e.g. using a commercial standard compound, does not guarantee the same solute behaviour when it is extracted from its biological matrix. The extractability of hyoscyamine in its pure salt form i.e. hyoscyamine hydrochloride and from its plant matrix *Scopolia japonica* Maxim showed good agreement when 10%- v/v of DEA-MeOH was used as modifier at 34 Mpa, 60^{o}C. While, scopolamine showed a different behaviour; scopolamine salts showeds good extractability both at 10%-v/v water and 10%-v/v DEA/MeOH. Whereas, its free base was only soluble with 10%-v/v DEA/MeOH [6]. Therefore, in the case of polar alkaloids, the utilization of modifiers is greatly suggested for enhancing targeted compounds solubility in SFs. However, every plant matrixes has its particular characteristics consequently it is not possible to apply one and the same modifier for all applications.

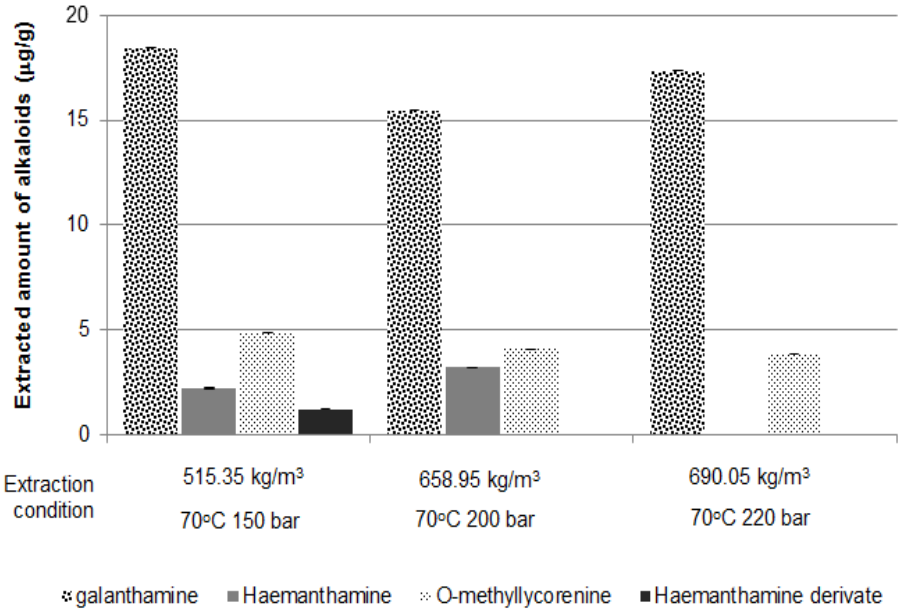

Figure 3. Extractability of *Narcissus pseudonarcissus* alkaloids treated with $NaHCO_3$ solution (Other identified alkaloids besides galanthamine are expressed as µg galanthamine/g of dry weight of plant material. Each experiment was performed twice, and reported values are the averages).

Alkaloids in the plant material are present in the protonated salts form; moistening with either by DEA or ammonium hydroxide solution (25% NH_3) may result in better solubility of the alkaloids by converting the salts into the free bases which are less polar and thus increase their solubility in SC CO_2. A surprising result was observed in the alkaloid extraction when methanol (MeOH) was used as modifier. When small amounts of MeOH were continuously mixed into the SC CO_2 line during the extraction peaks of palmitic and linoleic methyl ester were observed. They gave significant quantities as coextractives in the extract according to their peak height in the GC-MS chromatogram. Whereas only a small amount of galanthamine was obtained, 3.3055±0.0159 µg/g.

Table 1. Summary of the selected published works on the alkaloids extraction by SFE in the period 2000-2013.

Treatment and SFE conditions	Plant materials	Compound of interest	Results[α]	Pharmacological uses	Ref.
SFE was conducted at 34 MPa, 80°C, 1 mL/min of CO_2 flow rate during the dynamic extraction time. Static extraction time was 15 min and total CO_2 consumed was 10 mL. CO_2-MeOH-DEA (80:18:2) was used as modifier.	Aerial parts *of Ephedra sinica*	Benzylamine alkaloids: ephedrine derivatives (e.g. methylephedrine (ME), norephedrine (NE), ephedrine (E), and pseudoephedrine (PE)).	SCFE was yielded 0.37, 0.046, 3.44, and 0.40 mg/g of ME, NE, E, and PE respectively.	*Ephedrine sinica* have been used in traditional medicine as a diaphoretic, anti-asthmatic and diuretic as well as for the treatment of bronchitis and acute nephritic oedema. While the methylephedrine, norephedrine, ephedrine, and pseudoephedrine have been used for asthma, influenza and some types of inflammation.	[7]
SFE was conducted at 280 bar, 62°C, 1.5 L/min of CO_2 flow, 6.6%-v of MeOH was used as modifier for 40 min.	Dried leaves of *Catharanthus roseus* Petrus.	Indole alkaloids: catharanthine (CTR), vindoline (VDL), vinblastine (AVLB), and vincristine.	Yield 198.8 µg/g, 208.2 µg/g, and 77.8 µg/g for CTR, VDL, and AVLB respectively.	Vinblastine and vincristine are well-known anti-cancer drugs for treating Hodgkin's disease, and acute leukaemia.	[10]
Dried and pulverized powder of fruit was extracted at 250 bar, 80°C, 2 L/min of CO_2 flow, and 0.4 mL/min of MeOH as modifier. Static extraction time was 5 min and 78 min for dynamic time.	Unripe fruit of *Evodia rutaecarpa*	Indolequinazoline alkaloids: evodiamine and rutaecarpine	Yield 1.205 mg/g of evodiamine and 0.949 mg/g of rutaecarpine.	Evodiamine shows anti-tumour activities, anti-obesity, protection against myocardial ischemia-reperfusion injury, and inhibition expression. While rutaecarpine can induce CYP1A1 expression.	[11]
SFE at 20°C and 150 bar (902 kg/m^3) with 1 mL/min of constant flow rate of CO_2 for 1 h was conducted.	Leaves of mate *Ilex paraguariensis*	Purine alkaloids: caffeine and theobromine	Carbon dioxide extraction was more selective compared to 70% v/v ethanol extraction for the products	Saponins and alkaloids of mate extract are credited anti-oedematogenic activity on induced oedemas and stimulant effects on the central nervous, muscle, and circulatory systems in humans.	[21]
SFE was conducted at density of CO_2 0.9 g/mL (247 bar), 3% MeOH as modifier. Static time was 25 min and dynamic time was 30 min, while CO_2 flux was maintained at 1.5 mL/min.	Seeds of plants of *Colchicum autumnale* L. (Colchicaceae).	Colchicine, 3-demethylcolchicine, and colchicoside	The percentages recovery was greater than 78% for colchicine and colchicoside while almost 77% for demethylcolchicine; both were referred to dry weight.	Colchicine possesses anti-inflammatory properties, anti-mitotic agent, anti-tumour activity, and anti-fibrotic drug.	[8]
Powder of rhizome which passed 250 µm filter was used. SFE was conducted at 200 bar, 70°C, 2 L/min of CO_2 flow, 1,2-propanediol was used as modifier at 0.4 mL/min of flow rate. Static time was 5 min and 1.5 h for dynamic time.	Rhizome of *Corydalis yanhusuo* W.T. Wang	An isoquinoline alkaloid: dl-tetrahydropalmatine (dl-THP)	Yield 1.324 mg/g of dl-THP	Analgesics, antiepileptogenic and anticonvulsant action. Inhibition of epileptic attack, antioxidative activity and anxiolytic-like action.	[22]

Table 1. Summary of the selected published works on the alkaloids extraction by SFE in the period 2000-2013 (cont.)

Treatment and SFE conditions	Plant materials	Compound of interest	Results[α]	Pharmacological uses	Ref.
Powder of *Sinomenium acutum* was wetted with ammonia water (10%) and then was dried at 60°C. SFE was conducted at 300 bar, 60°C, 0.5 L/min of CO_2 flow, and 0.4 mL/min of methanol as modifier. Static extraction time was 5 min and 1 h for dynamic time.	Vine stem of *Sinomenium acutum (thumb) Rehd et Wils*	Sinomenine	Yield 7.34 mg/g of sinomenine.	Sinomenine shows some action of immunosuppression, anti-inflammation, arthritis amelioration, block of tissue remodelling, protection, and against hepatitis.	[9]
SFE at 45°C and 250 bar with CO_2-EtOH (4.6% w/w) as modifier was conducted for 3 h. The obtained extract then was fractionated.	Branches and leaves of *Tabernaemontana catharinensis*.	Indole alkaloids: coronaridine and voacangine	Coronaridine and voacangine was obtained at Alkaloid Fraction 3 (AF3). They were7% and 53% of AF3, respectively.	*Tabernaemontana catharinensis* is popular known for the treatment for the protozoan diseases. Alkaloid purified from its extract demonstrated anti-tumour, anti-microbial, anti-inflammatory, analgesic activity, anti leishmanial and anti-HIV-1 activities.	[13, 23]
Raw material in 60-80 mesh size was treated first by dipping in 0.1 mL/L ammonia-EtOH (1:4, v/v) for 24 h. SFE was conducted at 25 MPa, 50°C, 2 L/min of CO_2 flow rate, 0.04 mL/min of modifier (75% EtOH and 25% H_2O). Static time was 1 h and 2 h for dynamic time. Crude extracts was purified by high-speed counter-current chromatography (by chloroform-methanol- NaH_2PO_4).	Leguminosae-root of *Sophora flavescens* Ait. (Kushen)	Quinolizidine alkaloids (matrine (MT), oxysophocarpine (OSC), and oxymatrine (OMT))	Yield 85.8 g/g, 221.60 g/g, and 670.23 g/g for MT, OSC, and OMT respectively.	MT and OMT is reported to exhibit sedative, depressant, anti-tumour, antipyretic, cardiotonic activities, and anti-hepatitis B virus. While OSC suppressed the biosynthesis of leukotriene.	[12]
Dried leaves passed 40 mesh/inch sieve and were wetted first with ammonia water (10%) prior extraction. Then it was dried at 60°C and ready for further use for extraction. The SFE was carried out for 2 h at 70 °C under 30 MPa, with 10% (v/v) DEA and 1% (v/v) water in DEA as the modifier which kept a CO_2 flow rate of 1.2 mL/min.	Leaves of *Nelumbo nucifera* (N. nucifera)	Isoquinoline alkaloids: Dehydronuciferine, N-nornuciferine, O-nornuciferine, Nuciferine, and Roemerine.	Yield nuciferine 325.54 µg/g	Extract of *Nelumbo nucifera* leaves has been found to inhibit digestion, slower absorption of lipids and carbohydrates, accelerate lipid metabolism, up-regulate energy expenditure.	[15]

[α]reported yields were based on dry weight

4. Conclusions

SFE is widely used in pharmaceutical and food applications. However, for the application in alkaloid extraction not much work has been done yet. In our studies on alkaloids extraction from *Narcissus pseudonarcissus* cv. Carlton we found that SC CO_2 extraction yield is highly dependent on chemical treatment of the plant material. Selectivity for galanthamine and *O*-methyllycorenine was found at 690.05 kg/m^3 of CO_2 density (70^{o}C, 220 bar) by using $NaHCO_3$ as modifier. Others chemicals treatment i.e. diethylamine, water, and 25% NH_3 gave a broader range of extracted alkaloids. While using methanol as a modifier, fatty acids and their corresponding methyl esters were extracted as the major compound while galanthamine was only extracted in minor. Haemanthamine and *O*-methyllycorenine were always found as accompanying alkaloids in the products. Considering safety, selectivity and reliable yield of galanthamine as a major product, $NaHCO_3$ solution is the most appropriate modifier for extracting galanthamine from its plant matrix using SC CO_2 extraction.

Acknowledgments

Leenen B.V. is acknowledged for supplying powder of *Narcissus pseudonarcissus* cv. Carlton, Tiofarma B.V. for their donation of galanthamine-hydrobromide standard, and FeyeCon Carbon Dioxide Technologies for their kindness to lend the author theirs supercritical fluid extractor system. Andrea Lubbe and Justin Fischedik are appreciated for the GC-MS discussions. Directorate General for Higher Education (DGHE), Ministry of National Education of The Republic of Indonesia is also gratefully acknowledged for the DIKTI fellowship (Batch IV-2010) of O.R.

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