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Low-field NMR for quality control and fraud detection : application to the analysis of dietary supplements and e-liquids

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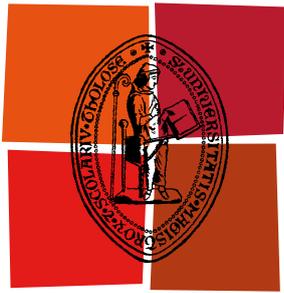
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Université
de Toulouse

THÈSE

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List of Abbreviations

General Abbreviations

API	Active pharmaceutical ingredients
<i>C. cassia</i>	<i>Cinnamomum cassia</i>
<i>C. verum</i>	<i>Cinnamomum verum</i>
CNS	Central nervous system
DS	Dietary supplements
E-liquid	Liquid mixture for electronic cigarette
EI	Electron ionization
ESI	Electrospray ionization
FA	Fatty acids
FDA	Food and Drug Administration
GC	Gas chromatography
HPLC	High performance liquid chromatography
MS	Mass spectrometry
NIST	National Institute of Standards and Technology
PG	Propylene glycol
PCA	Principal component analysis
PLS-DA	Partial least squares to latent structures-discriminant analysis
OPLS-DA	Orthogonal PLS-DA
OTC	Over the counter
RT	Retention time
SCs	Synthetic cannabinoids
THC	Tetrahydrocannabinol
TLC	Thin layer chromatography
TDI	Tolerable daily intake
TOF	Time of flight
UNODC	United Nations Office on Drugs and Crime
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet spectroscopy
IR	Infrared spectroscopy
VG	Vegetable glycerol

NMR-Specific abbreviations

AQ	Acquisition time
Cr(ACAC) ₃	Chromium(III) acetylacetonate
COSY	Correlation spectroscopy
D1	Relaxation delay
DOSY	Diffusion ordered spectroscopy
FID	Free induction decay
HF	High field
HETCOR	Heteronuclear correlation
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence spectroscopy
LB	Line broadening
LF	Low field
LOD	Limit of detection
LOQ	Limit of quantification
NMR	Nuclear magnetic resonance
NS	Scan numbers
P90	Pulse 90°
ppm	Parts per million
qNMR	Quantitative NMR
RF	Radio frequency
S/N	Signal to noise ratio
SW	Spectral width
T1	Longitudinal relaxation time
T2	Transverse relaxation time
TD	Time domain
TSP	3-(Trimethylsilyl)propionic-2,2,3,3-d ₄ acid sodium salt
TXO	Triple resonance probe with specific nuclei
TFT	α,α,α -Trifluorotoluene

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General Introduction

Nuclear magnetic resonance (NMR) is a spectroscopic technique and a non-selective analytical method. Comparing NMR techniques with routine chromatography methods, NMR can afford both structural and quantitative information concerning molecules in complex samples. Furthermore, the fact that NMR is a holistic (non-selective) and robust method and has properties like non-destructive and easy preparation without isolation regarding the samples make this method well suited for the analysis of food, herbals, drugs or other formulations [1, 2].

However, the routine application of NMR technique is limited by the high cost of spectrometers and maintenance expenses, especially for chemists in regulatory control laboratories or less developed countries. The recent introduction of benchtop cryogen-free low-field NMR (LF) spectrometers on the market could play an important role to supplement this drawback. Modern LF spectrometers are commercially available with relatively low magnetic field strengths (typically 40-100 MHz), and offer NMR performance and convenience for analysts. Even though LF NMR has a relatively lower sensitivity, it still can give us valuable signals and useful information in the spectra. For more complex samples, the application of multivariate statistical analysis can help to better extract useful information from LF NMR data. Indeed, with the combination of chemometric analysis, including unsupervised and supervised methods, the samples diversity and their similarities (*i.e.* samples grouping and outlier samples), the identification of markers and the establishment of predicted models could nevertheless be evaluated for complex samples. Recently, a few publications have described the method of LF NMR for quality control of various products including edible oil [3], meat [4], dietary supplements [5], and drugs [6].

Herbal dietary supplements are highly valued for various functional activities and usually considered as non-harmful for health. However, some of these products could be fake with, for instance, erroneous label information on their origin or ingredients. In this thesis, we first focused on cinnamon samples. Cinnamon is a famous spice that has a long history of use in traditional medicine for its health benefits and is also nowadays produced as herbal dietary supplements. *Cinnamomum cassia* (*C. cassia*) is the most

widespread cinnamon species that has occupied the major cinnamon supply in the market for the cheap price [7]. But *C. cassia* contains a significantly higher content of hepatotoxic coumarin compared to other species that would pose a health risk to consumers [8]. The cinnamon example illustrates the commercialization of herbal products for which the claimed origin or species can be wrong. In some cases, irregularities can be more acute. Indeed, unscrupulous manufacturers fraudulently added synthetic active pharmaceutical ingredients (API) to improve functional efficiency or reduce manufacturing costs [9, 10]. For example, anorexic drug sibutramine and laxative substance phenolphthalein have been detected in slimming herbal dietary supplements [5]. These problematic products are not only misleading consumers, but mostly the adulterants could cause serious health problems [11]. Therefore, their safety and efficacy, as well as quality control, have become issues with growing concern.

E-liquids are mixtures that can vapor aerosol for inhalation and widely used with the popularity of e-cigarettes. E-liquids usually contain propylene glycol, glycerin, and flavorings; most often, nicotine is added as the psychoactive ingredient [12]. Since e-cigarettes are not well regulated or controlled in many countries, the composition of the e-liquids can contain various substances including psychoactive ingredients. Synthetic cannabinoids (SCs) are the most rapidly growing class of recreational designer drugs adulterated in e-liquids to bring a psychoactive effect. SCs can be designed to have a similar structure to natural cannabinoids (*e.g.* THC in *cannabis*), but their structures are quite diverse and a large number of new SCs have appeared on sale to evade legal regulations, thus the quality control of smoking mixtures with SCs adulterated becomes more difficult. Moreover, compared to natural cannabinoids, SCs often have a greater binding affinity and potency to the cannabinoid receptors in brain [13, 14], many lethal cases caused by SCs abuses are reported in Europe [15].

Aim of the thesis

This doctoral thesis aims to explore the capabilities and limitations of benchtop LF NMR spectroscopy and further evaluate its perspective for routine analysis in quality control and fraud detection. For the analysis of complex herbal products, we particularly focused on the combination of LF NMR data with chemometric analysis, which was applied for global exploration of NMR data to obtain relevant and latent information. In e-liquids study, LF fluorine NMR was investigated as a selective analytical approach in these complex matrices.

Outline of the thesis

In this thesis, we begin with a general introduction. *Chapter I* is the bibliographic section that reviews the application of LF NMR for quality control of herbal products and fraud detection; it presents the classification and composition of cinnamon species, introduces the problem of adulterations in slimming DS and also SCs detected in recreational products. These topics are introduced along with the related analytical methods. *Chapter II* reports the quality control of two main cinnamon species (*C. verum* and *C. cassia*) in commercial culinary products and dietary supplements *via* both HF and LF ^1H NMR. The combination of chemometric analysis with LF ^1H NMR data is used for the classification of cinnamon samples and an approximate quantification is discussed. *Chapter III* explores the LF ^1H NMR-chemometrics analysis for quality control of herbal slimming supplements adulterated with API. *Chapter IV* reports the detection of SCs in e-liquids by using both proton and fluorine nuclei NMR and demonstrates that LF ^{19}F NMR can be an interesting approach to identify adulterated SCs in e-liquids. At last, a general conclusion with perspective ends this manuscript.

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Chapter I Bibliography

1. Introduction

Dietary supplements (DS) are products that intend to add nutrients to diet or to lower the risk of health problems. These products are usually labeled to have functional activities, such as antioxidant, antidiabetic, anticancer, etc. Herbal dietary supplements as the most popular DS which are highly valued for a wide range of natural function components, such as polyphenols, polysaccharide and fiber, and consumers usually accept them as non-harmful for their natural origin. However, in order to improve the functional efficiency of herbal products or reduce manufacturing costs; some manufacturers illegally add synthetic drugs in their products or replace claimed herbals with low-quality herbal DS (misleading origin) and even directly mislabelling [1, 2].

E-cigarettes were invented as an alternative way for quitting smoking and spread rapidly during recent years, e-liquid or smoke juice is the mixture that can vapor aerosol for inhalation when e-cigarette is activated [3, 4]. However, in order to increase the psychoactive effect, recreational designer drugs including synthetic cannabinoids (SCs) are more frequently adulterated in e-liquid products. As the prosperity of e-liquids, there are many cases and reports about the abuse of SCs [5, 6]. Thus, the safety of these products has gained a great deal of attention due to the quality problems and adverse cases reported [6-8].

Nuclear Magnetic Resonance (NMR) is considered as a universal and quantitative analytical method for screening complex mixture, like herbal products, plants, pharmaceuticals, etc. Being an unbiased identical and structural tool, NMR is highly reproducible, robust, inherently quantitative without specific reference demanded, no damage to sample and non-selective for comprehensive analysis [9, 10]. In recent years, ^1H NMR has become a valuable technique for quality control and fraud detection in complex herbal products or other mixtures. The combination of ^1H NMR and multivariate statistical analysis is often used in quality control of bulk products, it leads to a spectral fingerprint that can be used for metabolite markers detection, herbal species identification and discrimination of adulterated samples [9, 11, 12]. However, the expensive cost of equipment and maintenance limits the application of NMR in

routine chemical analysis, whereas the release of recent benchtop low-field (LF) NMR can be a great supplement to these drawbacks. Modern LF spectroscopies have been introduced commercially as a less expensive alternative to higher magnetic field, that are available in the discrimination of agricultural species and also the screening of adulterants within commercial herbal products [2, 13, 14].

This chapter presents a bibliographic review focusing of the keys points of my studies: (i) the ability of LF NMR for quality control of herbal products and fraud detection, and also the application of chemometrics treatment to LF NMR data (ii) the composition and classification of cinnamon (iii) the problematic of adulterations in herbal slimming DS (iv) the presentation of novel SCs and their detection in commercial products. Analytical methods and particularly NMR studies related to these topics will be reviewed.

2. Quality control assessed by NMR, and the use of low-field spectroscopy

Because of the rapid popularity of herbal products and e-cigarettes, many illegal manufactures choose to add synthetic drugs (no marked) into their products to improve the effects [15-18]. For consumers, these adulterated products pose a great threat to health because the content of adulterants or synthetic drugs is unknown. Furthermore, many novel drugs or analogues are adulterated in these products to evade governmental control, but their pharmacokinetics and pharmacology properties are not clear in the body. Therefore, the quality control of these products is quite important to ensure the public health and consumer rights [19].

2.1 Analytical methods used for quality control of complex samples and the application of NMR

The routine analytical methods for screening of complex mixture are chromatography methods, such as Thin Layer Chromatography (TLC) [20-22], High-Performance Liquid Chromatography (HPLC) [16, 21, 23-26] or Gas Chromatography (GC) [7, 27, 28] combined with Mass Spectrometry (MS), or capillary electrophoresis.

These techniques are widely used in analytical labs and have high sensitivity, especially for mass spectrometry with the limit of detection in the pM range [16, 17, 29].

However, there are still some drawbacks to these gold-standard methods in quality control of complex samples. The primary purpose of chromatography methods is the separation of components, whereas herbal products usually contain a variety of ingredients, the separation process would be complicated and time and solvent consuming. Moreover, the pre-purification steps would lose targeted constituents, and the samples tested would be damaged or hard to recycle. Furthermore, many illegal products contain novel synthetic compounds, and also some unexpected herbal metabolites are not easy to be identified by conventional approaches without standard compounds. Additionally, these methods are highly selective, if only a few components are emphasized, some other compounds would be neglected [10].

The application of NMR can generally complement these drawbacks. NMR as a high-throughput analytical method can be used for comprehensive components analysis in mixtures, it has become a routine tool for the structural elucidation of natural and synthetic compounds. Qualitative and quantitative analysis can be simultaneously evaluated, quantitative results are acquired by comparison of the proportional ratio of resonating nuclei and signal intensity. Even though NMR has relatively lower sensitivity than mass spectrometry, it provides many advantages other than chromatography-based methods, including simple method development, easy sample process, relatively short analysis time, identical reference-free and no damage to samples [8, 10, 30, 31].

Large numbers of publications have reported the application of NMR for quality control of herbal products, by using (1) conventional 1D & 2D NMR, (2) hyphenated NMR, (3) quantitative NMR (qNMR), (4) NMR-based metabolomics, and (5) Diffusion Ordered Spectroscopy (DOSY) NMR [10]. Among these NMR approaches, ^1H NMR as a rapid, simple, output method is most frequently applied for the screening of herbal products [31].

NMR, usually referred to as high-field (HF) NMR, although it can provide a more sophisticated answer to the classical chemical questions than many other analytical techniques. But it is often considered as complicated, space-consuming and expensive

to maintain. The release of recent low-field (LF) NMR can be a great supplement to HF NMR and offer NMR performance and convenience for analysts. Although the spectra are less resolved, the properties about low maintenance, robustness, small-size and user-friendly are more valued [2, 30].

2.2 Recent Low-Field NMR

Modern benchtop spectrometers are commercially available with lower magnetic field strengths, there are several typical brands of LF NMR spectrometers available on market, including Fourier 80 FT-NMR (80 MHz, Bruker), Spinsolve (43, 60 & 80 MHz, Magritek), NMReady-60Pro, 100Pro (60 & 100 MHz Nanalysis), X-Pulse (60 MHz, Oxford Instruments) and picoSpin 45II, 80II (45 & 80 MHz, ThermoFisher) [32]. Comparing LF NMR with traditional HF NMR, of course, the signals of LF NMR appear broader and more likely to be overlapped [13], but recent LF NMR is better suitable than its bulky predecessors from 40 years ago in chemical analysis. Modern LF spectroscopy can measure multiple nuclei and record two-dimensional (2D) NMR spectra. Furthermore, novel algorithms and data treatments have been developed to treat the common S/N problems (sensitivity barriers) associated with LF instrumentations. For instance, spin effects enhancing methods like hyperpolarisation of nuclear spins can greatly enhance signals intensities observed in LF NMR application; ultra-fast LF NMR analysis is also achieved depending on the availability of high-performance gradient coils. Novel data processing involving lineshape, phase and baseline automatic corrections without manual input has been reported to provide more interpretable results [33]. Thanks to the progress of superior electronics and advanced sequences, LF NMR sensitivity is sufficient to analyze bio-metabolites and to serve as a chemical detector in size-exclusion chromatography [14].

For daily chemical analysis, this technique has several advantages like (1) the less expensive instrument and maintenance cost than HF NMR (without requiring any infrastructure or liquid cryogenics); (2) the small bench-top size of spectrometer means it can be placed near the work environment or in-house use; (3) the simple operation and troubleshooting of the spectrometers [2, 14]. LF NMR was mainly employed on relaxation (T1 & T2) and diffusion measurements in the early period with very low

magnetic fields, and now it has been utilized in a wide range of research sectors, like food, herbal products, pharmaceutical and bio-fluid analysis [2, 14, 33, 34].

2.3 Low-field NMR spectroscopy in food, pharmacy and forensic

LF NMR has been used for quality control of natural products as an alternative to conventional analytical methods, these researches were mainly performed in the field of food, pharmacy and forensics analysis [32, 33]. By using LF NMR, the botanical species, effective natural components and adulterants in natural products were successfully evaluated [2, 30, 35].

Recently, benchtop NMR spectroscopy has been available in rapid discrimination of the species of agricultural products, such as oil [35-38], beef [39] and hops [40]. During these experiments, typical signals of botanical metabolites or special components of different species can be identified in LF NMR spectra. Kim *et al* developed a method for perilla oil authenticity by using a 43 MHz LF ^1H NMR [35]. Significant differences of integration values were acquired through 6 peaks found in the spectra from both authentic and adulterated perilla oil samples; 4 peaks containing allylic or olefinic protons present in all unsaturated fatty acids (FA) and methylene protons present in all FA were integrated as the best variables for oil species authenticity. A model established by the mixture of perilla oil and a range concentration of soybean oil (0~100 %) also verified the possibility of this method, soybean with a concentration higher than 6 vol% can be correctly discriminated.

LF NMR analysis was also applied to distinguish the species of ground roast coffees. As we have known, there are two main species of commercial coffee beans in the world, *Coffea arabica* L. (around 70% of the market) and *Coffea canephora* Pierre *ex* A (variety robusta). Arabica coffee beans are prized for their smooth taste and rounded flavor, this species usually have higher prices on sale; while robusta plants exhibit better anti-disease ability, and robusta beans have a lower price because of the relatively poor quality. Thus it is potential that unscrupulous manufacturers replace or adulterate arabica coffee with robusta coffee beans. Defernez *et al* explored a method to discriminate these two species of coffee by using a 60 MHz benchtop ^1H NMR [13]. They monitored the signal of 16-O-methylcafestol (16-OMC, a recognized marker compound for robusta beans), even though signals at 6.20 and 3.75 ppm were

overlapped with kahweol and caffeine respectively in low field spectra, but singlet at 3.16 ppm was successfully isolated and marked as the best potential signal, and further integrated. An authentication protocol was established by 81 authentic coffees and mixtures, from which the detection limit of robusta coffee in Arabica was estimated between 10% and 20% w/w. All 27 retail sourced coffee samples were confirmed as “100% arabica”. These publications indicate that LF NMR can be used as a cost-effective approach for species discrimination the authenticity of natural products.

LF NMR as an economic and user-friendly method was recently applied for forensic propose and synthetic drug detection. Several publications described the identification of psychoactive adulterants in seized drugs by LF NMR [30, 41, 42]. Assemat *et al* detected nine different SCs in forty-one herbal blend samples seized by French customs, both HF and LF NMR were applied in this study, the ability of LF NMR for rapid identification of SCs was first explored [30]. Antonides *et al* screened 432 seized samples including herbal blends through LF NMR data combined with an algorithm analysis, the results were then matched to a library of over 300 reference spectra form known substances. Samples contained controlled drugs like SCs, cocaine, amphetamine, heroin, etc were identified with > 99% correct for classification and > 95% correct for the compound. The LF NMR results were also supported by GC-MS analysis [43]. Zhong *et al* analyzed 12 known illegal drugs and their derivatives including morphine and codeine on 600 MHz ¹H NMR and used to create the data library, illegal substances were partially recorded on 82 MHz LF NMR as standard spectra. Then 2 real case samples were recorded on LF NMR and compared with the existed standard spectra on both HF and LF NMR, the results showed that one sample contained morphine and acetylcodeine, while another contained methamphetamine (MAM) and 3,4-methylenedioxymethamphetamine (MDMA). The authors suggested that LF NMR is a reliable and promising approach to rapidly identify illegal drugs in case scenes [42].

Pages *et al* screened “100% natural” named DS through benchtop LF NMR spectroscopy (60 MHz), pharmaceutical substances including desmethylcarbodenafil, dithiodesmethylcarbodenafil, sildenafil, tadalafil, etc in sexual enhancement DS, as well as sibutramine and phenolphthalein in weight loss DS were efficiently detected. Compared to HF ¹H NMR, even LF NMR has lower spectral resolution and sensitivity,

but valuable signals of synthetic drugs still can be acquired, and quantitative analysis was also explored [2]. Singh *et al* identified strychnine and its counterions by exploring the chemical shift as a signature in different 1D ^1H and ^{13}C experiments, the results on 43 MHz NMR spectrometer were confirmed by combination with 2D experiments (COSY, HETCOR, HSQC, HMBC and J-resolved) [44].

LF NMR can be also used for quality control of substandard and falsified medical products, Keizers *et al* applied LF NMR to assess medical product quality, qualitative analysis was explored through chemometric in combination with spectral database, active ingredients containing acetaminophen, aspirin, caffeine, diclofenac, ibuprofen, naproxen, sildenafil, tadalafil, sibutramine, cocaine, and gamma hydroxybutyric acid (GHB) were identified in products, with a limit of detection of about 1 mg/mL [41].

2.4 Application of chemometrics in LF benchtop analysis

^1H NMR can be used for screening components in herbal mixtures, but signals from all resonating protons make the spectra even complicated. Especially for signals overlaps in LF NMR, when the chemical composition of signals is unknown, spectra analysis becomes much more difficult. Hence, for the explanation of high-throughput and complex ^1H NMR spectra, how to improve the processing efficiency is important.

2.4.1 Chemometric tools and NMR

In ^1H NMR spectra of complex mixtures, the entire information about signals in the overlapped region couldn't be directly obtained, and it is often necessary to analyze the data by combining correct mathematical models and effective calculation methods [10]. Chemometrics is the science of extracting information from chemical systems by data-driven means. Chemometrics is inherently interdisciplinary, using methods frequently employed in core data-analytic disciplines such as multivariate statistics, applied mathematics, and computer science, in order to address problems in chemistry, biochemistry, medicine, biology and chemical engineering. On the other hand, chemometrics is a discipline that establishes the relationship between measured values and the state of chemical system and applied to both descriptive and predictive problems in fields, the essence is the basic theory and methodology of chemical measurement [45]. ^1H NMR combined with chemometrics can comprehensively analyze the spectra data, it maximally extracts the componential and structural

information of samples from complex NMR data, obtains effective characteristic data and establishes mathematical models [10, 46]. This method can be used for interpretation, discrimination, and prediction of measurement data, with the advantages of fast calculation speed and good recognition function.

Generally, chemometric methods include two categories: unsupervised and supervised pattern recognition, a supervised scheme identifies an unknown pattern as a member of a predefined class, while an unsupervised scheme groups input patterns into a number of clusters defined as classes [45]. Principal component analysis (PCA) is one of the most commonly used unsupervised exploratory techniques, PCA is usually applied to evidence outliers or natural grouping of samples. It visually reflects the differences between samples by reducing the data dimensionally with the minimum loss of data information. The similarities and differences among samples can be observed in specific plot with the new principal components through scores plots with group information in 2 or 3-dimensional plots; and the loading plot reveals the influencing variables and significant components (NMR signals) concerning scattering behavior. PCA is one of the most widely used methods for finding patterns and trends, but it is not suitable to assign out the discrimination of unknown test samples [10, 45].

While commonly used supervised pattern recognition such as partial least squares to latent structures-discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) are required to create classification or prediction models. PLS-DA as a reliable and robust multivariate statistical analysis method is well suited for quality control and category prediction of complex samples. OPLS-DA is based on PLS-DA with an orthogonal signal correction (OSC) filter. Comparing with PLS-DA, the main benefit of OPLS-DA is that it can separate predictive from non-predictive (orthogonal) variation. Thus it can decrease the impact of NMR data variation that is unrelated to the sample class (*e.g.* age of herbal plant). PLS-DA and OPLS-DA are supervised models in which the users assign the identity of each group of samples such that the maximum variance of the groups can be attained in the hyperspace. The analytical results are also observed as scores and loading plots. Furthermore, the validation is regarded as the critical step to ensure the reliability of PLS and OPLS predictable model. Cross-validation method is routinely employed, with R^2Y and Q^2 are calculated. The R^2Y metric describes the percentage of variation explained by the model; Q^2 shows the

predictive ability of the model. Other validation methods, like CV-ANOVA uses the cross-validated predictive residuals of a model as a basis for hypothesis testing [45, 47].

For quality control of herbal products, a large number of samples are usually collected from the market. Because of the complex composition, other abundant constituents or unknown compounds present in the extract can affect the identification and discrimination of adulterants and active components. Thus, quality control of herbal products becomes an even hard task, in facing the huge and complex data, how to improve its efficiency becomes even important. ^1H NMR together with multivariate statistical analysis is therefore attractive to address these issues [10]. Recently, ^1H NMR combined with chemometrics has been widely used for the characterization of herbal species and different plant organs [9, 11], authentication of edible oil species [48, 49], geographical origin of wines [50, 51], chemotaxonomy [40, 46], saffron (*Crocus sativus* L.) [52], etc.

2.4.2 Chemometrics and LF NMR

Recently, the combination of ^1H NMR and chemometric analysis has been applied for species authentication and detection adulteration in herbal products [10, 11, 53]. Benefit from the development of modern benchtop NMR, publications concerning LF NMR combined with chemometrics began to be reported in the field of food quality control and fraud detection. Compared to routine HF ^1H NMR, LF NMR has lower sensitivity and the assignment of compounds becomes hard for complex herbals, but valuable signals still can be obtained in the spectra. While considering the advantages of economic and handling considerations, with the application of chemometrics, the lack of resolution of the LF spectra can be overtaken. This method could be a breakthrough for the NMR application in quality control of herbal products and fraud detection.

In the early stage of LF NMR development, few publications reported the use of ultra LF NMR for screening of edible oil (22 MHz for ^1H resonance) and honey samples (13 MHz) with chemometrics [54-56], but these LF ^1H NMR experiments are based on relaxation times measurement, for detecting T2 differences variations with adulterants. Because relaxation time is highly related to physico-chemical parameters (pH, water activity and moisture content), thus pure samples can be discriminated by processing

T2 values with chemometrics [56]. Insofar this method has limitations, it is only suitable for special liquid formed samples authentication, like edible oil and honey products. Furthermore, concerning the samples' components, no chemical structures identification nor quantitative analysis were carried out by LF NMR.

Parker *et al* evaluated the ability of a 60 MHz pulsar benchtop NMR for detection of the adulteration of olive oil with hazelnut oil. By comparing a single internal ratio of two isolated groups of peaks, the adulteration of hazelnut oil could be identified in olive oil samples with a detection limit at 13 %w/w. Whereas with the combination of whole-spectrum chemometric analysis, the detection limit can decrease to 11.2 %w/w. The authors also proposed that recent benchtop NMR delivered comparable sensitivity and improved specificity, making it a superior screening tool by comparison to FTIR [36]. Frause *et al* applied 60 MHz NMR to detect 17 adulterants in patchouli essential oils, and 14 adulterants could be identified by visual inspection in spectra. With the application of automated chemometric evaluation, 15 adulterants were recognized in LF spectra. Furthermore, for quality control of essential oils containing non-volatile adulterants, this research proves that LF NMR is more complimentary than GC-MS which is only compatible with volatile components [38]. Jakes *et al* demonstrated that 60 MHz LF NMR can distinguish beef and horse meat by comparison of triglyceride compositions. Bis-allylic, olefinic and the terminal CH₃ peak signals were proved to be the most significant differences between two meats. Depending on these signals, a PCA model was built to further compare training samples, the results showed that 106 samples of 107 meat extracts were correctly authenticated, thus concluded that LF NMR represented a feasible high-throughput approach for screening raw meat [39].

Recently, Killeen *et al* screened different bitter acids in batch hops (important beer ingredients). Qualitative and quantitative analyses were explored through various approaches, including 400 MHz NMR and a Magritek Spinsolve® 43 MHz NMR spectrometer. Compared to HF ¹H NMR, LF signals of α -acids including humulone, adhumulone and cohumulone; β -acids including lupulone, adlupulone and colupulone were well resolved in 18.0-19.4 ppm, and also the ratio of different type acids could be calculated. Moreover, the LF signal of cohumulone cannot resolve from adhumulone, but the chemometrics process can well separate these signals, and PCA analysis was used to separate these compounds and shown in the loading plot. At last, the author

suggested that LF NMR combined with chemometrics could rapidly discriminate different hops chemotypes, and highlight plants with potential for new flavour cultivars [40]. Keizers *et al* developed an analytical method for the determination of active substances in illegal street drugs by 1.4T (60 MHz) benchtop NMR spectroscopy. Sildenafil, tadalafil, sibutramine, cocaine, etc were successfully identified and further quantified with an error of 10%. Additionally, a chemometric procedure was used to identify products, the spectra of reference standards and products were collected to build the dataset. New spectra were ranked on similarity and having determined the top three candidates, their distance, consensus and tail probability were determined. Then the active substance in products can be predicted by the training data obtained. The author claimed that LF NMR data combined with chemometric analysis can help to identify the active compounds in illegal samples, reducing the need for expert interpretation of spectra acquired [41].

3. Cinnamon: From the composition to the authentication

Cinnamon is an aged-old spice that has been used for thousands of years. Cinnamon is originated from the dried inner bark of *Cinnamomum* which is a genus of evergreen aromatic trees and shrubs belonging to the laurel family, distributed over South East Asia (around 250 species), China and Australia, many of which are aromatic and flavoring [57, 58]. Cinnamon is daily used as an aromatic condiment in cuisines and flavor additive for food, like sweet and savory dishes, pastry, snack food, confectionery, wine, tea, and traditional food. Furthermore, Cinnamon as one of the most important and popular spices not only for its aromatic flavor but also widely applied for its health beneficications in traditional and modern medicines [58]. The functional activities of cinnamon are attributed to a wide variety of secondary metabolites like other herbal plants. These compounds include essential oil components, monoterpenes, sesquiterpenes, phenyl propenes, flavonoids, and other polyphenols. Cinnamaldehyde is defined as one of the most important bioactive compounds which is rich existed in essential oil (75%) as the trans format. During these two decades, cinnamaldehyde and other components in cinnamon have been reported for their healthy beneficications, such as antioxidant [59], antidiabetic [57], anti-inflammatory [60], antibacterial [61], antifungal [62], and anticancer [63] effects.

3.1 Overview on species of cinnamon

Cinnamon is a spice obtained from the dried inner bark of genus *Cinnamomum*. There are more than 300 species of *Cinnamomum* distributed in tropical and subtropical regions [64]. Volatile oil as the chief ingredient amounts to 1% of the bark, and principle components cinnamaldehyde, cinnamyl acetate, as well as other constituents, including eugenol and toxic coumarin [65]. But only a few species of *Cinnamomum* in the family Lauraceae can be used for spices depending on the special flavor and sweet taste, there are 2 major cinnamon species used worldwide.

3.1.1 Ceylon cinnamon (*C. verum*)

Ceylon cinnamon (*C. verum*) is referred to as true cinnamon which is made from the inner bark of *Cinnamomum verum* tree native to Sri Lanka. Ceylon also indicates the species' center of origin. Even though there are several species of commercial cinnamon on sale, *C. verum* is usually considered as superior quality and with a higher price compared with other species [66]. According to the report from the Food and Agriculture Organization of the United Nations, Sri Lanka still produces around 80-90% of *C. verum* in the world market, and also cultivated in other origins like Seychelles and Madagascar [67].

3.1.2 Cassia cinnamon (*C. cassia*)

Cassia cinnamon is made from the tree bark of *Cinnamomum cassia*. *C. cassia* is the most widely cultivated *Cinnamomum* in southern China and south-east Asia. *Chinese cassia*, *Indonesian cassia*, and *Saigon or Vietnam cassia* are collectively known as cassia cinnamon, and also marketed as cinnamon on sale [64].

Compared to *C. verum*, *C. cassia* is native to many countries and regions which have already occupied the most commercial supply of cinnamon in the world. Because of the huge export and lower price, Indonesia and China produced 75% of the world's supply of cinnamon in 2016 [68]. For commercial cinnamon products, *C. cassia* has replaced *C. verum* as the species most widely used [69].

3.1.3 Difference between two cinnamon species

Even though *C. verum* and *C. cassia* are both sold as cinnamon in the market, but there are still different features between these two species. Primary, the shape and color

of these two species are different. Fig I - 1 shows that *C. cassia* is generally medium to light reddish-brown in color, hard and woody in texture, and thicker (2–3 mm (0.079–0.118 in) thick), as all of the layers of bark are used. *C. verum*, using only the thin inner bark, has a lighter brown color, a finer, less dense and more crumbly texture; it is considered to be subtle and more aromatic in flavor than *C. cassia* that can release much of its flavor during cooking. *C. cassia* is usually sold as broken pieces of thick bark, as the bark is not supple enough to be rolled into quills, whereas *C. verum* is usually sold as a cigar shape as rolled inner bark [66, 69, 70].

The taste and flavor of two species also have differences, *C. cassia* has a strong and exciting aroma flavor because of the higher content of cinnamon oil, and also made a more spicy taste for people. Considering *C. verum*, because of mild subtle taste with hints sweetness as well as it is easy to crash, it is usually used in beverages, such as coffee, tea [71].



Fig I - 1. Two species of cinnamon, *C. verum* (left) and *C. cassia* (right).

The toxicity of cinnamon is also concerned in these years, in 2018, Hajimonfarednejad *et al* reported a systematic review of adverse events caused by cinnamon [72]. Coumarin as a secondary metabolite is dominantly discussed, research has confirmed that coumarin is toxic to liver and kidney for overtaking and prolonged use. The attention that *C. cassia* contains a significantly higher concentration of coumarin. However, *C. verum* only contains around 0.004% of coumarin (trace), which is regarded as generally safe. Due to the different origins of cassia cinnamon, and other factors, such as cultivate methods, climate, etc, there is a wide range of coumarin concentration in *C. cassia*, usually, *C. cassia* contains over 1.0mg/g of coumarin and

sometimes up to 12 times more[73]. For example, the coumarin content can reach 6.97 mg/g in the authentic Saigon cinnamon sample (*C. loureiroi*) [69].

Wang *et al* screened 4 authentic cinnamon species and 40 commercial sourced cinnamons by UPLC-MS in the American market. Cinnamaldehyde is the dominant component in all species; the content of cinnamaldehyde in *C. cassia* is significantly higher than *C. verum*, reaches to 55.8 mg/g to 16.8 mg/g for authentic cinnamon. The same result was also required in another research, Farag *et al* analyzed 10 cinnamon species found that *C. cassia* has a relatively higher content of essential oil, and which contributes to the more strong flavor and spicy taste [9]. Quantitative analysis of coumarin showed that *C. cassia* (Vietnam cinnamon) had coumarin content can reach to 9.3 mg/g (around 0.54 g/day for 50 Kg body weight) [69]. In contrast, *C. verum* has only trace amounts of coumarin, whereas cinnamon from Vietnam (*C. loureiroi*) and Indonesia (*C. burmannii*) contained substantial amounts.

Other bioactive compounds like cinnamaldehyde and styrene in the cinnamon bark, powder or essential oil can also be toxic for health if taken at a high dose [74].

3.2 Functional activities of cinnamon

Cinnamon as a worldwide spice and traditional herbal medicine has been used for thousands of years. Depending on plenty of researches in vitro and in vivo, cinnamon extracts or its components exhibit a wide variety of functional activities, such as anti-diabetes, anti-oxidant, anti-inflammatory, anti-microbial, anticancer, lipid level lowering effects. Moreover, cinnamon was reported to have ability against neurological disorders, like Parkinson's and Alzheimer's diseases, and also treating cardiovascular diseases. The bio-activities of cinnamon extracts or compounds isolated were summarized in Table I - 1 below.

Table I - 1. Functional activities of cinnamon species.

Functional activities	Cinnamon species	Material/compounds	References
anti-diabetes	<i>C. verum</i> <i>C. cassia</i>	extracts, cinnamon oil, water extracts, flavonol and other polyphenols compounds, procyanidin oligomers, polyphenol type-A polymers	[57, 75-79]

anti-oxidant	<i>C. verum</i> <i>C. cassia</i>	ether, methanol and water extracts, essential oil, polysaccharides, phenolic constituents, Lignan	[59, 65, 80-84] [71, 85-87]
anti-inflammatory	<i>C. cassia</i> <i>C. verum</i>	water extract, essential oil, ethanol extract, cinnamaldehyde	[60, 88-92]
anti-microbial	unspecified <i>C. cassia</i> <i>C. verum</i>	essential oils, cinnamaldehyde,	[61, 93-95]
anti-cancer	<i>C. cassia</i>	water extract (cinnamaldehyde mainly), cinnamaldehyde, cinnamic acid	[63, 96, 97]
lipid level lowering effects	<i>C. verum</i> <i>C. cassia</i>	ethanol extracts, aqueous extract,	[76]
against neurological disorders	<i>C. verum</i> <i>C. cassia</i> unspecised	cinnamon capsule, aqueous extracts,	[98, 99]
treat cardiovascular diseases	<i>C. cassia</i>	2-methoxycinnamaldehyde,	[100]

3.3 Chemical compositions of cinnamon

3.3.1 Main essential oil components

The knowledge on cinnamon powder composition is well established for essential oil, cinnamon primarily contains essential oils which consist of a variety of compounds, such as cinnamaldehyde, eugenol, cinnamic acid, and cinnamate, these resinous compounds contribute to the special spicy aroma flavor and taste [101]. The main compound in cinnamon essential oil is cinnamaldehyde which can reach more than 90% [101], and it is also the main target ingredient for cinnamon in Chinese pharmacopeia [102]. Cinnamaldehyde is also the main active compound in cinnamon, occurring naturally as predominantly the trans (E) isomer, but different districts and species of cinnamon have a significant variety of concentrations. For example, *C. loureiroi* (Vietnamese cinnamon) had a relatively higher concentration of cinnamaldehyde which varied from 12.5 to 76.1 mg/g, whereas *C. verum* determined from 3.1-22 mg/g [69]. Cinnamic acid is also one of the major effective components, the concentration of cinnamic acid can reach to 47.60 mg/g in the powder of *C. loureiroi* [103]. The content

of cinnamic acid can be increased with prolonging storage time because of the oxidation of cinnamaldehyde [104]. Eugenol has a pleasant and cloves like scent, that is marked as a symbol of *C. verum* and only detected in *C. verum*, which contributes to a special gentle aromatic and sweet taste different to *C. cassia* [9]. Fig I - 2 shows the chemical structures of major compounds in cinnamon oil [27, 95, 105].

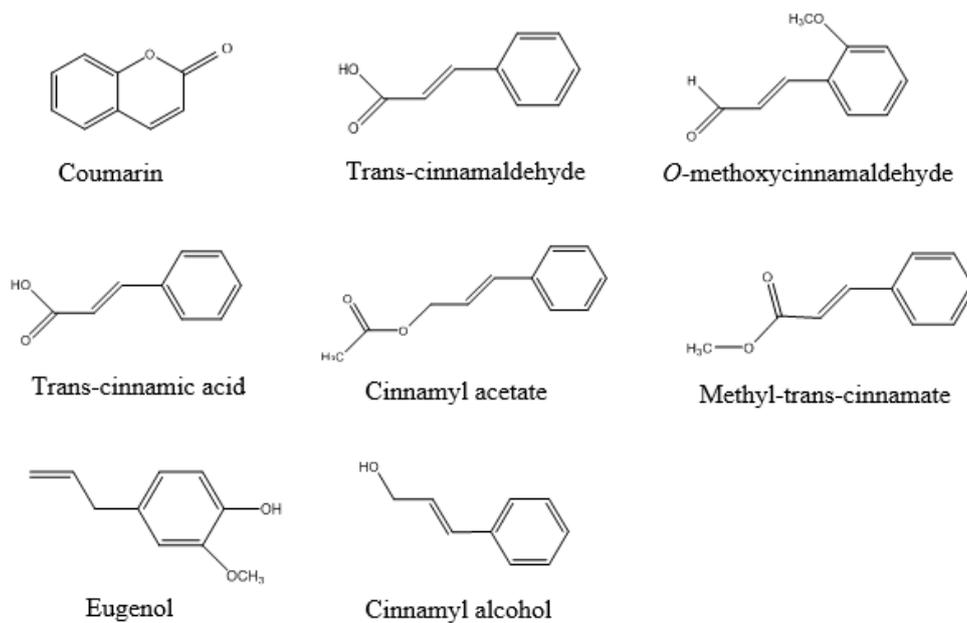


Fig I - 2. Chemical structures of major compounds present in cinnamon.

There are also other constituents isolated from cinnamon essential oil, like terpenoids, aromatic alcohol, phenols, and other compounds, the classification of functional groups and components isolated from essential oil are listed in Table I - 2.

Table I - 2. Classification of functional groups and components isolated from Cinnamon Essential Oil.

Aldehydes	Cinnamaldehyde	Terpenoids	α -Terpineol
	Methoxy-cinnamaldehyde		Phellandrene
	Hydroxy-cinnamaldehyde		Borneol
	Benzaldehyde		Decanal
	Acetaldehyde		Linalool
	Phenyl-acetaldehyde		Caryophyllene
	Salicylaldehyde		α -humulene
	Cumminic aldehyde		α -cadinol
	4-Anisaldehyde		Terpinolene
	Isovaleric aldehyde		γ -Cadinene

Phenols	Cinnamyl acetate		Copaene
	Phenylpropyl acetate		α -Pinene
	Cinnamic aldehyde	Alcohols	2-Phenylethyl alcohol
	Eugenol		Cinnamic alcohol
	Methyl eugenol		Cinnamyl alcohol
	Isoeugenol		Benzyl alcohol
	2-Vinylphenol		Cuminic alcohol
	Eugenol methyl ether	Esters	Benzyl benzoate
Paeonol	Phenylethyl benzoate		
Acids	Cinnamic acid		Methyl cinnamate
	Methoxycinnamic acid		Ethyl 2-methoxycinnamate
	Benzoic acid		Benzyl cinnamate
	Caproic acid	Other	Sterols
	β -Hydroxybutyric acid		
	Isovaleric acid		
	Lauric acid		
	Myristic acid		
	Propionic acid		
	Salicylic acid		
Tannic acid			

3.3.2 Other cinnamon chemicals

Cinnamon contains several flavonoid and flavanol compounds, such as gossypin, gnaphalin, hesperidin, hibifolin, hypolaetin, oroxindin, quercetin, epicatechin, catechin, and procyanidin B2. These compounds have been successfully isolated from cinnamon species and proved to be crucial in anti-inflammatory activities and antioxidant activities of cinnamon [106]. The presence of a wide range of other compounds, like lignans [87], furocoumarins [69], phenolic acid [84], polysaccharide [83], trace inorganic elements [107] were also reported.

3.4 Analytical approaches for analysis and authentication of raw cinnamon

Concerning the high content of coumarin in *C. cassia*, the tolerable daily intake (TDI) of coumarin is 0.1 mg of per Kg body-weight in Europe [108]. But consumers couldn't visually distinguish the cinnamon species in cinnamon powder products or food, and *C. cassia* is more often used as the ingredient for the cheaper price and wide source. Therefore, the determination of coumarin content and cinnamon species is quite important for quality control of cinnamon products.

3.4.1 Chromatography methods

Chromatography method (HPLC or GC) coupled with or without mass spectrometry as a routine analytical method, is mostly applied for the analysis of components in cinnamon products. Ballin *et al* analyzed 74 cinnamon food samples in Danish market by UPLC-PDA method, the result indicated that nearly 50% of samples collected had coumarin content exceeded the EU limit, including one sample even reached to 3 times higher than the limit. The author suggested that this exceedance maybe because the manufactures were lack of information about the regulatory limits about coumarin in food and how to comply with the regulation [73]. Wang *et al* used UPLC-UV/MS analyzed cinnamon and other flavouring compounds in authenticated cinnamon bark samples and locally bought cinnamon samples, cinnamon-flavored foods as well as cinnamon dietary supplements. The result showed that *C. verum* bark sample contained only a trace of coumarin while *C. cassia* contained substantial amounts, *e.g.* Vietnamese cinnamon (*C. loureiroi*) can reach the content of coumarin at 6.97 g/Kg in the authentic bark sample, and Indonesian cinnamon (*C. burmannii*) can reach to 9.30 g/Kg in commercial source bark sample. Coumarin was detected in all locally bought products, their chemical profiles indicated that the cinnamon samples and the cinnamon in food supplements and flavored foods were probably *C. burmannii* [69].

Li *et al* screened the volatile compounds of nine cinnamon barks from three species in different Chinese habitats. GC-MS results indicated that trans-cinnamaldehyde was the main compound in the volatile oil of all samples (66.3-82.0%). Vietnamese cinnamon (*C. loureiroi*) contained the highest concentration of volatile oil (3.1%), and which consists of a higher percentage of trans-cinnamaldehyde at 82.0%. Moreover, the volatile profiles combined with chemometric analysis proved an effective way to identify the species and geographical distribution of these cinnamon bark samples [27]. Geng *et al* extracted essential oil from *C. cassia* bark with different tree growth age and position (*i.e.* 1–3 years old for the branch bark; 5–12 years old for the stem bark). These oil samples were screened through GC-MS, the results showed that the branch bark fraction had a higher volatile oil yield (2.70-3.11%, w/w) than stem bark (0.41-2.61%, w/w), the percentage various because of the age and segment (top, center and lower) of the tree. Forty-one volatile compounds were identified in essential

oils, the percentages of trans-cinnamaldehyde composition didn't present high fluctuations in both different growth stages and segments, whereas other compounds changed significantly. These results can be a good reference to improve the extraction efficiency for industrial use [109].

3.4.2 NMR approaches

Few studies reported the application of NMR for screening complex cinnamon samples. Killday *et al* extracted cinnamon samples by CDCl₃, extracts of cinnamon barks and dietary supplements were then recorded on a Bruker 600 MHz spectrometer. Flavouring compounds like cinnamaldehyde, cinnamyl alcohol, cinnamic acid, eugenol, cinnamyl acetate and coumarin were identified and further quantified. The result showed that *C. cassia* samples contained significantly higher amounts of coumarin than *C. verum* (nearly trace), with a considerable concentration range from 0.5-3.1 mg/g. Cinnamaldehyde as the major flavor compound was highly variable in these cinnamon samples with the concentration from 0.8 to 23.9 mg/g [110].

The combination of ¹H NMR and chemometric analysis was performed to distinguish two major species of cinnamon (*C. verum* & *C. cassia*) used for food products [9]. Ten barks samples from different geographical origins and one cassia oil were employed for ¹H NMR metabolomics analysis, nine key secondary metabolites were identified and quantified, with cinnamaldehyde as the main component. Multivariate PCA of NMR data revealed that specimens belonging to each species can be slightly differentiated through PC2 axes in score plots. The metabolite loading plot of PCA which revealed the most significant clues for scattering behavior, exposed that ¹H NMR signals of eugenol, cinnamaldehyde and fatty acids were accounted as the most relative components for samples segregation. OPLS-DA was further applied to identify metabolic patterns that are correlated with each species. The OPLS-DA score plot showed clear discrimination between two different cinnamon species. The loading S-plot correlated with ¹H NMR signals indicated that eugenol was a marker of *C. verum* for -OCH₃ signal at 3.84 ppm and typical aromatic signal at 7.68 ppm, which was mostly found in *C. verum* samples. Otherwise, fatty acids were detected abundance in *C. cassia*, signals at 1.28 and 1.32 ppm of w-2 and w-1 fatty acids were the most significant marker in the loading plot. This research successfully explored the NMR metabolites fingerprinting of two major cinnamon resources, and provided a novel

insight for species authentication of spicy or herbal products through ^1H NMR with chemometric tools. But there are also limitations about this research, only 9 bark samples are involved, the multivariate analysis results would be affected with a small sample size; moreover, no NMR quantitative analysis nor a statistical prediction of cinnamon metabolites processed concerning the bark samples.

4. Herbal slimming supplements and adulteration

Obesity has become a global problem associated with many negative effects on health, such as cardiovascular diseases, type II diabetes, certain types of cancer, osteoarthritis, and depression [111]. Depending on the report of WHO, more than 1.9 billion adults were overweight and 650 million were obese, with the nearly tripled number since 1975 [112].

Excepting illnesses like endocrine disorders, medications or mental disorders and genetic defects, lack of physical activity and excessive food intake are commonly considered as the main cause of obesity. Changes to diet and exercising are the main approach to lose weight; diet quality can be improved by increasing dietary fiber other than high fat and sugars food [112]. To reduce appetite and fat absorption, medicines can also be used with a suitable diet [113]. Currently, few drugs are approved by countries for the treatment of obesity. Orlistat, liraglutide, naltrexone/bupropion have evidence for long term use about losing weight in both Europe and USA, as well as lorcaserin and phentermine-topiramate can be used for long term treatment only in USA (3 months), a data showed that the weight loss range from 3.0 to 6.7 kg after one year of placebo [114]. Moreover, phentermine and diethylpropion are also allowed for short duration treatment. In Europe, lorcaserin and phentermine-topiramate were not approved by European regulatory authorities, because lorcaserin is associated with heart valve problems and phentermine-topiramate for more general heart and blood vessel problems [115]. Orlistat was also reported for the high rates of gastrointestinal side effects and concerns have been raised about negative effects on the liver [116, 117]. Even though these medicines can be used for obesity treatment under medical supervision, there is still no exact information on how these drugs affect longer-term complications of obesity like cardiovascular disease or death [113].

Because of the serious adverse effects of these medicines above and also strict regulations (only orlistat is available as OTC drug at low dosage) [118], herbal slimming dietary supplements (DS) are emerging and becoming popular on the market. Herbal slimming products are usually claimed as 100% natural products, easy weight loss and usually considered as totally safe for health, as they are freely purchased through numerous online/offline shops [119]. Slimming or weight loss supplements are forms of capsules, tablets, liquid, powders sold in pharmacies, health food stores and the Internet, that contain many ingredients like herbs, fiber, and minerals in a variety amounts or combinations. The ads of products are usually claimed that the herbal ingredients can help people lose weight by blocking the absorption of fat and carbohydrates, curbing your appetite, or speeding up your metabolism. Hence, the overweight group is tending to buy herbal slimming products to self-treat or prevent obesity [120].

However, there is little scientific evidence to support the ability slimming DS in weight loss work, and also three main areas of concern are provided: (1) Misleading claims are proposed to improve the selling, (2) absention of labeled ingredients, (3) many products may be adulterated with synthetic active pharmaceutical ingredients (API) to improve efficiency, especially for products sold on the Internet. While the first two areas only cause economic loss for consumers, whereas the use of API without supervision could cause serious health problems [19].

4.1 Illegal adulterations in herbal slimming supplements

Generally, consumers are led to believe that herbal slimming DS are totally safe compared to synthetic drugs. Actually, the anti-obesity activity of real herbals products is lower than synthetic anorexic compounds. Therefore, with the growing dietary supplement market, illegal manufactures frequently added API in slimming agents to enhance efficiency and reduce the treatment period [120]. According to literature, there are more than 110 illegal drugs detected in slimming DS in 2017 [15]. Currently, the leading type of illegal adulterants in slimming DS are anorexic (sibutramine and its analogues, rimonabant, fenfluramine, amfepramone, benzocaine, amphetamine, et al), anxiolytic (diazepam, bromazepam, flurazepam, midazolam, and chlordiazepoxide), antidepressant (fluoxetine, bupropion, paroxetine, citalopram, nefazodone), laxative and diuretic (phenolphthalein, spironolactone, furosemide, althiazide, &

hydrochlorothiazide) chemicals [15-17].

Even though products containing these compounds have significant high potent for anti-obesity, but the rebound effect is also very obvious after quitting intake. More importantly, many reports revealed the side effects and clinical problems from consumers who took adulterated slimming DS, like cardiovascular disease, liver and renal failure, strokes, mental/mood changes (*i.e.* excitement, restlessness, confusion, depression and occasionally even thoughts of suicide). The development of the most frequent synthetic drugs and their adverse effects are briefly introduced below [15-18].

4.2 Sibutramine and its analogues

Sibutramine is a typical anorexic compound derived from amphetamines, the principle is that sibutramine acts as a serotonin-noradrenalin reuptake inhibitor can stably promote weight loss in the obese group [20]. The main pharmacological effects of sibutramine are inducing satiety with reducing appetite and also thermogenesis. Thus, sibutramine was widely used for obesity treatment since 1997 and proved by US-FDA. However, besides the weight loss ability, sibutramine was reported for the side effects that it over-stimulates the central nervous system, and causes headache, xerostomia, numbness, nervousness, paraesthesia and increased cardiovascular events such as stroke and heart attack in patients (US-FDA, Schedule IV) [20, 119]. A case in China reported that users related symptoms of headache, vertigo, and numbness after taking the herbal slimming capsules adulterated with sibutramine, at the same time, sibutramine components was confirmed in patients' urine and also the capsules [121]. Research also proved that sibutramine can substantially increase blood pressures and heart rates in patients after long-term intake of weight-reducing drugs [23]. Due to the adverse effects, sibutramine was withdrawn from China, Europe, and America for obesity treatment since 2010 [17, 18].

However, illegal manufactures intend to modify the structure of sibutramine to evade the enforce control. Recently, many publications reported the adulteration of sibutramine and its analogues like desmethylsibutramine, didesmethylsibutramine, benzylsibutr-amine, chlorosibutramine, and homosibutramine in slimming DS [24-26, 122, 123].

4.3 Phenolphthalein

Phenolphthalein as a traditional laxative drug has been used for over a century [124]. Since the early 1990s, phenolphthalein has been detected as a laxative component in slimming food and herbal products [16]. However, the abuse of phenolphthalein can cause electrolyte imbalance, severe constipation, lethal cardiac arrhythmia, and most serious, it can induce cancer [16, 124, 125]. Because of these adverse effects, phenolphthalein was forbidden in slimming products in the late 1990s, and currently is being removed from OTC laxatives [126]. In Europe, phenolphthalein has been added to the European Chemicals Agency's candidate list for Substances of Very High Concern (SVHC) [127].

Even though the application of phenolphthalein is controlled in the slimming products, but it is usually detected as an adulterant in slimming DS [15-18, 120]. An interesting phenomenon is that phenolphthalein as the only laxative substance identified in these formulations, which is usually detected associated with sibutramine [17]. Hachem *et al* screened 160 herbal food supplements on the market, phenolphthalein was detected in 36 samples, the concentration ranged from 0.05-51 mg per formulation unit, and 25 samples were identified with both phenolphthalein and sibutramine adulterated [120]. The reason is that phenolphthalein can stimulate the colon and increase bowel movements; furthermore, sibutramine as an anorexic reagent can reduce appetite and lowering food consumption. Thus, the combination of two adulterants could induce a significant effect leading to weight loss in the short term. However, consumers undertake a huge risk because of the side effects for health and weight would rebound quickly after quitting ingesting.

4.4 Other synthetic slimming adulterants

There are also many other synthetic compounds detected in slimming DS without the label, like fluoxetine [21, 120, 128], orlistat [1, 120], caffeine [16, 18, 19], fenfluramine [16, 17, 29], sildenafil [17, 29, 120], lorcaserin [8, 19, 120], etc. These reports also reveal the poor quality control in herbal products manufacturing practices.

4.5 Analytical approaches for screening adulterants in herbal slimming supplements.

According to the abuse of synthetic drugs in a considerable proportion of slimming DS, such as products named “super slim”, “loss weight fast”, “extra slim”, and so on, various analytical methods are applied to detect adulterants. Conventional chromatography methods like HPLC, GC and hyphenated-mass spectrometric are widely used for separation and detection of target adulterants in slimming DS. Furthermore, the relevance of NMR as a spectroscopic method without complex sample preparation and providing structural information has been demonstrated in quality control of slimming DS, especially for the identification of novel analogues of synthetic drugs.

4.5.1 Chromatography hyphenated-mass spectrometry techniques

Recently, Shi *et al* screened seventeen slimming DS by using liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS), 15 key chemicals were detected, including the strictly restricted adulterants (sibutramine, fenfluramine, phenolphthalein, caffeine, etc) [16]. Wang *et al* applied HPLC-ESI-MS to screen adulterants in herbal weight-reducing dietary supplements, six synthetic adulterants, including sibutramine, N-di-desmethyl sibutramine, N-mono-desmethyl sibutramine, fenfluramine, phenolphthalein, and orlistat were successfully detected and the limits of detection for the six synthetic adulterants ranged from 0.0018 to 0.73 mg/g [1]. Zou *et al* identified sibutramine and two metabolites plus one analogue in slimming DS through liquid chromatography triple quadrupole mass spectrometry (HPLC-TQ-MS) and time-of-flight mass spectrometry (HPLC-TOF-MS) [26]. Shekari *et al* analyzed toxic or carcinogen adulterants in herbal slimming pills through gas chromatography-mass spectrometry (GC-MS) fingerprinting assisted with chemometric methods. Undeclared ingredients, such as phenolphthalein, amfepramone, caffeine and sibutramine were effectively identified in the mixture samples [129]. Khazan *et al* analyzed eight slimming DS from Iran and five synthetic drugs were identified. Sibutramine, phenolphthalein and phenytoin were investigated and quantified by GC-EI-MS; bumetanide and rimonabant by HPLC-ESI-MS [18].

4.5.2 ^1H NMR approach

HF NMR especially ^1H NMR is routinely used for detection, structural characterization and quantification of adulterants in slimming DS. Hachem *et al* detected six active pharmaceutical ingredients among 164 slimming DS by ^1H NMR (one DS contains 5 different batches in the same packing box), forty-three samples (26%) were adulterated by sibutramine only, 9 with phenolphthalein (6%) and 23 samples (14%) with the mixture of these two adulterants. Results were confirmed by mass spectrometry, the quantitative analysis was performed by ^1H qNMR [120]. Monakhova *et al* screened sixteen slimming DS from online stores by using 400 MHz ^1H NMR, both qualitative and quantitative analyses were explored with peak-area comparison with TSP reference. A wide range of adulterants (unlabelled) was rapidly detected, including sibutramine, caffeine, mesterolone, vinpocetine, oxymetholone, monacolin K, evodiamine, kavain, and dehydroepiandrosterone. Diffusion ordered spectroscopy (DOSY) is a well-established NMR method that reports diffusion coefficients for individual resonances in NMR spectra. DOSY is primarily used to analyze mixtures of small molecules and the oligomeric state of biomolecules depending on the size and shape of the molecules. In this research, DOSY ^1H NMR allows the virtual separation of multi-components in mixture depending on the difference of diffusion coefficients. This method is demonstrated to provide a fingerprint of all components in slimming DS. Vaysse *et al* analyzed twenty slimming DS by DOSY ^1H and quantitative NMR, both active and in-active components were detected, only two samples were strictly herbals and four had correctly declared ingredient. Other samples were all adulterated with sibutramine alone (8 samples, 4.4-30.5 mg/capsule), both sibutramine (5.0-19.6 mg/capsule) and phenolphthalein (4.4-66.1 mg/capsule) in five samples, the last one left was adulterated with synephrine (19.5 mg/capsule) [130].

For detection of novel adulterations or drug analogues, NMR and other analytical approaches like HPLC or GC hyphenated with MS are usually explored as a multi-step method for reliable and quick analysis. Csupor *et al* analyzed sibutramine from slimming DS by color tests, TLC, HPLC-DAD, MS and NMR [22]. Yun *et al* identified a novel sibutramine analogue – chlorosipentramine in slimming DS, target compounds was first isolated by a preparative-LC system, and structural analysis was further carried

out by Fourier transform infrared (FT-IR) and NMR [24]. Kim *et al* detected a suspected sibutramine analogue (chloro-sibutramine) in slimming DS by UPLC-ESI-TOF/MS method, and structural analysis was explored by NMR after isolation by semi-preparative column chromatography [119]. Hachem *et al* first identified lorcaserin, a schedule IV anorectic drug in slimming DS. The existence of lorcaserin was proved by using LC-UV, NMR, MS, and IR, further quantitative analysis was carried out by NMR leading to a content of 6.6 mg/capsule [8].

LF NMR was recently applied for the rapid screening of adulterants in commercial herbal products. Herbal supplements claimed “100% natural” were analyzed by using the first implementation of LF ^1H NMR. Pagès *et al* evaluated the capabilities of a 60 MHz benchtop NMR to identify pharmaceutical adulterants in weight-loss dietary supplements [2]. Adulterants including sibutramine and phenolphthalein in slimming herbal products were readily detected after rapid and simple sample preparation. The authors proved that LF NMR provided valuable clues on the chemical structure of adulterants. 22.5 min acquisition time was sufficient to identify signals of adulterants with a LOD of 0.9 mM. Quantification analysis of internal sildenafil was also employed by LF ^1H qNMR with a LOQ around 2 mM, 45 min recording time acquired similar results compared to HF ^1H qNMR.

4.5.3 Other methods

High-performance thin-layer chromatography (HPTLC) is an alternative, easy handling and low-cost method for the identification of illegal adulterants in slimming DS [20]. Ariburnua *et al* developed HPTLC-densitometry as a new method, the qualification and quantification of adulterated sibutramine were employed at 225nm [20]. Capillary electrophoresis (CE) is a rapid, high resolution and low chemical consumption analytical technique for separation and determination of adulterants in herbal products [17]. Carvalho *et al* detected eight adulterants in slimming DS through capillary electrophoresis with contactless conductivity detection, including (sibutramine, fluoxetine, amfepramone, fenproporex, bupropion, sertraline, paroxetine, and flurazepam) [128]. A study by Deconinck *et al* described the detection of sibutramine in slimming DS by using attenuated total reflectance-infrared (ATR-IR) spectroscopy combined with chemometric techniques, results showed that this method

can be able to screen out all adulterated samples with a minimum of false-positive results [131].

5. Synthetic cannabinoids: Illicit drugs in commercial products

Cannabis (*marijuana*) has been used long times in history for the treatment of headaches, fever reduction, appetite stimulation, and so on [132]. Even though cannabis can be applied for medicine use in some countries and areas, many of its non-medical users use it extensively, thereby abusing drugs [132, 133]. Until now, Cannabis was still classified as a Schedule I controlled drug by the Drug Enforcement Agency (DEA, USA) since 1970, along with other drugs include heroin, LSD, peyote and ecstasy in the Schedule I meaning “a drug with no currently accepted medical use and a high potential for abuse” [134].

Cannabis contains a group of cannabinoids which produce mental and physical effects when consumed, the most notable cannabinoid is the phytocannabinoid tetrahydrocannabinol (THC), as the primary psychoactive compounds in cannabis (*i.e.* responsible for “body high” and “head high” feeling). THC and other cannabinoids can bind with cannabinoid receptors 1 and/or 2 (CB1 and/or CB2) in the central nervous system [135]. These receptors can inhibit neurotransmitter release from presynaptic neurons through retrograde synaptic signaling mechanisms in postsynaptic neuron. Whereas the administration of exogenous cannabinoids such as THC or synthetic cannabinoids disrupt the subtle endocannabinoid signaling process and may result in the common THC tetrad of delusions, hallucinations, paranoia, and sedation [135, 136].

Until now, cannabis and cannabis-related extracts are still not permitted for marketing authorizations by both European Medicines Agency (EMA) and the United States Food and Drug Administration (US-FDA). Besides the strict medical use of cannabis, its abuse of cannabis for recreation can bring many adverse effects, such as the risk of addiction, adverse effects on brain development especially for teenagers, inducing mental illness, etc [137].

As cannabis is controlled or forbidden during countries, synthetic cannabinoids (SCs) have come into sight and appealed attention. SCs are a group of artificial

compounds which are designed to mimic cannabis bind to cannabinoid receptors as THC, and originally used as therapeutic drugs for its anti-convulsant, anti-nociceptive, and anti-psychotic properties [138].

Basing on THC structure modification, SCs can be designed to have more potent than THC to strongly interact cannabinoid receptors in the brain, and they may also bind to other receptors that THC or other natural cannabinoids do not. In the last decade, SCs as new designer drugs were detected in e-cigarettes, herbal blends and even beverages [139]. Until 2015, 177 SCs were reported by UNODC (United Nations Office on Drugs and Crime) early warning system with reports from 58 countries and territories. SCs as a new kind of psychoactive substances are often abused as cannabis substances in marijuana forbidden or restricted regions, many lethal cases caused by SCs abuses were reported in Europe. The Centers for Disease Control and Prevention (CDC) found that the number of deaths for SCs abuse tripled between 2014 and 2015 [6, 139, 140].

5.1 Introduction of SCs;

SCs as a group of THC similar compounds were firstly synthesized for legitimate scientific and medical research purposes since the 1970s [138]. But SCs were never designed to be abused as they are today. On the other hand, SCs are a chemically diverse group of compounds functionally similar to THC to mimic marijuana to induce a marijuana-like high. Thus, SCs also have the potential for recreational use as cannabis.

Unfortunately, clandestine manufacturers began illegally synthesizing some of these compounds and distributing them for illicit use in the past decade. The abuse of SCs started to impact public health around the world. The first public report about SCs was the abuse of JWH-series in the herbal blends for smoking in Germany; as illegal manufacture sprayed SCs to the surface of herbals to improve mental stimulation and sold as “natural herbals” or “spice” [141]. According to the official report, the number of SCs has increased to 257 on the list of UNDOC in September 2018 [140]. SCs have become one of the largest and most diverse of NPS (psychoactive substances) reported by UNODC with over 251 of the 803 total NPS between 2017-2019 [142]. Because of the easy purchase of recreational products, such as ‘Spice’ and other ‘fake pot’ products in retail stores and online shops, 17% of responders declared to use SCs products

according to a global survey [143]. In 2017, Palamar *et al* reported that 10% of US high school students have used SCs products in the past several years, and 3% admitted to used during the past months [144]. Even though SCs have many adverse effects reported, like regarding seizures, acute kidney injury, cardiotoxicity and sudden deaths [6]; US-DEA has also labeled many SCs as “Schedule I” substances [145]. However, it is quite difficult to control the spread of SCs because illegal manufactures already can synthesize novel cannabinoids with modified chemical structures to evade regulation [146].

SCs were designed to have similar structures to natural cannabinoids in the early period. In fact, SCs are more dangerous than cannabis. SCs could be designed to have the ability to more strongly bind with cannabinoid receptors comparing with THC. As a kind of full receptor agonists, illegal SCs can exhibit 4-5 times or even magnitude higher binding affinity to the cannabinoid CB1 receptor, and also SCs usually possess CB2 receptor affinity [147]; moreover, they may interact with other receptors in the brain that natural cannabinoids do not [139]. As the structures of SCs are changing all the time in order to escape the restrict laws, thus makes the screening of SCs even more difficult without pure reference samples. Consumers do not know the content they have taken, the absorption, toxicity, and metabolism of most novel SCs are still unknown in humans because of the rapidly changing structures, especially the consequence for long time use. Most important, SCs are easily obtained from recreation products in the street, like e-liquid for e-cigarette and herbal blends with street names, and also less imitation for young people [139].

5.2 Structure of SCs and their development

For the rapid structural evolution of SCs, structure characterization becomes crucial for the screening of SCs in commercial products.

5.2.1 SCs structural classifications

Scientists have classified several groups from popular SCs according to their structures: classical cannabinoids, non-classical cannabinoids, hybrid cannabinoids, aminoalkylindoles, carboxamideindazoles, eicosanoids, and other unclassified SCs, the structures of different groups are given in Fig I - 3 [141, 148].

Classical cannabinoids usually have nabilone or dronabinol skeleton structure with strong never stimulation activity. However, they are difficult to synthesize, especially for the home productive workshop. As a consequence, this kind of SCs has not been often detected in psychoactive products because of the high production cost [149].

Non-classical cannabinoids are a group of cyclohexylphenols based SCs, like CP-47,497, which is the first designed cannabinoids, and the primary purpose was used in scientific research [150].

Hybrid cannabinoids are a group of SCs combined with the structural features of both classical cannabinoids and non-classical cannabinoids. For example, AM-4030 as a derivative of HU-210, has the dibenzopyran ring and an aliphatic hydroxyl group which is common in classical cannabinoids and nonclassical cannabinoids (CP family) respectively [151].

Aminoalkylindoles are the most common SCs found in illegal herbal blends so far, they contain a wide variety of cannabinoid 3-indole derivatives, and the core structures are not similar to THC, including benzoyl derivatives (*e.g.* RCS-4), naphthoyl derivatives (*e.g.* JWH-018), phenylacetyl derivatives (*e.g.* JWH-250), carboxamide derivatives (*e.g.* ADBICA), ect [141, 152].

Carboxamideindazoles is the most popular group of SCs recently, many recent publications didn't independently regulate this group of newer SCs. Whereas it is rather important that as the rapid evolution of SCs, many carboxamide-indazoles have replaced the old SCs detected in illegal products to evade the control [153, 154]. Carboxamideindazoles have a similar structure to aminoalkylindoles, with an indazole carboxamide structure instead. 5F-ADB is one typical indazole-based synthetic cannabinoid from carboxamide indazole derivatives, which is believed to be extremely potent based on the very low levels detected in tissue samples, and exhibits more significant toxic to humans than previously SCs [155]. Most clandestine manufacturers can directly make small changes in the structure to synthesize this group of SCs, such as changing an indole to indazole structure (AM-2201 to THJ-2201) or terminal fluorine replacement (AKB48 to 5F-AKB48) [156].

Eicosanoids represent a group of endocannabinoid analogs [141]. Endocannabinoids are natural cannabinoids from the animal body, such as anandamide.

Methanandamide as one of the best-known eicosanoids, its psychoactive effect has been proved in different organisms, like mammals, fish, etc [157].

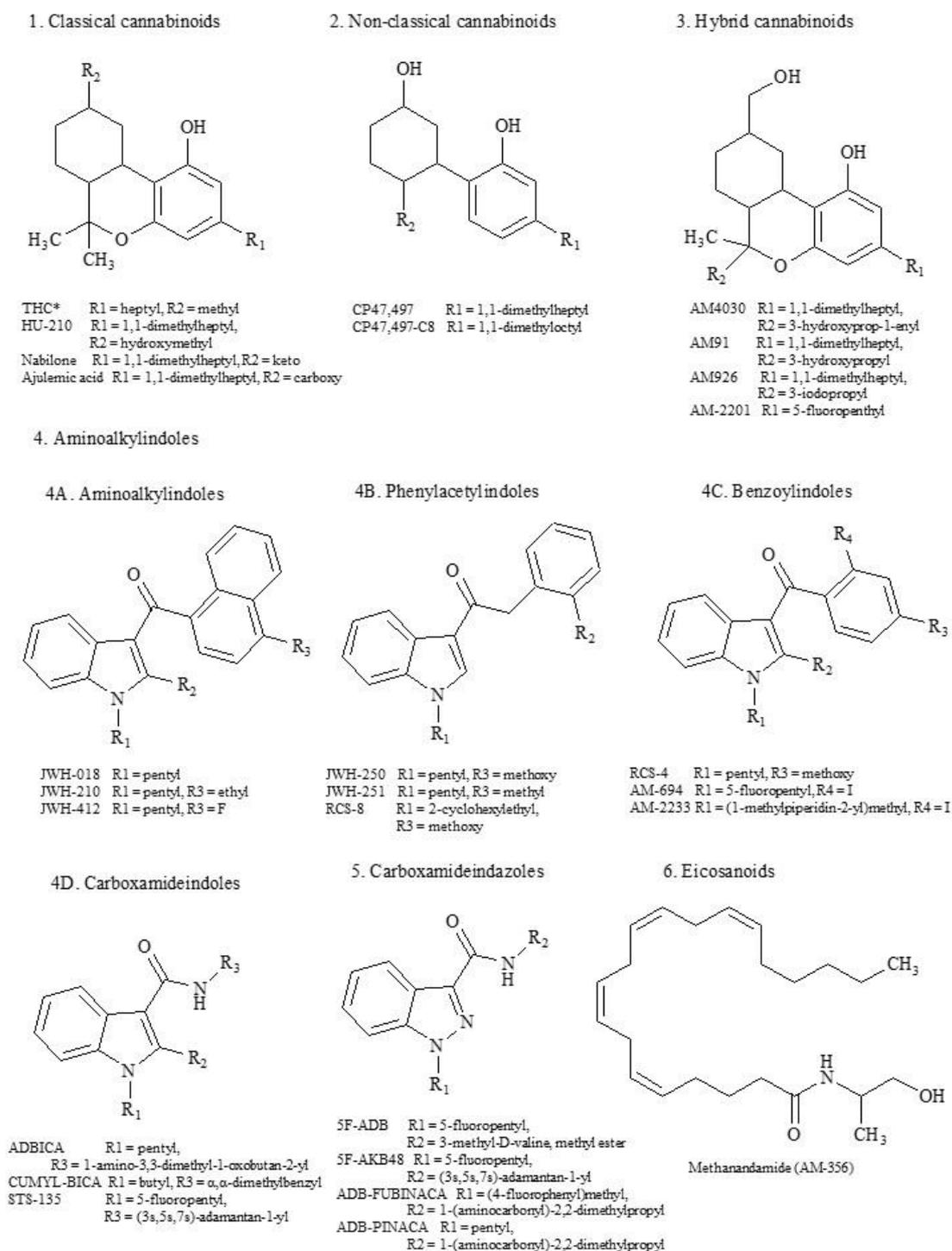


Fig I - 3. Chemical structures of the main SCs

Apart from the SCs group above, there are still many other classified SCs. These SCs are usually detected in recent years, with new structures or slight structural changes. Like naphthylmethylenes, naphthoyl pyrroles, and carboxylate indazole derivatives have been detected as a designer drug in illegal products [158].

5.2.2 Generations of SCs

Since the first identification of a CP-47,497 analog in commercial “spice” products by German researchers in 2008 [159], Shanks *et al* have regulated the identified SCs into 3 generations during the past decade, these generations are highly relative to legislation [160, 161].

At the end of 2010, US-DEA first announced five JWH compounds (JWH-018, JWH-250, JWH-073, as well as CP-47,497 and its C8 homologue) into controlled Schedule I classification, because the cases reported by these compounds, many of SCs contained products were very popular among teenagers and adults under name of “spice” and “K2” [162, 163]. After that, to evade legal supervision, the second generation of SCs have come into public vision. Compounds like JWH-210, JWH-122, and AM-2201 were detected in commercial products and occupied the main illegal market, fifteen SCs were added in Schedule I with 3 times increase than 2010, these SCs were controlled after first ban products and referred as the second generation [163].

After 2012, the third generation of SCs caused serious cases, sixteen kidney injury cases were reported due to XLR-11 and this compound entered Schedule I list in 2013. In 2014, indazole based ADB-PINACA and AB-FUBINACA were detected, and also indazole SCs AB-CHMINACA and ADB-CHMINACA, US-DEA scheduled these compounds in 2015 [164-166]. Another obvious feature of the third generation SCs is that a large part of these compounds contains fluorine nuclei in the structure. The addition of fluorine substituent was purposed to evade legal restrictions imposed on specified SCs and also can enhance psychoactive potency [167, 168]. The evolution of SCs indicates that newer SCs are usually emerged after old SCs been controlled, whereas the number of cases caused by SCs is continually increasing. The typical structures of different generations of SCs are shown in Fig I - 4.

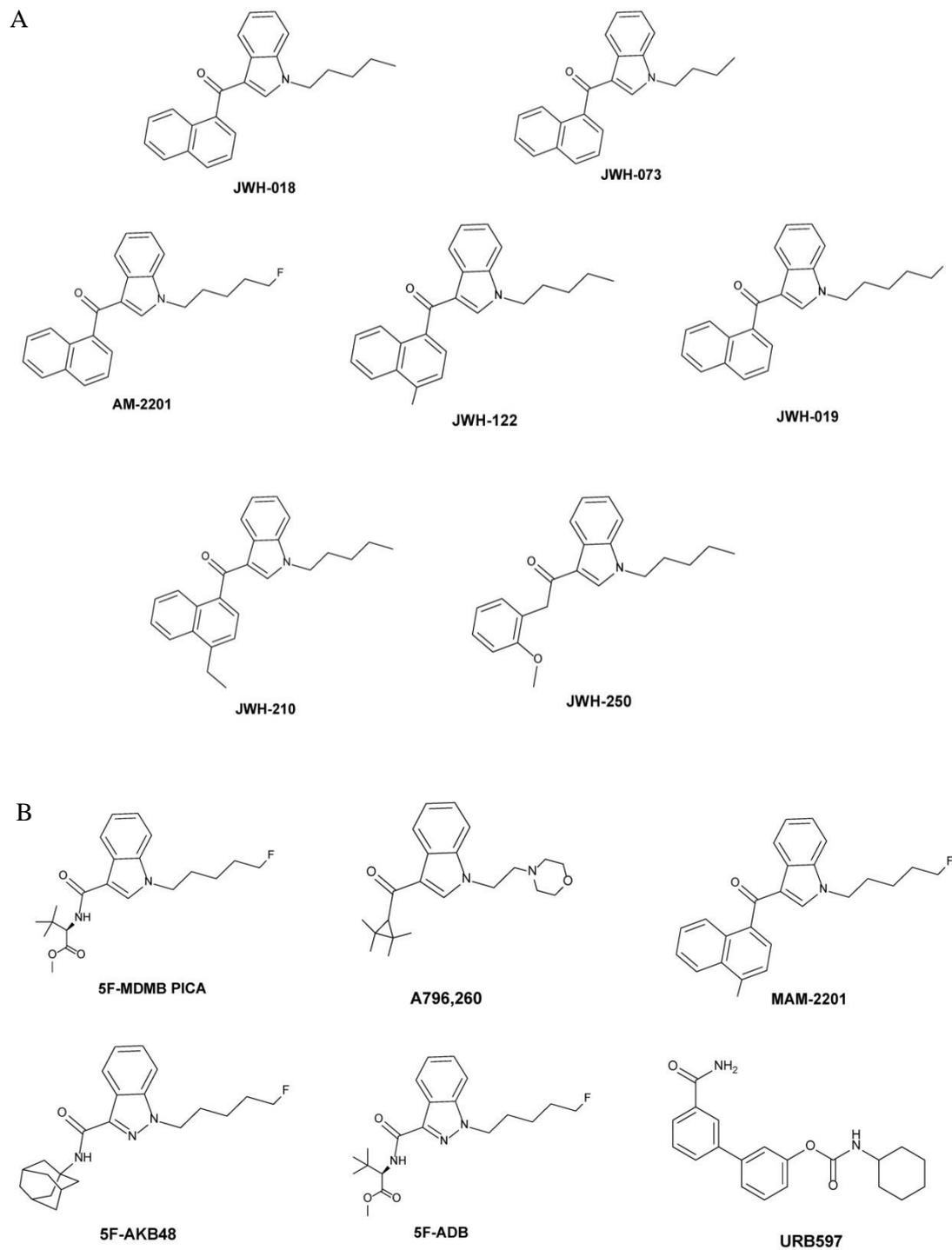


Fig I - 4. Structure of selected SCs: first and second generations(A), third generation (B).

5.3 Formulations of SCs commercial products

Even though SCs are scheduled for recreational use, there are still identified as psychoactive adulterants in commercial products, like smoking herbal blends, electronic-liquid (e-liquid), beverage, cookie and so on; the first two formulations are most popular among young people [139, 144, 146], and typical products are shown in Fig I - 5.



Fig I - 5. Two typical commercials with potential SCs adulterated: left, herbal blends [169]; right, e-liquid formulations [154].

The first application of SCs in herbal blends was reported in 2009, while illegal manufacturers sprayed SCs solution on the surface of herbals to improve the excitement and easily to be addictive [159]. These kinds of herbal blends usually named “K2” or “Spice” have been sold on the Internet and specialized shops since at least 2006, some reports suggested that these illegal products may be already sold around 2004, with similar effects as *cannabis* [170]. Until 2011, 5 of the first-generation SCs were scheduled by US-DEA, but more than 140 different spice products have been identified on sale; these products were easily available on the Internet and legal in many countries, usually marked as natural sources and non-*cannabis* [171]. After that, even though governments have banned laws and regulations to control the abuse of SCs in commercial recreations, newer SCs usually detected in herbal blends have replaced the banned SCs to evade legal control [153, 162].

E-cigarettes were first invented in China as an alternative way for quitting smoking and spread rapidly during recent years. There is no clear evidence that e-cigarettes could help people quit smoking (WHO) but compared with traditional tobacco, they are inexpensive, easy to use and described as a safer role for public health

[3, 172]. Electronic liquids (e-liquids) are formulations used in e-cigarettes which can vapor aerosol for inhalation. E-liquids usually contain a ratio of propylene glycol (PG) and vegetable glycerol (VG), flavors, and in some case pharmaceutical agents and/or herbal remedy inside [3, 4]. SCs are the most rapidly growing class of recreational designer drugs as psychoactive ingredients used in e-liquids [173, 174]. As the prosperity of e-liquids, there are many cases and reports about the abuse of SCs in e-liquids [145]. Because the wide variety of e-liquid products on sale (*i.e.* different kinds of flavor, color, ingredients), these products are easily purchased on retail and online shops, many young people are unknown to buy vaping liquid that selling as a natural cannabis-based “CBD vape”, but which is in fact “spice” [175, 176]. Thus the regulation of e-liquid is even harder than herbal blends.

5.4 Analytical methods for identification SCs in commercials

Because of the rapid structural evolution of SCs and the absence of published data, the analytical detection of SCs in adulterated commercials becomes even difficult for analysts. LC and GC-MS are the most widely used methods for screening of SCs in illegal products. Although their identification and quantitative analysis are limited by the availability of pure standards samples, with the open database like SWGDRUG 3.3 (www.swgdrug.org), verified SCs can be detected even at relatively low concentrations. Besides these routine methods above, other methods like immunoassay technique and NMR are also applied for screening of SCs in biological specimens and novel SCs structural confirmation respectively [140, 152]. The analytical used for identification of SCs in herbal blends and e-liquids are summarized in Table I -3.

Table I - 3. Analytical methods for quality control of commercial herbal blend and e-liquid samples.

Formulation Type	SCs	Analytical method	Ref
Herbal blends	JWH-018; CP 47.497; CP 47.497 analogues	GC-MS, LC-MS/MS, ¹³ C NMR	[159]
Herbal blends	6-APB, AM-2201, JWH-122, JWH-210, JWH-250, etc	UPLC-MS	[160]
Herbal blends	A796,260, MAM-2201, UR-144, URB597 and XLR-11	UPLC-MS	[177]
Herbal blends	QUPIC, QUCHIC, ADB-FUBINACA, ADBICA, etc	GC-MS, LC-MS/MS, ¹ H & ¹³ C NMR	[158]
Herbal blends	AM-2201, JWH-122, JWH-203, JWH-210, and RCS-4	DART-MS	[178]
Herbal blends	5F-AKB48, AB-FUBINACA, BB-22, MAM-2201 and UR-144	GC-MS, ¹ H NMR	[179]
Herbal blends	AM-2201, JWH-122, JWH-203, RCS-04, etc	DART-MS, ¹ H NMR	[180]
Herbal blends	MAM-2201, JWH-210, UR-144, XLR-11, 5F-AKB48, etc	¹ H NMR, LF ¹ H NMR	[30]
Herbal blends	5F-ADB and Cumyl-PeGaClone	GC-MS, EI/MS, NMR	[140]
Herbal blends	5F-ADB, FUB-AMB, 5CI-AKB48, etc	GC-MS and LC-MS	[28]
Herbal blends	AM-694 and 5F-ADB	¹⁹ F & ¹ H NMR	[168]
Herbal blends & E-liquid	AMB-FUBINACA, 5F-AKB48, 5F-CUMYL-PINACA, FUB-AKB48, etc	HPLC-DAD, HRMS, ¹ H NMR and GC-MS	[181]
E-liquid	5F-ADB	DART-MS; GC-MS	[153]
E-liquid	MDMB-FUBINACA	DART-MS, GC-MS	[154]
E-liquid	5F-Cumyl-PINACA	GC-MS, ¹ H & ¹³ C NMR	[182]

5.4.1 Identification of SCs in herbal blends

The identification of SCs in herbal blends and named as “spice” since 2004 in European countries [159], these researchers also investigated the rapid evolution of SCs [7, 28, 140]. GC-MS and LC/UPLC-MS are routinely used for the detection of SCs in herbal blends [28, 160, 177], and additional with NMR analysis to ensure their structures [140, 158, 159]. Other methods like direct analysis in real-time mass spectrometry (DART-MS) as a relatively simple and rapid approach was also used [178, 180].

^1H qNMR was applied to quantify SCs in illegal products [179], Assemat *et al* first used both HF and LF ^1H NMR spectroscopies to identify the existence of SCs, and the ability of benchtop NMR in quality control of mixtures was discussed [30]. ^{19}F NMR as a rapid NMR approach with very low background interference was first applied to screen and further quantify SCs in herbal blends in 2019 [168].

5.4.2 Identification of SCs in e-liquids

As the recent popularity of e-cigarette among the world [183, 184], the content of psychoactive ingredients including SCs inside e-liquid is quite concerned [185-187]. Peace *et al* identified MDMB-FUBINACA in three available commercial e-liquid by using DART-MS and GC-MS, other ingredients like flavor profiles and alcohol were also detected in these samples [154]. Poklis *et al* screened nine CBD labeled e-liquids by using DART-MS and GC-MS, 5F-ADB in four samples and dextromethorphan (DXM) in one product were unintentionally detected [153].

Angerer *et al* identified 5F-Cumyl-PINACA in e-liquid and its metabolites *in vitro* and *in vivo* though HPLC-MS and NMR [182]. Apirakkana *et al* identified 6 SCs in both herbal blends and e-liquids, the results proved the emerging trends of adulteration of SCs in alternative formulations, like e-liquids [181].

6. Conclusion

In this chapter, the quality control applications of benchtop LF NMR are systemically reviewed. Even though LF NMR has a lower sensitivity and the spectra are more likely to be overlapped, useful signals could be nevertheless identified. Moreover, with the application of statistical analysis, the latent information from LF data can be effectively figured out.

In the following experimental chapters, LF NMR is first applied for quality control of cinnamon products, the botanical species among cinnamon samples would be distinguished by the detection of metabolites signals and the statistical analysis of LF NMR data. Second, the ability of LF NMR for adulterants detection is also explored, LF NMR data combined with chemometric analysis can be used to rapidly identify slimming DS adulterated with synthetic substances or drugs. At last, the efficiency of

LF NMR for quality control of e-liquid products is evaluated, LF ^1H and ^{19}F NMR are both applied to characterize SCs adulterated in e-liquid products.

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Chapter II Evaluation of Low-field versus High-field Proton NMR Spectroscopy for Quality Control of Cinnamon Samples

1. Introduction

As we reported in *chapter I (section 3.1)*, there are two main cinnamon species including *C. verum* and *C. cassia* used worldwide. Besides the flavor and appearance differences, *C. cassia* contains a significantly higher content of hepatotoxic coumarin than *C. verum*. Thus, it's a great interest to propose the quality control methods for cinnamon species and specially coumarin content in commercial products.

HPLC, GC or UPLC coupled with MS analysis were usually applied for quality control of cinnamon products, related publications are reviewed in *chapter I (section 3.4)*. Compared to chromatographic methods, NMR was also applied to screen complex cinnamon, with the key components were structurally characterized and further quantified [1, 2].

In this study, we identified the key chemicals of cinnamon products by NMR, and further classified the two major species of cinnamon in both culinary products and dietary supplements *via* both HF and LF NMR. LF NMR was first explored to screen cinnamon products by comparison with HF results, and the LF capabilities and limits were assessed and discussed. At last, the statistical model based on LF NMR data was successfully established to classify commercial cinnamon samples and further predict quantification.

2. Materials and Methods

2.1 Materials

(E)-cinnamaldehyde (Acros Organic, 99%), coumarin (Acros Organic, 99%), cinnamyl alcohol (Acros Organic, 99%), dimethyl maleate (Sigma-Aldrich, 99%), o-methoxy-cinnamaldehyde (Sigma-Aldrich, 98%), (E)-cinnamic acid (Acros Organic, 98%), eugenol (Sigma-Aldrich, 99%), 3-(trimethylsilyl) propionic-2,2,3,3-d₄ acid

sodium salt (TSP, Acros Organic, 99.8% D), tetramethylsilane (TMS, Acros Organic, 99% D), Eu(fod)₃ (Sigma-Aldrich, 99%), EuCl₃ (Sigma-Aldrich, 99.9%), Eu(NO₃)₃·5H₂O (Sigma-Aldrich, 99.9%), dimethyl sulfone (Sigma-Aldrich, 98%) were used as received without any further purification unless otherwise described. All deuterated solvents (99.80% D) were supplied from Eurisotop. An authentic sample of *Cinnamomum verum* was obtained from Extrasynthese (Lyon, France).

The fourteen samples of cinnamon for cooking were bought from French malls or markets, and the fourteen dietary supplements were purchased online or in French specialized shops like organic groceries or dietetic stores. All samples were analyzed before their expiry date.

2.2 Samples Preparation for NMR analysis

2.2.1 Extraction methodology

All the preliminary extraction experiments described in this section were analyzed with the HF NMR spectrometer. In order to choose the best solvent to extract commercial samples, solid-liquid extractions with deuterated methanol (MeOH-d₄), deuterated chloroform (CDCl₃), heavy water (D₂O) and mixtures of solvents MeOH-d₄:D₂O (50%:50%; 80%:20%) were investigated. A liquid-liquid extraction of an aqueous suspension of cinnamon powder with CDCl₃ was also tested.

Briefly, around 100 mg of the powdered commercial cinnamon sample **25** were weighed and mixed with 1 mL of the chosen solvent, shaken for 20 s with a vortexer, sonicated for 10 min in a stoppered tube and centrifuged at 5000 g for 5 min. For the liquid-liquid extraction, 0.5 mL D₂O were added to the powdered sample followed by 1 mL CDCl₃. The sample was then mixed, sonicated and centrifuged as described above. After centrifugation, the CDCl₃ phase was submitted to NMR analysis. In the final step, 700 μL of supernatant were put into a 5 mm NMR tube and 60 μL of TSP were added (10 mM in MeOH-d₄) for NMR analysis. For the samples extracted with CDCl₃, 10 μL of a solution (120 mM) of dimethylsulfone as internal reference were mixed with 500 μL of supernatant. Each extraction condition was tested in duplicate.

Experiments with successive extractions of the powdered samples were also implemented. For the first step, the extraction protocol was the same as previously described, *i.e.* around 100 mg of samples **10** (n=2) and **25** (n=2) were mixed with 1 mL MeOH-d₄, shaken for 20 s with a vortexer, sonicated for 10 min and centrifuged. The supernatant was removed and 700 μ L were mixed with 60 μ L TSP (10 mM, MeOH-d₄) for NMR analysis. The residual powder was dried on a filter paper to absorb the residual liquid and then re-extracted leading to the second extraction sample. This step was repeated once to obtain the third extraction sample. The supernatants from the second and third extractions were then analyzed by mixing 700 μ L of supernatant with 60 μ L TSP (10 mM, MeOH-d₄). The mean results of extraction provided a recovery of $88.2 \pm 0.9\%$ and $86.1 \pm 1.8\%$ for (E)-cinnamaldehyde and coumarin respectively in the first extraction. Residual active compounds were measured at $10.6 \pm 0.8\%$ and $12.4 \pm 1.6\%$ in the second extraction and $1.2 \pm 0.1\%$ and $1.5 \pm 0.2\%$ in the third extraction. The same procedure of three successive extractions was repeated for the two samples **10** and **25** with only 15 mg of powder. The recoveries were $96.8 \pm 0.9\%$ and $97.5 \pm 0.7\%$ for (E)-cinnamaldehyde and coumarin respectively in the first extraction, $3.2 \pm 0.9\%$ and $2.5 \pm 0.7\%$ in the second extraction and nothing in the third one.

2.2.2 Preparation of commercial samples for NMR analysis

1 mL of MeOH-d₄ was added to around 15 or 100 mg exactly weighed of each powdered commercial cinnamon sample placed in a stoppered tube. After 20 s of vortex mixing and 10 min of sonication, the mixture was centrifuged at 5000 g for 5 min. 700 μ L of supernatant were then collected, mixed with 60 μ L of TSP (10 mM) and transferred into a 5 mm NMR tube. The extracts from 100 mg of cinnamon powder were analyzed with both HF and LF NMR and those from 15 mg only with HF NMR for the absolute quantification of (E)-cinnamaldehyde and coumarin. All samples were prepared and analyzed in duplicate for both HF and LF NMR.

2.2.3 Experiments with chemical shift reagents

100 mg of sample **25** were extracted with MeOD-d₄ as described in section 2.2.2. The supernatant (700 μ L) was mixed with 20 μ L of a solution of Eu(fod)₃, EuCl₃, or Eu(NO₃)₃ in MeOH-d₄ at a concentration of 750 mM. TSP (60 μ L, 10 mM) was added

as an internal reference. The final concentrations for europium salts were 20 mM. For the blank sample, 20 μ L of MeOD-d4 were used instead of the europium salt solution.

2.2.4 Standard solutions for statistical analysis

Solutions of standard (E)-cinnamaldehyde and coumarin in MeOH-d4 at different concentrations were prepared as follows. Stock solutions (5 mg/mL) of each standard were diluted to obtain concentrations corresponding to real amounts in commercial samples: 3, 5, 8, 10, 12, 15, 17, 20, 25 and 30 mg/g for (E)-cinnamaldehyde and 2, 4 and 6 mg/g for coumarin. The dilution process is explained as follows: *i.e.* 100mg of powder sample (3 mg/g) are extracted by 1 mL of MeOH-d4, 700 μ L supernatant are taken for test, so the content of (E)-cinnamaldehyde is 0.21 mg in tube. To imitate the supernatant above, for the standard (E)-cinnamaldehyde solution, it's equal to 42 μ L of stock solution mixed with 658 μ L of solvent.

These values take into account a correction factor linked to the extraction yield of real samples (see *section 3.2.1*). A blank MeOH-d4 solution was also analyzed. The solution of TSP as internal reference was added for NMR analysis as previously described.

2.3 NMR analysis

2.3.1 HF NMR analysis

HF ^1H NMR experiments were performed on a Bruker Avance 400 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) equipped with a TXO probe at 298 K. For qualitative experiments, HF ^1H NMR spectra were recorded with the following parameters: pulse width 13.5 μ s (flip angle 90°), acquisition time 2.56 s, spectral width 16 ppm, 32 K data points and relaxation delay 1.0 s. The number of scans was 32 or 128, corresponding to a recording time of *ca.* 2 or 8 min.

Typical acquisition parameters for quantitative experiments were as follows: pulse width 4.53 μ s (flip angle 30°), acquisition time 5.12 s (AQ), spectral width 16 ppm, 64 K data points, and a relaxation delay of 10 s (D1); the number of scans was 256 or 512 giving a recording time of *ca.* 65 or 130 min. The signal of TSP set at 0 ppm was used as an internal reference for chemical shift measurement and quantification.

Before quantitative analyses, the longitudinal relaxation times T1 of protons of (E)-cinnamaldehyde, coumarin, and TSP in standard solutions and the commercial samples **10** and **25** were measured by the inversion-recovery pulse sequence method with recovery delays from 0.001 to 50 s. The T1s found were less than 6 s (*i.e.* H1 (E)-cinnamaldehyde 5.0 s, H4 coumarin 5.8 s), whereas the TSP protons have a relaxation time of 3.7 s. All ¹H resonances were thus considered as fully relaxed since 99% of the signal intensity of the proton with the longest T1 (5.8 s) were recovered with a repetition time of 15.1 s.

Need to pay attention that the use of a 90° pulse angle and a 5×T1 relaxation delay is also a classical way to proceed for quantitative NMR. Whereas the 30° pulse saves recording time because the relaxation delay is lower, the recovery of the signals is explained below:

$$\% \text{ relaxation of the signal intensity} = \frac{1 - e^{-\frac{RT}{T1}}}{1 - (e^{-\frac{RT}{T1}}) \times \cos \theta}$$

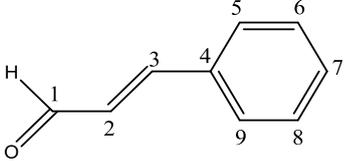
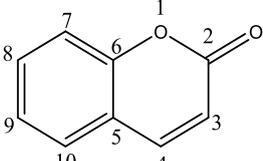
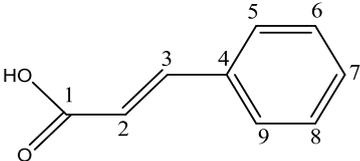
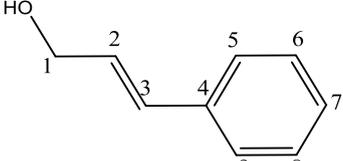
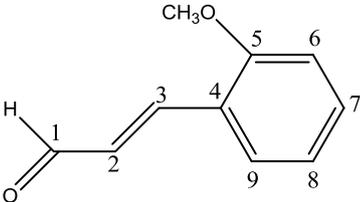
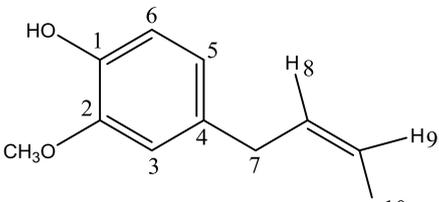
RT (s): acquisition time + relaxation delay in the pulse sequence

T1 (s): longitudinal relaxation time of considered proton

θ (rad): pulse angle (Ernst Angle)

In our case, RT = 15.12 s (AQ = 5.12 s and D1 = 10 s) and T1 = 5.8 s (proton with the longest T1). If $\theta = 90^\circ$, the percentage of relaxation of the proton with the longest T1 is 92.62%; with $\theta = 30^\circ$, it is 98.94%. For the other protons with T1 lower than 5.8s, the relaxation is of course superior to 99%.

Table II - 1. ¹H NMR (400 MHz) characteristics (solvent CD₃OD) of standard (E)-cinnamaldehyde, coumarin, (E)-cinnamic acid, (E)-cinnamyl alcohol, o-methoxy-cinnamaldehyde and eugenol.

(E)-cinnamaldehyde			Coumarin		
					
δ (ppm)	m ^(a) (J) ^(b)	assignment	δ (ppm)	m ^(a) (J) ^(b)	assignment
9.66	d (7.7)	H1	7.96	d (9.5)	H4
7.67	m	H3, H5, H9	7.62	m	H10, H8
7.45	m	H6, H7, H8	7.35	m	H7, H9
6.77	dd (16.0, 7.7)	H2	6.44	d (9.5)	H3
(E)-cinnamic acid			Cinnamyl alcohol		
					
δ (ppm)	m ^(a) (J) ^(b)	assignment	δ (ppm)	m ^(a) (J) ^(b)	assignment
7.65	d (16.0)	H3	7.40	m	H5, H9
7.57	m	H5, H9	7.29	m	H6, H8
7.38	m	H6, H7, H8	7.21	m	H7
6.47	d (16.0)	H2	6.60	dt (15.9, 1.5)	H3
			6.36	dt (15.9, 5.6)	H2
			4.22	dd (5.6, 1.5)	H1
o-methoxy-cinnamaldehyde			Eugenol		
					
δ (ppm)	m ^(a) (J) ^(b)	assignment	δ (ppm)	m ^(a) (J) ^(b)	assignment
9.62	d (7.9)	H1	6.74	d (1.9)	H3
7.93	d (16.0)	H3	6.70	d (8.0)	H6
7.64	dd (7.7, 1.7)	H9	6.60	dd (8.0, 1.9)	H5
7.45	m	H7	5.94	ddt (17.0, 10.1, 6.7)	H8
7.09	d (8.4)	H6	5.04	dm (17.0)	H10
7.01	bt (7.6)	H8	5.00	dm (10.1)	H9
6.83	dd (16.0, 7.9)	H2	3.82	s	-OCH ₃
3.93	s	-OCH ₃	3.28	dt (6.7, 1.4)	H7

(a) m, multiplicity; d, doublet; t, triplet; dd, doublet of doublet; dt, doublet of triplet; ddt, doublet of doublet of triplet; bt, broad triplet; m, multiplet; dm, doublet of multiplet.

(b) J: coupling constant (Hz).

NMR assignments of (E)-cinnamaldehyde, coumarin, (E)-cinnamic acid, o-methoxy-cinnamaldehyde, (E)-cinnamyl alcohol and eugenol were achieved by comparison with spectra of standard compounds and with 2D experiments (gCOSY, gHSQC, gHMBC and ^{13}C) to clear up ambiguities. Data are given in Table II -1.

1D ^1H NMR data were processed using the TOPSPIN 3.1 software with one level of zero-filling and Fourier transformation after multiplying FIDs by an exponential line-broadening function of 0.3 Hz, then phasing and baseline correction were applied. T1 values were calculated with the T1/T2 relaxation module included in the TOPSPIN software.

2.3.2 LF NMR analysis

Spectra were acquired on a PulsarTM benchtop NMR spectrometer (Oxford Instruments, Abingdon, UK) operating at a frequency of 59.7 MHz for ^1H . The temperature inside the spectrometer was 310 K. The acquisition was performed with the SpinFlow 1.2.0.1 software (Oxford Instruments) and the processing with MNova 11.0 (Mestrelab Research, Santiago de Compostela, Spain). FIDs were recorded with a flip angle of 90° (12.0 μs), a spectral width of 5000 Hz, and 8K complex points (acquisition time of 1.64 s). The relaxation delay was set at 2 s, and 2000 transients were recorded leading to a total acquisition time of 2 h. For data processing, the FIDs with one level of zero-filling were apodized with an exponential (0.3 Hz) filter and automatic Whittaker smoother baseline correction was applied.

The T1s of standard (E)-cinnamaldehyde (H1), coumarin (left part of the H4 doublet) and TSP were measured (n=4) in MeOH-d4 by the inversion recovery pulse sequence method with 20 recovery delays from 0.001 to 40 s. Values were 3.8 ± 0.1 s, 4.3 ± 0.2 s and 3.1 ± 0.1 s, respectively. T1 measurements were also performed on the commercial samples **10** and **25** in duplicate. Values obtained were 3.5 ± 0.2 s, 3.9 ± 0.4 s and 3.1 ± 0.1 s for T1 of (E)-cinnamaldehyde (H1), coumarin (H4) and TSP, respectively. From these T1 measurements, it was possible to calculate the recovery of the signals compared to TSP in our recording conditions. It was 90% for (E)-cinnamaldehyde and 82% for coumarin with RSD <2% for standard samples but reaching 5% for commercial samples for which variability is greater.

At last, the LOD and the apparent LOQ were determined by analyzing the diluted standard solutions of known concentration for signal-to-noise ratios (S/R) of 3 and 10, respectively. We mention here an "apparent" LOQ insofar as we will show later that the experimental conditions used were not fully quantitative. The SNR was estimated using the SNR peak calculator tool included in the MNova software. The LOD of the left signal of H4 of coumarin was 0.096 mg/mL and the apparent LOQ was 0.29 mg/mL for the same signal. For the H1 of (E)-cinnamaldehyde, the LOD and apparent LOQ values were 0.048 mg/mL and 0.19 mg/mL respectively. The limits of apparent quantification thus corresponded to 1.9 mg/g and 2.9 mg/g for (E)-cinnamaldehyde and coumarin, respectively, in real samples.

2.4 HF NMR quantification

Concentrations were measured by comparing the signal areas of targeted protons [*i.e.* H1 (δ 9.66 ppm, doublet (d)) of (E)-cinnamaldehyde, H4 (δ 7.96 ppm, d) of coumarin, H2 (δ 6.47 ppm, d) of (E)-cinnamic acid, right part of the H1 d at 9.62 ppm of o-methoxy-cinnamaldehyde, H2 (δ 6.36 ppm, doublet triplet) of (E)-cinnamyl alcohol, and -O-CH₃ (δ 3.82 ppm, singlet) of eugenol (Table II - 1)] to the area of the reference signal, the area of each NMR peak being directly proportional to the number of protons giving rise to it.

The amount of each compound expressed in mg/g of sample was determined using the following general equation:

$$\text{Amount (mg/g)} = [\text{REF}] \times \frac{A_x}{A_{\text{REF}}} \times \frac{N_{\text{REF}}}{N_x} \times V \times \frac{M_{W_x}}{W_{\text{sample}}} \quad (1)$$

[REF] (mM) is the concentration of the internal reference (TSP),

A_x and A_{REF} the integrated signal areas of the targeted compound and TSP, respectively,

N_x and N_{REF} the number of protons giving rise to the signal considered,

V (mL) the volume of solution,

M_{W_x} (g/mol) the molecular weight of each compound,

W_{sample} (mg) the mass of cinnamon sample used for the NMR assay.

2.5 Chemometric analysis of LF spectra

After Fourier transform and phasing of the LF spectra (commercial samples and standard compounds) as described above, a manual multipoint baseline correction including the free selection of control points was performed with the algorithm smooth segments and a RMS (Root Mean Square) calculation span of 300.

Next, a total of 19 ± 2 control points was used: 7-9 from 7.96 to 11.00 ppm, 4-6 around TSP at 0 ppm and 6 specific points at 7.96, 6.38, 6.28, 5.75, 3.00 and 2.48 ppm. Data matrix generation was finished by the Chemometrics module (Mnova) with the automatic binning approach using the sum method and a bin width of 0.01 ppm. Then buckets were normalized by dividing their areas by that of the internal standard TSP for standard solutions and also by the correction factor linked to the extraction yield (see section 3.2.1) for commercial samples. The new matrix was subjected to multivariate statistical analyses using the SIMCA-P+ 13.0 software (Umetrics, Umea, Sweden). Principal Component Analysis (PCA) with Pareto scaling on the selected variables was first performed with all commercial samples (56 spectra).

In order to better differentiating the cinnamon products based on the variety contents of (E)-cinnamaldehyde and coumarin, a range concentration of standard solutions was used to build the PCA training set, *i.e.* LF NMR spectra of solutions of standard (E)-cinnamaldehyde (22 spectra) and coumarin (8 spectra) with known concentrations. Then the test data set constituted of all commercial samples (56 spectra) was projected therein and the score plots were generated. In the score plots, samples with different contents of (E)-cinnamaldehyde or coumarin could be ranked by comparison to standard scattering behavior.

Partial least squares regression (PLS1) were built with the areas of specific LF NMR signals of standard (E)-cinnamaldehyde (22 spectra) and coumarin (8 spectra) solutions as the set of independent variables (X) and their known concentrations as the set of dependent variables (Y). Models were validated using cross-validation method (Q2) and CV-ANOVA. The quantification of (E)-cinnamaldehyde and coumarin in commercial samples was then predicted according to their respective PLS1 models. The predicted results based on PLS1s were compared to quantitative values obtained by HF NMR.

3. Results and discussion

3.1 HF and LF NMR assignment of signals

Fig II - 1 compares the spectra of the same NMR tube containing a solution of standard (E)-cinnamaldehyde (A and B) or coumarin (C and D) recorded at 400 (A and C) and 60 (B and D) MHz. As the coupling constants are independent of the magnetic field, the signals appear obviously much more spread with more overlaps in the spectrum at LF. For instance, the H2 doublet of doublet of (E)-cinnamaldehyde becomes a second order system with a slight "roof" effect at LF. Likewise, H3 and H4 of coumarin give two doublets at HF ($\Delta\nu/J > 60$) but tend to an AB system at LF with $\Delta\nu/J = 9.4$. Besides, some signals overlap, *i.e.* H7, 8, 9 and 10 of coumarin co-resonate as a broad multiplet that spreads from 7.1 to 7.7 ppm at LF. The NMR assignments of spectra recorded at 400 MHz are reported in Table II - 1.

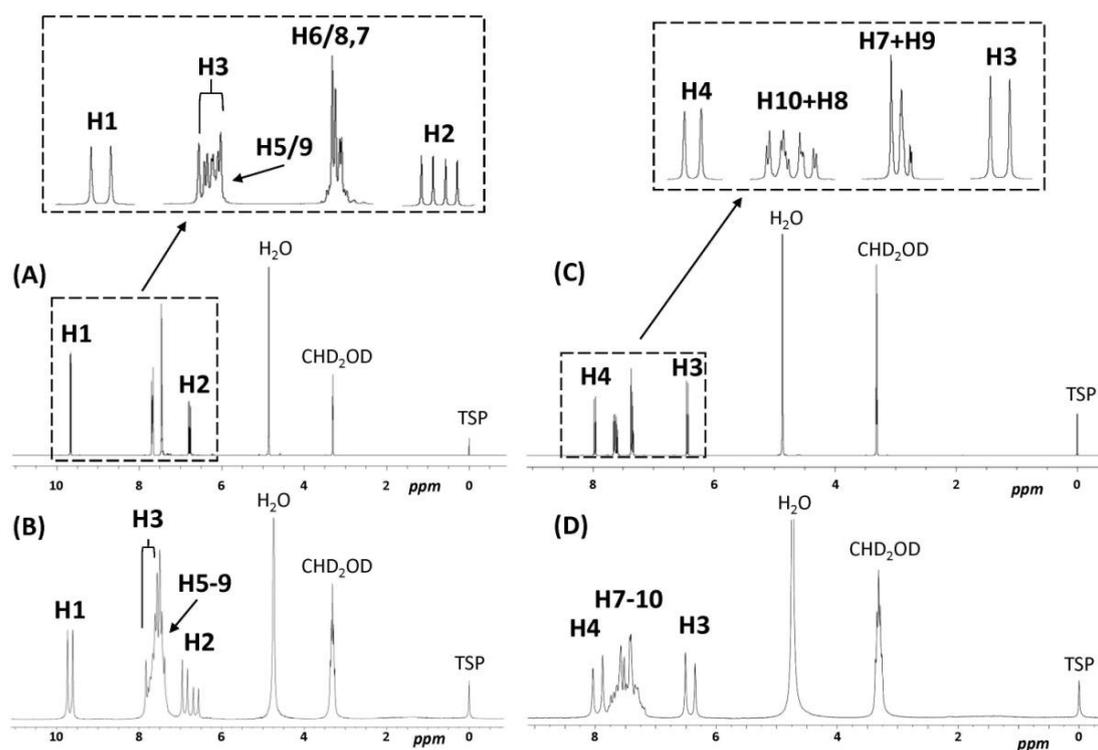


Fig II - 1. ¹H NMR spectra of solutions of standard (E)-cinnamaldehyde (A and B) and coumarin (C and D) in MeOH-d₄. (A) and (C) spectra are recorded at HF and (B) and (D) at LF. Recording times were 2 min at HF and 2 h at LF. See Fig II - 2 for chemical structures and proton numbering of (E)-cinnamaldehyde and coumarin.

Fig II - 2 illustrates the ^1H NMR profiles of the cinnamon food sample **2**. The HF spectrum shows, in addition to signals of coumarin and (E)-cinnamaldehyde, some typical resonances of o-methoxy-cinnamaldehyde, cinnamyl alcohol and (E)-cinnamic acid that were assigned by comparison to previous work [2] and by addition of standard compounds in the medium. NMR assignments of spectra of standard compounds recorded at 400 MHz are reported in Table II - 1. Another flavouring compound, eugenol, was detected in HF spectra of some samples (not present in the spectrum shown Fig II - 2A) thanks to its characteristic singlet at 3.82 ppm (O-CH₃) [2] and other signals from its aromatic ring (δ (ppm) 6.72, d 2 Hz; 6.69, d 8.0 Hz; 6.58, dd 8.0, 2.0 Hz). Only (E)-cinnamaldehyde (H1, δ 9.66 ppm and H2, δ 6.73 ppm) and coumarin (H3, δ 6.42 ppm and H4, δ 7.94 ppm) signals are readily observed in the LF spectrum of the cinnamon food sample **2** (Fig II - 2B). To complete the samples screening, the HF ^1H NMR spectrum of an additional sample of an authentic standard of *Cinnamomum verum* is reported in Fig II -3 and compared with sample **2**. In this spectrum, signals of (E)-cinnamaldehyde, o-methoxy-cinnamaldehyde, cinnamyl alcohol, (E)-cinnamic acid and eugenol were observed whereas those of coumarin could not be detected. Eugenol as the symbol of this cinnamon species at a low content, the singlet of O-CH₃ is overlapped with other signals in the spectra (Fig II - 3).

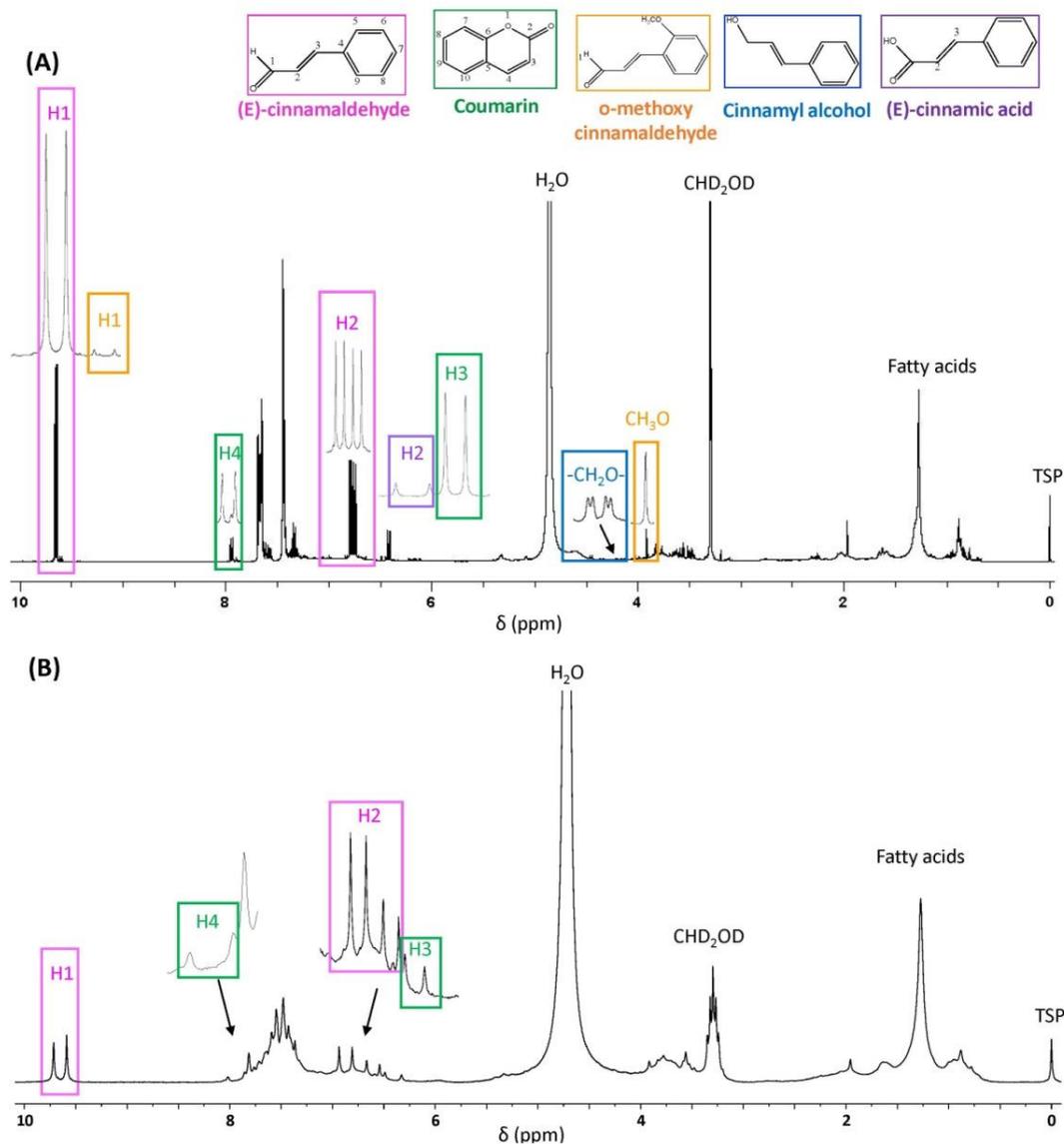


Fig II - 2. (A) HF and (B) LF ^1H NMR spectra of cinnamon food (sample 2) highlight typical signals of the main components. Recording times were 8 min at HF and 2 h at LF.

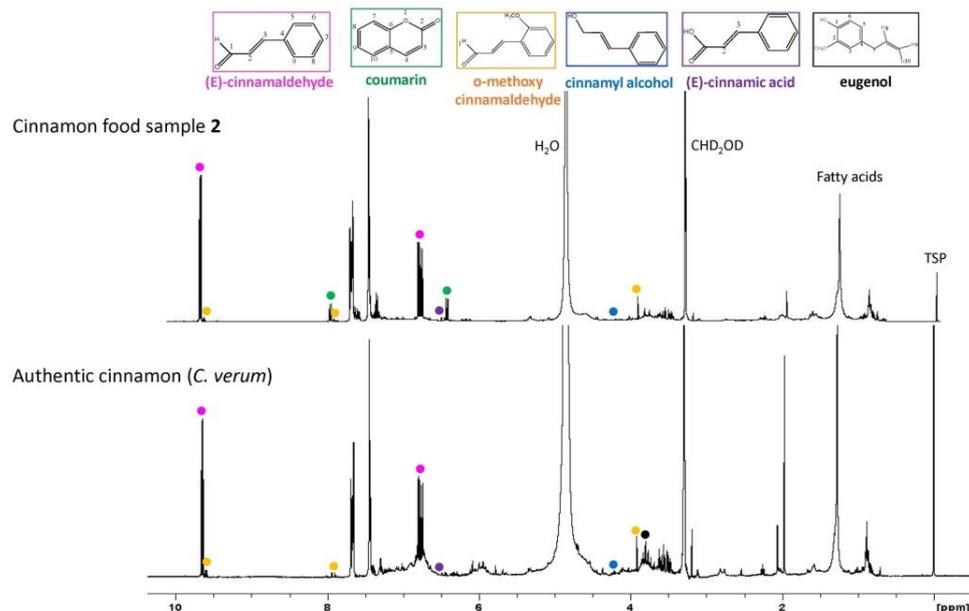


Fig II - 3. The comparison of HF ^1H NMR spectra of the authentic *Cinnamomum verum* sample and a cinnamon food (sample 2).

3.2 Implementation of the experimental conditions

3.2.1 Extraction tests and solvent choice

To select the best extraction procedure, different extraction tests were performed on the commercial dietary supplement **25** which contains significant amounts of (E)-cinnamaldehyde and coumarin as shown later.

In the literature different solvents have been proposed for the quantification of active compounds of cinnamon. The most common are methanol [2-4] and methanol/water or ethanol/water mixtures [5-9]. Only one study carried out by NMR used CDCl_3 as extracting solvent [1].

A first set of experiments was thus dedicated to the screening of different solvents. MeOH-d_4 , mixtures $\text{MeOH-d}_4:\text{D}_2\text{O}$ (80:20 and 50:50) and CDCl_3 were tested with direct extraction of 100 mg of powder. Moreover, a liquid-liquid extraction of a D_2O suspension of powder by CDCl_3 was also explored. The extraction yields were evaluated by integration of the H1 signal of (E)-cinnamaldehyde and H3 and H4 signals of coumarin on the HF ^1H NMR spectra. Results are reported in Fig II - 4. The solvents that extracted the highest quantities of (E)-cinnamaldehyde and coumarin were MeOH-d_4 and the mixture $\text{MeOH-d}_4:\text{D}_2\text{O}$ in the 80:20 ratio. This better extraction yield with

methanol is in agreement with most published studies [2, 5, 6]. The extraction yields found in CDCl_3 relatively to MeOH-d_4 were poor (10.6 and 9.5% for (E)-cinnamaldehyde and coumarin, respectively) demonstrating that it was not possible to implement a quantitative analysis of cinnamon samples in this solvent unlike previously reported [1]. For the next steps of the study, since our objective was to propose a method on a LF NMR spectrometer, we chose to use MeOH-d_4 and not a $\text{MeOH-d}_4:\text{D}_2\text{O}$ mixture as extraction solvent because a single solvent facilitates the implementation of the LF NMR experiments. Indeed, the lock procedure is more convenient and less additional signals due to residual non-deuterated solvents are observed.

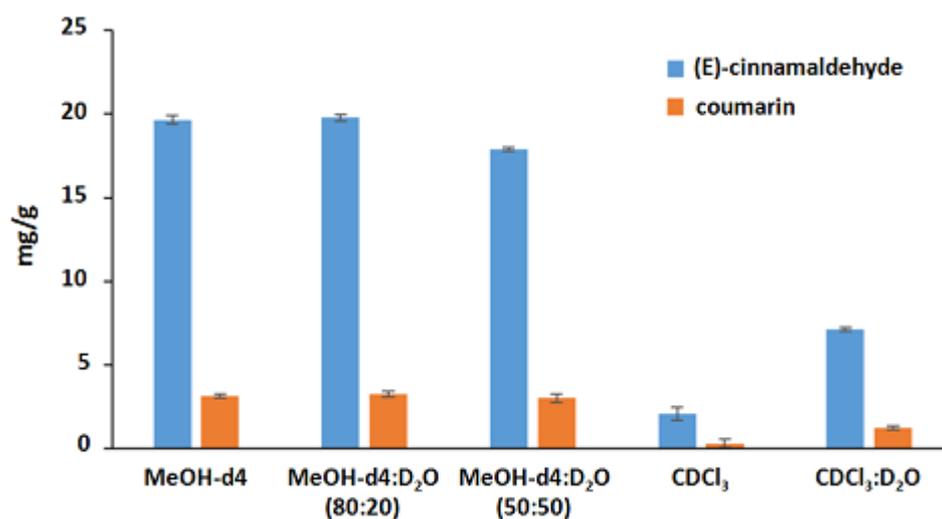


Fig II - 4. Comparison of the amounts of (E)-cinnamaldehyde and coumarin extracted from the commercial sample **25** depending on the extraction solvents. The vertical bars represent the standard error of the mean.

In order to establish the extraction procedure, three successive extractions were performed on 100 mg or 15 mg of samples **10** and **25**. From these preliminary experiments (see results in *section 2.2.1*), we chose to analyze all the commercial samples after a single extraction in MeOH-d_4 . HF NMR analyses were done from both 15 and 100 mg of powder. The 15 mg samples were considered for the assay of (E)-cinnamaldehyde and coumarin as we showed that a lower amount of powder ensures a better extraction yield, and the 100 mg samples for the assay of the other chemicals (eugenol, o-methoxy-cinnamaldehyde, (E)-cinnamyl alcohol, (E)-cinnamic acid) whose concentrations are much lower. The LF capabilities were assessed with 100 mg of powder only (discussed in *section 3.2.3*).

3.2.2 LF NMR exploratory experiments with Chemical Shift Reagents (CSR)

CSR have been explored extensively in 1960-1970s when NMR spectrometers operated with ^1H frequencies around or under 100 MHz. Recently, the use of CSR was reported for the new generation of compact LF NMR spectrometers to increase the frequency dispersion of the targeted NMR signals [10]. In most experiments with CSR, the best solvent for improving spectral resolution is CDCl_3 . In the context of cinnamon analysis, we showed above that CDCl_3 did not provide a good extraction yield and that MeOH-d_4 was the best solvent. Although the interaction of the paramagnetic ion with the oxygen atom of MeOH-d_4 reduces its availability for interacting with other species, we nevertheless decided to test CSR in our experimental conditions.

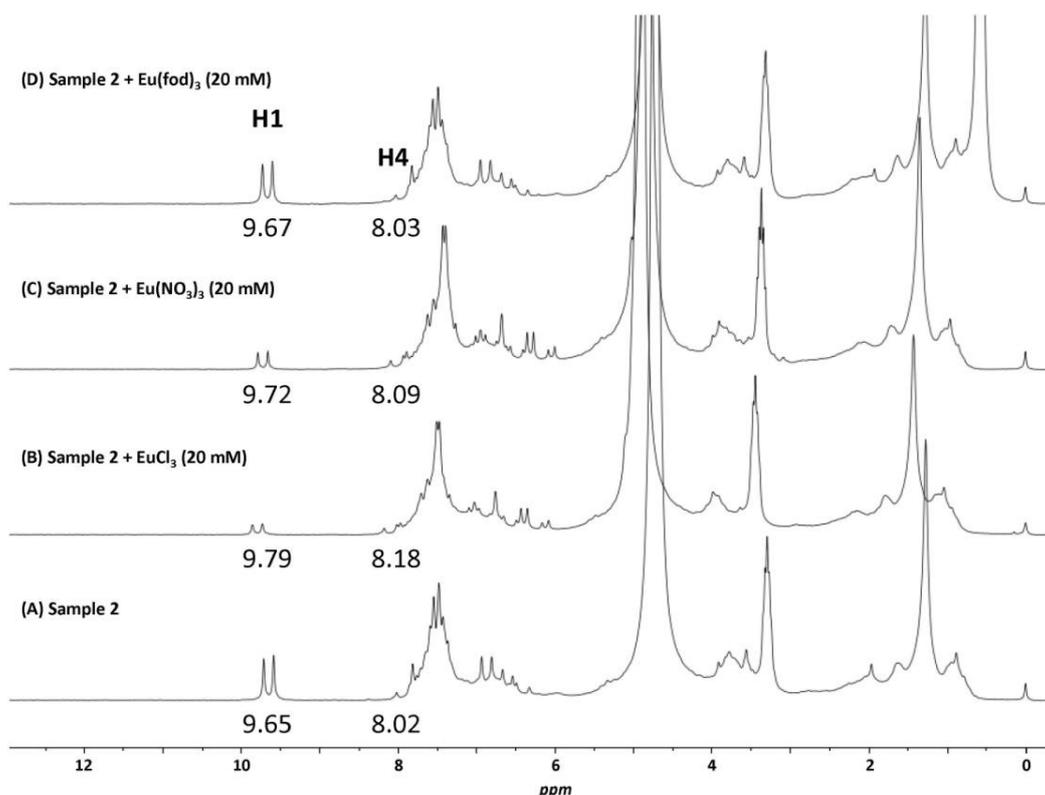


Fig II - 5. LF ^1H NMR spectra of a cinnamon food (sample 2) without CSR (A) and after addition of CSR at a final concentration of 20 mM, EuCl_3 (B), $\text{Eu}(\text{NO}_3)_3$ (C) and $\text{Eu}(\text{fod})_3$ (D). Chemical shifts (ppm) of the H1 of (E)-cinnamaldehyde and of the left part of the doublet of the H4 of coumarin are reported on the spectra.

Our aim was to spread the signals and particularly to downfield slightly (≈ 20 Hz) the H4 signal of coumarin thus making its area easier to measure. Three CSR ($\text{Eu}(\text{fod})_3$, $\text{Eu}(\text{NO}_3)_3$ and EuCl_3) were tested (Fig II -5). For a TSP calibrated at 0 ppm, the

chemical shift of the H4 signal did not shift significantly. Indeed, the left part of the doublet moves from 8.02 ppm without CSR to 8.03 ($\Delta\delta = 0.5$ Hz), 8.09 ($\Delta\delta = 4.4$ Hz) and 8.18 ppm ($\Delta\delta = 9.7$ Hz) with the addition of $\text{Eu}(\text{fod})_3$, $\text{Eu}(\text{NO}_3)_3$ and EuCl_3 respectively. Moreover, for EuCl_3 which induces the highest chemical shift modification of the coumarin resonance, a broadening of the signals, as well as a sharp decrease of the (E)-cinnamaldehyde signal, were observed because EuCl_3 induced strongest interaction with the chemicals. From these experiments, it appears that the use of CSR for the analysis of cinnamon extracts under our experimental conditions did not constitute an interesting tool for better discrimination of signals. Experiments with CSR were not thus deeply investigated due to the restricted solvent choice imposed by the extraction step.

3.2.3 LF quantitative analysis: attempt to implement and limitations

Quantitative HF NMR is nowadays a recognized method for the analysis of complex samples [11-13] and was taken as the standard reference method in this study. However, the implementation of LF quantitative analysis remains a challenging task [14]. In this section, we discuss the attempt to set up and encountered limitations for cinnamon analysis.

The first option was to quantify the samples prepared with a correct extraction yield, *i.e.* using the same solution for both HF and LF NMR quantifications. For HF ^1H NMR quantitative analysis, 15 mg of commercial sample were sufficient to measure accurate concentrations in a reasonable recording time of 1 h with a LOQ of 0.09 mg/g and 0.15 mg/g for (E)-cinnamaldehyde and coumarin respectively. With the same parameters and recording time on the LF spectrometer, the LOQ was estimated at 6 mg/g and 10 mg/g due to the decrease of the SNR per unit time by a factor of ≈ 60 . This means that if we wanted to obtain a correct SNR ratio for coumarin quantification, the experimental time would have been superior to 100 h for each analysis of commercial samples. Even with extracts from 100 mg of commercial powder, at least 17 h were necessary to obtain a SNR of ≈ 10 for samples containing ≈ 3 mg/g of coumarin (data not shown). It thus appeared that it was not possible to implement correct quantitative measurements of coumarin with both a full relaxation of the signals and under a reasonable recording time compatible with routine analyses.

Another way to proceed was to run LF NMR analysis using concentrated samples, *i.e.* samples extracted from 100 mg of powder, recorded in a reasonable experiment time of 2 h in non-fully relaxed conditions, and to consider several correction factors as described below:

(i) The first factor linked to the extraction yield, corresponding to extraction recovery of (E)-cinnamaldehyde and coumarin respectively (see experimental *section 2.2.1*).

(ii) A second integrating the fact that signals were not fully relaxed in 2 h of recording conditions [15]. Indeed, the signal recoveries of (E)-cinnamaldehyde and coumarin compared to TSP are discussed in *section 2.3.2*, with furthermore a great variability of the T1 relaxation time between commercial samples due to matrix difference.

(iii) A third applied to coumarin only, linked to the roof effect of the second order system of H4 and H3 protons. On the LF spectra, only one component of the “doublet” of H4, the left part, could be measured due to the overlap of signals in this region of the spectrum (Fig II - 2B). Considering that at the spin system tends to an AB system at LF (see *section 3.1*), the ratio between the large (internal) and the small (external) signals was experimentally evaluated on the spectra of commercial samples. It was found at $124 \pm 3 \%$, in agreement with the ratio calculated from the LF spectra of standard coumarin.

(iv) Finally, LF quantifications were altered by the automatic baseline correction procedure that introduces experimental errors related to the SNR of the considered signal. This error was experimentally estimated at 10% for an SNR close to 10.

Taking into account all the correction factors described above, the global resulting RSD for quantification would be $>15\%$. We thus concluded that cinnamon samples could not be accurately quantified at LF in our experimental conditions. Nevertheless, we will further investigate in *section 3.3.2*, a statistical treatment of LF NMR spectra obtained from commercial samples recorded for 2 h after extraction of 100 mg samples.

3.3 Analysis of commercial samples

3.3.1 HF NMR analysis: from profiling to quantitative analysis

Amounts of (E)-cinnamaldehyde, coumarin, eugenol, o-methoxy-cinnamaldehyde, cinnamyl alcohol and (E)-cinnamic acid in cinnamon commercial samples measured from HF ^1H NMR spectra are reported in Table II - 2. These data allow distinguishing several categories of samples. In four dietary supplements (**16**, **19**, **21** and **24**), neither (E)-cinnamaldehyde nor coumarin was detected but only signals of fatty acids and/or sugars. None of the typical chemicals of cinnamon being detected, not even (E)-cinnamaldehyde, we assess that these dietary supplements did not contain cinnamon powder (or at trace level) contrary to what was announced on the label. Eugenol which was previously reported as a marker of *Cinnamomum verum* [2, 4] was observed and quantified in samples **5**, **6**, **9**, **17** and **18**. Moreover, coumarin was not detected in these samples which could be thus classified as "true cinnamon" (*Cinnamomum verum*) samples. Indeed, the amount of coumarin in *Cinnamomum verum* (0.04 mg/g) is under the LOQ of HF NMR for this compound (0.15 mg/g). Two samples of food cinnamon, **2** and **11**, contained the highest amounts of both (E)-cinnamaldehyde and coumarin in agreement with the Vietnamese origin claimed by the producers and previously reported data on Vietnamese cinnamon (*Cinnamomum loureirii*) [4]. Samples containing detectable levels of coumarin could be classified as cassia cinnamon (samples **1-4**, **8**, **10-15**, **20**, **25-28**) without a more precise classification according to geographical origin or species (*burmanii*, *zeylanicum*...). Classification of samples **7**, **22** and **23** could not be assessed from NMR data insofar as only (E)-cinnamaldehyde was detected. The proposed classification of samples from HF NMR data is reported in Table II - 2. Moreover, we can observe that the two samples **22** and **23** contain high levels of (E)-cinnamic acid with 4.1 and 3.7 mg/g respectively, which can be due to a longer storage of these samples that leads to the oxidation of (E)-cinnamaldehyde [16].

Table II - 2. Quantitative data (mg/g \pm SD) of cinnamon products from HF NMR analysis (n=2) and comparison of labelled information to the proposed classification.

Sample	(E)- cinnamaldehyde	Coumarin	Eugenol	o-methoxy- cinnamaldehyde	Cinnamyl alcohol	(E)-cinnamic acid	Commercial information about origin (when available)	Proposed classification	Daily dose of coumarin ^a /mg	
FOOD	1	15.8 \pm 0.3	1.7 \pm 0.1	-	0.16 \pm 0.01	0.24 \pm 0.02	0.53 \pm 0.03	Southeast Asia Cinnamon	Cassia	
	2	32.5 \pm 0.2	6.1 \pm 0.1	-	0.94 \pm 0.01	0.24 \pm 0.03	0.98 \pm 0.07	Vietnam Cinnamon	Cassia	
	3	7.8 \pm 0.1	1.8 \pm 0.1	-	-	-	0.65 \pm 0.02	-	Cassia	
	4	4.7 \pm 0.1	1.3 \pm 0.1	-	-	-	0.51 \pm 0.01	-	Cassia	
	5	6.6 \pm 0.1	-	0.08 \pm 0.01	0.28 \pm 0.02	0.12 \pm 0.01	0.18 \pm 0.01	-	<i>C. verum</i>	
	6	6.0 \pm 0.1	-	0.12 \pm 0.01	0.23 \pm 0.01	0.12 \pm 0.01	0.19 \pm 0.01	Ceylon Cinnamon, Madagascar	<i>C. verum</i>	
	7	10.4 \pm 0.1	-	-	-	-	1.39 \pm 0.04	-	Unclassified	
	8	10.7 \pm 0.1	2.1 \pm 0.1	-	-	-	0.55 \pm 0.03	-	Cassia	
	9	5.2 \pm 0.3	-	0.11 \pm 0.02	0.22 \pm 0.01	-	0.21 \pm 0.01	Ceylon Cinnamon	<i>C. verum</i>	
	10	11.7 \pm 0.1	2.8 \pm 0.1	-	-	-	0.43 \pm 0.04	<i>Cinnamomum burmannii</i> , Indonesia	Cassia	
	11	36.1 \pm 0.8	5.4 \pm 0.1	-	1.70 \pm 0.10	0.20 \pm 0.01	0.78 \pm 0.07	Vietnam Cinnamon	Cassia	
	12	9.6 \pm 0.4	2.4 \pm 0.1	-	-	-	0.57 \pm 0.01	<i>Cinnamomum verum</i>	Cassia ^b	
	13	10.8 \pm 0.2	1.7 \pm 0.1	-	-	0.33 \pm 0.01	0.79 \pm 0.05	Ceylon Cinnamon	Cassia ^b	
	14	9.8 \pm 0.2	2.6 \pm 0.1	-	0.31 \pm 0.04	-	0.87 \pm 0.02	Cinnamon Cassia, Thailand	Cassia	
DIETARY SUPPLEMENTS	15	0.2 \pm 0.1	1.5 \pm 0.1	-	-	-	0.62 \pm 0.04	<i>Cinnamomum zeylanicum</i>	Cassia	2.4
	16	-	-	-	-	-	-	<i>Cinnamomum verum</i>	No cinnamon detected	
	17	6.4 \pm 0.1	-	0.08 \pm 0.01	0.27 \pm 0.02	-	0.28 \pm 0.02	<i>Cinnamomum verum</i>	<i>C. verum</i>	
	18	3.1 \pm 0.1	-	0.05 \pm 0.01	0.14 \pm 0.01	0.13 \pm 0.02	0.18 \pm 0.01	Ceylon Cinnamon	<i>C. verum</i>	
	19	-	-	-	-	-	-	<i>Cinnamomum zeylanicum</i> , <i>C. cassia</i>	No cinnamon detected	
	20	6.0 \pm 0.1	1.2 \pm 0.1	-	-	-	0.20 \pm 0.01	<i>Cinnamomum cassia</i>	Cassia	0.65
	21	-	-	-	-	-	-	Ceylon Cinnamon	No cinnamon detected	
	22	1.2 \pm 0.1	-	-	-	-	4.10 \pm 0.31	<i>Cinnamomum cassia Presl</i>	Unclassified	
	23	2.0 \pm 0.1	-	-	-	-	3.74 \pm 0.20	<i>Cinnamomum cassia Presl</i>	Unclassified	
	24	-	-	-	-	-	-	Ceylon Cinnamon	No cinnamon detected	
	25	20.0 \pm 0.7	3.4 \pm 0.1	-	-	-	0.50 \pm 0.08	<i>Cinnamomum burmannii</i> , Indonesia	Cassia	3.5
	26	12.9 \pm 0.2	2.5 \pm 0.1	-	-	0.20 \pm 0.03	0.43 \pm 0.02	Ceylon Cinnamon	Cassia ^b	4.8
	27	19.0 \pm 0.1	2.7 \pm 0.1	-	-	-	0.50 \pm 0.06	<i>Cinnamomum cassia</i>	Cassia	7.7
	28	17.1 \pm 0.1	3.2 \pm 0.1	-	-	0.19 \pm 0.01	0.67 \pm 0.03	<i>Cinnamomum cassia</i>	Cassia	8.4

^a Calculated from the HF NMR quantification according to the recommended dosage on the dietary supplement label.

^b For these 3 samples, the commercial information does not fit with the NMR analysis; Ceylon cinnamon was replaced by cassia cinnamon.

The toxicity of cinnamon samples is related to their coumarin content and the hepatotoxic level of 0.1 mg/kg body weight must be considered. Consumption of cinnamon in food is very variable and linked to dietary habits of each country. For instance, it was reported that in Germany the consumption of cinnamon present in various desserts can reach 17 g over the ten days of the Christmas period, so a mean value of 1.7 g/day [17]. In food cinnamon samples reported in Table II - 2, the highest concentration of coumarin found was 6.1 mg/g. This kind of spice sample may thus present a risk for the health of a big consumer of cinnamon cookies or other Christmas desserts.

The issue is different for dietary supplements for which a dosage is recommended by the manufacturer. Indeed, the coumarin content of dietary supplements can be discussed considering the daily amount ingested according to the recommended dosage indicated on the packaging. For samples **15**, **20**, **25-28**, it was calculated, from HF NMR data, between 0.65 and 8.43 mg (mean 4.6 mg). For the two dietary supplements **27** and **28**, the daily coumarin intake, 7.72 and 8.43 mg respectively, is over the hepatotoxic level of 0.1 mg/kg considering an average body weight of 70 kg. Therefore, their daily ingestion can be at risk for consumers.

3.3.2 LF NMR analysis: from statistical approach to quality control

As shown above (see *section 3.2.3*), the LF quantification of cinnamon samples could not be done correctly. We nevertheless chose to extract 100 mg of powder from commercial samples, to record the LF NMR spectra in non-fully relaxed conditions and then to apply a statistical treatment.

PCA is an unsupervised clustering method that decreases the dimensionality of the multivariate data. First, an PCA was built from the LF NMR spectra limited to the region of olefinic and aromatic protons (5.7-11 ppm) of all samples (Fig II - 6A). Other parts of NMR spectra were excluded in order to remove (i) areas affected by the residual signals of water and methanol at 3.8-5.7 ppm respectively, (ii) signals of fatty acids (0.5-2.5 ppm) and (iii) noise (2.5-2.8 ppm) (seen in Fig II - 2B). Based on the quantitative HF analysis, three classes of samples were colored on the PCA score plot: samples which contain neither (E)-cinnamaldehyde nor coumarin (**16**, **19**, **21**, **24**) in red, samples in agreement with Ceylan cinnamon *i.e.* without coumarin but containing

eugenol (**5, 6, 9, 17, 18**) in blue, and all the remaining samples in green. Two compact clusters were identified: the first one corresponds to the dietary supplements which contain no cinnamon (in red) and the second to samples containing true cinnamon (*Cinnamomum verum*) (in blue). The wide dispersion of the last group (in green) is related to the NMR signals at 6.4-7.0 ppm and 7.3-7.7 ppm corresponding to all the protons (except H1) of (E)-cinnamaldehyde (Fig II - 1) which is present in varying amounts (0.2 to 36 mg/g) (Table II - 2). The score plot of a PCA built from the sole H1 signal of (E)-cinnamaldehyde (data not shown) was similar to that shown in Fig II - 6A, thus demonstrating that it is mainly (E)-cinnamaldehyde which causes the statistical differentiation of the samples.

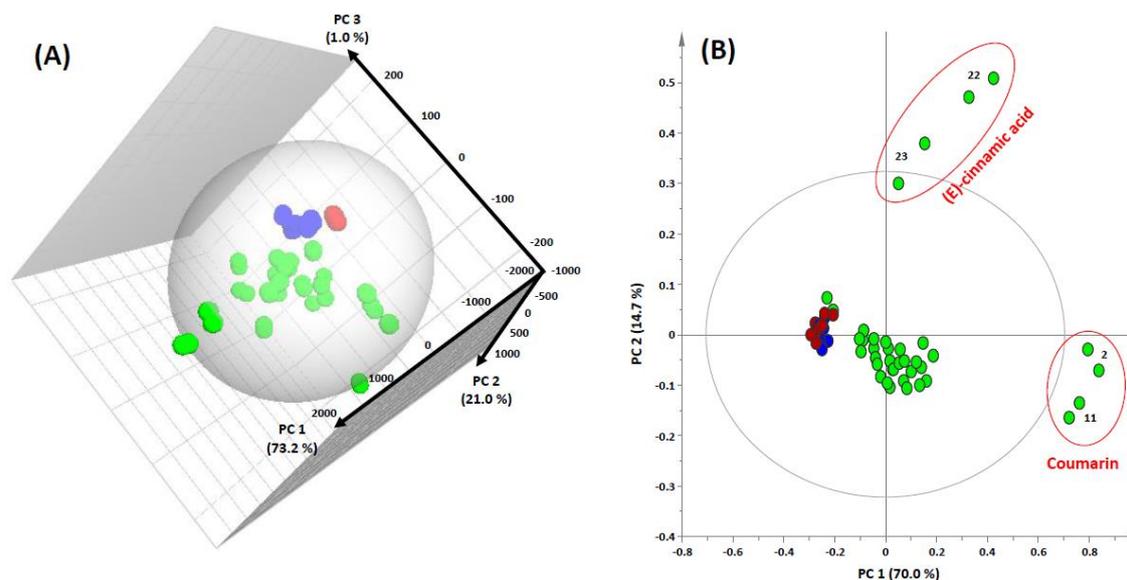


Fig II - 6. (A) Three-dimensional score plot of a PCA built from the LF NMR data of all the samples using signals in the range 5.7 to 11 ppm. (B) Score plot of a PCA built from the LF NMR data focusing on the signals of H3 (right component at 6.34 ppm) and H4 (left component at 8.03 ppm) of coumarin. Samples without cinnamon (**16, 19, 21, 24**) are colored in red, samples with true cinnamon (**5, 6, 9, 17, 18**) in blue and other samples in green.

For coumarin, in order to check if the whole doublet could be considered for the next step, a PCA score plot based on parts of the H3 (right component) and H4 (left component) doublets of coumarin was performed. It shows a clear grouping of samples where coumarin was not detected (red and blue in Fig II - 6B). Samples **2** and **11** with the highest levels of coumarin (6.1 and 5.4 mg/g respectively, Table II - 2) are located in the lower right part of the score plot. The two samples **22** and **23** seem atypical because they contain significant amounts of (E)-cinnamic acid (Table II - 2) whose H2 has the same chemical shift than the right resonance of the H3 of coumarin at LF (Fig II - 7). In the same way, the broad signal around 8 ppm is only detected in sample **22** and to a lesser extent in sample **23**. For the next steps, the two formulations **22** and **23** were no longer considered and the analysis was restricted to the left resonance of the H4 of coumarin.

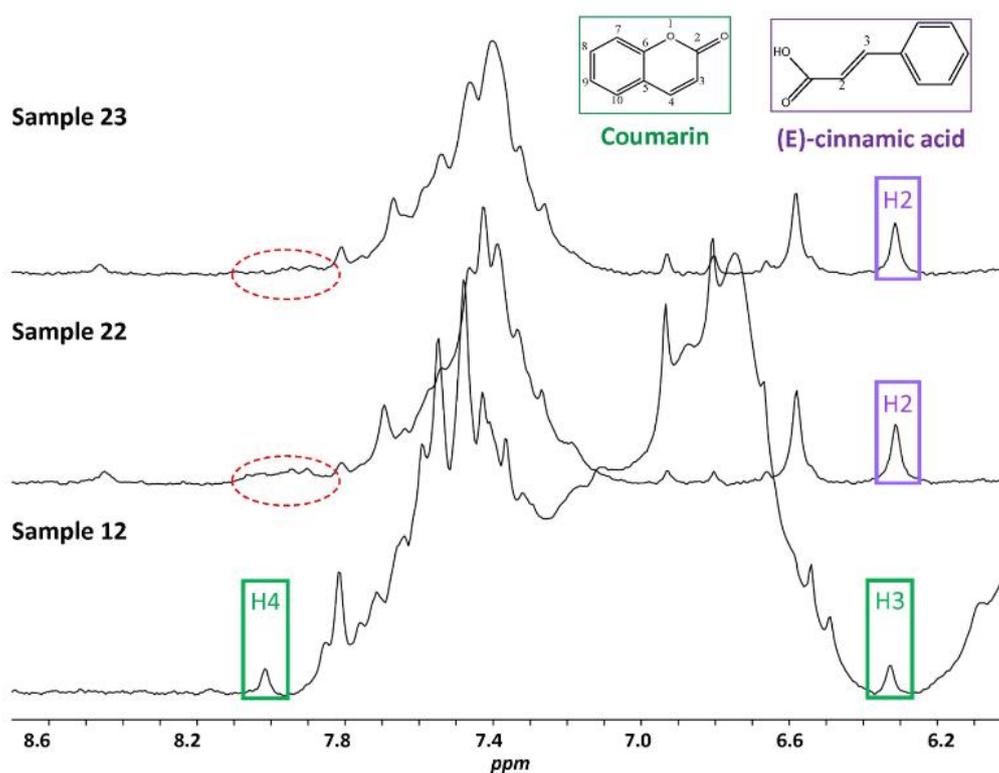


Fig II - 7. LF ¹H NMR spectra of the cinnamon samples **12**, **22** and **23** focused on the 6.0-8.7 ppm area. The red dotted ellipse identifies the broad signal around 8 ppm mentioned in the 3.3.2 section.

To provide better separation and further rank the samples according to the variety content of (E)-cinnamaldehyde and coumarin, Fig II - 8 proposes an approximate ranking based on the LF NMR data focused on typical signals of (E)-cinnamaldehyde (H1) or coumarin (left component of the H4 doublet). For this approach, spectra of standard and commercial samples were acquired and processed under the same conditions. Only the correction factor linked to the extraction yields of commercial samples was included in the normalization of the data. PCAs were first built with the training set, *i.e.* LF NMR spectra of solutions of standard (E)-cinnamaldehyde (Fig II - 8A) and coumarin (Fig II - 8B) with known concentrations correspond to real range in commercial samples.

The projections of the test dataset constituted of the commercial samples were then generated (Fig II - 8C and D). The score plot (C) represents a ranking of the samples based on their (E)-cinnamaldehyde content. The highest concentrated samples are located on the right and those containing no (E)-cinnamaldehyde (in red) on the left, aligned with the blank reference sample. Values reported on the scale of the score plot fit with the results obtained by quantitative HF NMR: for instance, samples **2** and **11** contain more than 30 mg of (E)-cinnamaldehyde whereas samples **13**, **1** and **25** contain 10.8, 15.8 and 20.0 mg respectively (Table II - 2). Ceylan cinnamon samples, in blue, are included in the range 0-10 mg/g, which is in good agreement with the real concentrations of (E)-cinnamaldehyde (3.1-6.5 mg/g, Table II - 2). The same approach was applied to coumarin (D). All samples with no coumarin (red and blue) are ranked on the left part of the score plot and aligned with the blank sample. All commercial samples contain less than 4 mg/g of coumarin except the two samples **2** and **11** that have concentrations close to or higher than 6 mg/g. These statistical data are well correlated with the quantitative values, *i.e.* 6.1 and 5.4 mg/g for sample **2** and **11** and between 1.2 and 3.4 mg/g for the other samples (Table II - 2).

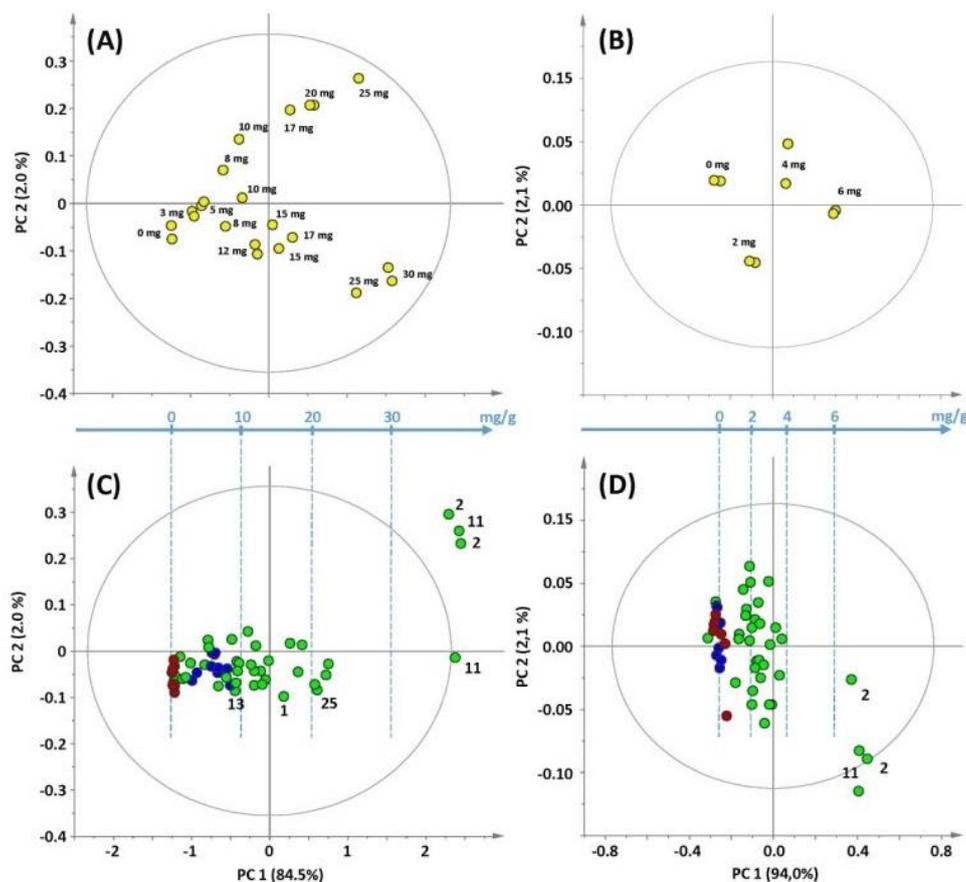
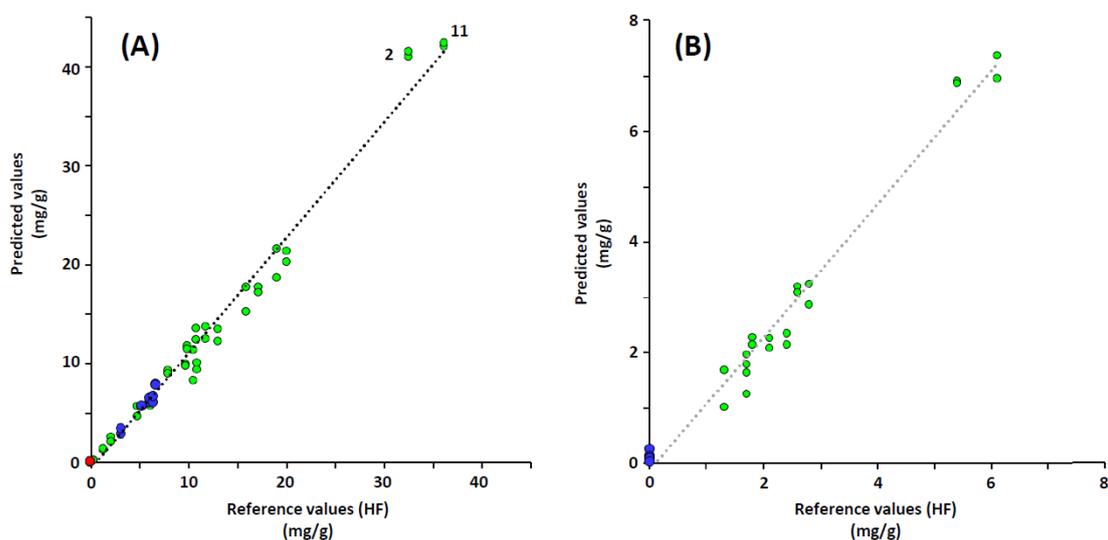


Fig II - 8. Statistics applied to rank the samples from LF NMR data. PCAs were built from LF NMR spectra of solutions of standard (E)-cinnamaldehyde (A) and coumarin (B). Concentrations correspond to real amounts in commercial samples. The score plots (C) and (D) show the projection of the commercial samples on the PCA score plots built for (E)-cinnamaldehyde and coumarin respectively.

After the PCA, a supervised PLS method was further applied to predict the content of (E)-cinnamaldehyde and coumarin in products. PLS1 models were first built with the same LF NMR spectra of standard (E)-cinnamaldehyde and coumarin solutions with known concentrations as the PCA above. Fig II - 9 shows that a quantification based on the LF NMR data focused on typical signals of (E)-cinnamaldehyde (H1) or coumarin (left component of the H4 doublet) can be predicted. Each model was validated ($Q^2 > 0.99$ and $CV\text{-ANOVA} < 10^{-8}$) and used to predict concentrations of these compounds in commercial samples. The first scatter plot (Fig II - 9A) shows the good

correlation ($R^2 = 0.98$) between the predicted concentrations of (E)-cinnamaldehyde and the real concentrations measured with HF NMR for the three classes of samples (red, blue and green) from 0 to ≈ 40 mg/g for the more concentrated samples **2** and **11**. The same result was obtained for coumarin in food samples (Fig II - 9B). Fig II - 9C shows the bar plot of the concentrations of coumarin predicted by the same PLS1 but restricted to dietary supplements (without samples **22** and **23**). Here, the maximal recommended daily dosage was considered for data treatment. This graph highlights that the two samples **27** and **28** containing ≈ 6 -8 mg/g of coumarin may lead to the ingestion of daily high levels of coumarin by the consumer. They can thus pose a health risk because they are located in the hepatotoxic range for a bodyweight of about 70 kg.

This chemometric approach demonstrates that key information on the quality of commercial cinnamon samples can be reached from LF NMR data obtained with a quick and easy sample preparation step and 2 h of NMR spectra recording. Moreover, it allows a classification of the samples depending on their concentrations in (E)-cinnamaldehyde and coumarin, and statistical process allows a predicted quantification of (E)-cinnamaldehyde and coumarin levels in commercial samples.



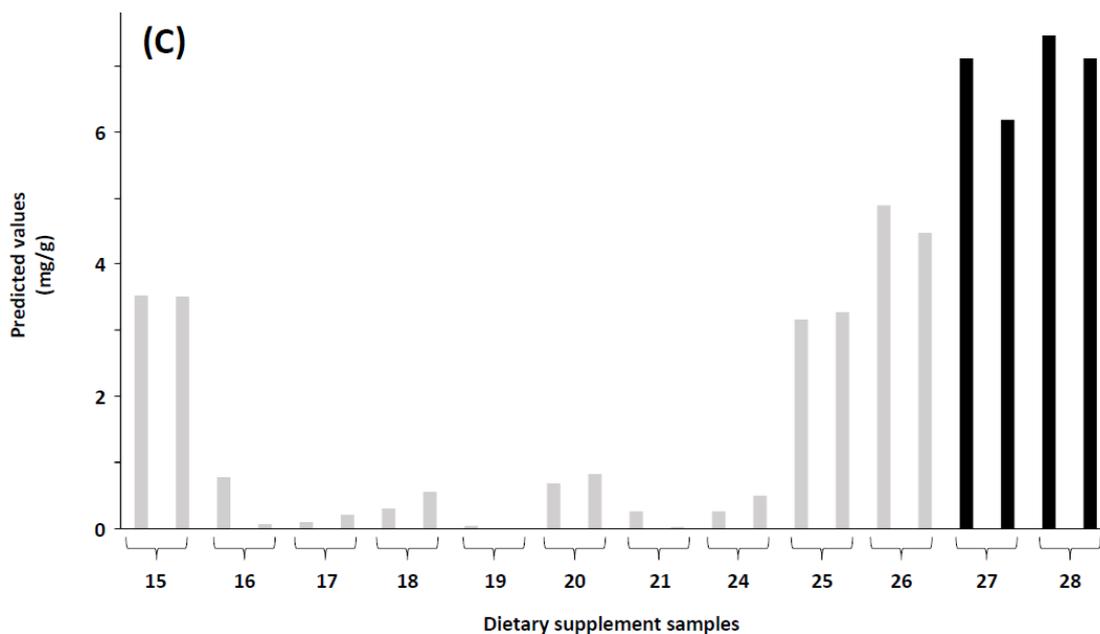


Fig II - 9. Chemometric analysis of LF NMR data. Representation of predicted values obtained from PLS1 models built with LF NMR data versus HF NMR quantitative data for (A) (E)-cinnamaldehyde in all samples and (B) coumarin in food samples. (C) Bar plot of predicted contents of coumarin in dietary supplement samples according to their recommended dosage.

4. Conclusion

The present study aimed to evaluate the pros and cons of LF NMR, a technique reported here for the first time for quality control of commercial cinnamon samples. Although LF NMR spectra of cinnamon samples are crowded and assignments tricky due to signal overlaps, characteristic resonances of (E)-cinnamaldehyde and coumarin can nevertheless be detected. LF absolute quantification can not be proposed as a routine control method for cinnamon analysis but the treatment of LF NMR data with a chemometric approach is a promising way to enhance the power of LF NMR and thus to consider it as a full analytical technique. These encouraging results must lead to continuing the evaluation of benchtop LF NMR spectrometers for quality control applications in herbal products and food analysis.

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Chapter III Chemometric Analysis of Low-field ^1H NMR Spectra for Unveiling Adulteration of Slimming Dietary Supplements by Pharmaceutical Compounds

1. Introduction

As already presented in *chapter I* (section 4.1-4.4), the illegal adulteration of synthetic drugs in slimming dietary supplements (DS) and their adverse effects have gained great attention nowadays [1, 2]. Various analytical methods including HPLC, GC, hyphenated-MS and NMR were applied for quality control of slimming DS with adulterations (*chapter I*, section 4.5).

Benchtop LF NMR is an emerging technique based on the new generation of compact NMR that can be used for quality control of adulterations in complex mixtures. Here, the two most common adulterants sibutramine and phenolphthalein were analyzed in slimming DS, alone or in combination [2-4]. We have thus analyzed adulterated and non-adulterated slimming DS, previously qualitatively and quantitatively characterized by HF ^1H NMR, with LF ^1H NMR to create statistical models in which the LF ^1H NMR data of new samples are injected. The interest and limitations of this approach are also discussed.

2. Materials and Methods

2.1 Sampling and preparation

Different groups of weight-loss DS were analyzed with LF ^1H NMR: (N) without adulteration, (S) adulteration with sibutramine, (P) adulteration with phenolphthalein, (PS) adulteration with both phenolphthalein and sibutramine, (T) test samples, and (X) two atypical samples (Table III - 1). 40 DS were used for building the statistical models ((N) (n = 19), (S) (n = 12), and (P) (n = 9)) as well as (PS) samples (n = 11), and the two DS (X) were previously qualitatively and quantitatively characterized in our lab by HF ^1H NMR, and would not test again in this study [4]. For testing the statistical models,

13 new DS (T1–T13) were bought on the Internet in November 2019 and were analyzed by LF and HF ¹H NMR upon receipt.

For sample preparation, around 100 mg of powdered samples were mixed with 1 mL of deuterated methanol under vortex agitation for 15 s and then sonicated for 5 min. The suspension was then centrifuged (5 min, 3000 rpm) and the supernatant (700 μL) analyzed. Thirty microliters of sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl) propanoate (TSP, 40 mM) as the internal chemical shift reference was added before the NMR analysis. Each DS was prepared in duplicate.

2.2 HF ¹H NMR analysis

Thirteen new slimming DS were analyzed by HF ¹H NMR. Qualitative experiments were performed on a Bruker Avance 400 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) equipped with a TXO probe at 298 K. For qualitative experiments, HF ¹H NMR spectra were recorded with the following parameters: pulse width 13.5 μs (flip angle 90°), acquisition time 2.93 s, spectral width 14 ppm, 32 K data points and relaxation delay 1.0 s. The number of scans was 64, corresponding to a recording time of ca. 4 min 29 sec. The signal of TSP set at 0 ppm was used as an internal reference for chemical shift measurement. 1D ¹H NMR data were processed using the TOPSPIN 3.1 software with one level of zero-filling and Fourier transformation after multiplying FIDs by an exponential line broadening function of 0.3 Hz, then phasing and baseline correction were applied.

2.3 LF ¹H NMR analysis

Qualitative LF ¹H NMR spectra were acquired on a Pulsar™ benchtop NMR spectrometer (Oxford Instruments, Abingdon, UK) operating at a frequency of 59.7 MHz for ¹H. The temperature inside the spectrometer was 310 K. The acquisition was performed by using the SpinFlow 1.2.0.1 software (Oxford Instruments) and the processing was done with MNova 11.0 (Mestrelab Research, Santiago de Compostela, Spain). Free induction decays (FIDs) were recorded with a flip angle of 90° (12.5 μs), a spectral width of 5000 Hz (83.75 ppm), and 8 K complex points (acquisition time of 1.64 s). The relaxation delay was set at 2 s, and 256 transients were recorded leading to a total acquisition time of 15.5 min. For data processing, the FIDs were apodized with an exponential filter (line broadening (LB) of 0.3 Hz), and a Whittaker smoother was

applied for automatic baseline correction. The number of points was increased to 16 K in Fourier transformed spectra. The signal of TSP set at 0 ppm was used as an internal reference for chemical shift (δ) measurement.

2.4 LF ^1H NMR Data analysis and chemometrics

First, the data matrix with all LF ^1H NMR spectra (132 spectra) of ((N), (S), (P), (PS), (T), and (X)) groups was generated in the chemometrics module included in the MNovo software with a spectral resolution of 0.01 ppm/point. Data were transferred to the Matlab[®] software (R2018a, The Mathworks Inc., Natick, MA, USA) for the alignment procedure using the Icoshift algorithm [5] with the following input arguments: PS9 as reference spectrum (target vector), data matrix with all spectra, a file for local alignment with three specific intervals 8.385–7.495, 7.495–7.275, and 7.275–6.055 ppm, an optional ‘f’ command for a fast search of the best alignment for each interval and no co-shift preprocessing step. Then, the bucketing procedure was performed with an optimized bucketing algorithm [6] and a fixed bin width of 0.01 ppm. Data were normalized by dividing their areas by that of the internal standard TSP signal and by the weight of powder in capsules, tablets, sachet, coffee or tea bags. Multivariate statistical analyses were done with the SIMCA-P+ 13.0 software (Umetrics, Umea, Sweden). PLS-DA with UV-scaling analyses were performed with two (80 spectra corresponding to 19 samples (N) and 21 adulterated samples (P) and (S)) or three qualitative variables (19 (N), 9 (P) and 12 (S)). Then (PS), (T) and (X) LF ^1H NMR data (52 spectra) were projected into the active model and predicted score plots were built. Predicted Y-values (YPredPS) were provided by the classification list included in the predict module of the SIMCA-P+ software.

3. Results and discussion

3.1 HF ^1H NMR analysis

The weight-loss DS used in this study, except the newly purchased test samples (T), were previously analyzed and fully characterized by HF ^1H NMR, *i.e.* the nature and amount of adulterants by unit (capsule, tablet, or sachet) were known [4]. The full list of DS is given in Table III - 1.

Table III - 1. Information on slimming dietary supplements analyzed in this study.

Identification ^a	Formulationname	Form	Adulterant	
			Sibutramine	Phenolphthalein
N1	7+1	Capsule		
N2	AF6	Capsule		
N3	CiblAction	Capsule		
N4	Colodetox	Capsule		
N5	KILO KO	Capsule		
N6	Kilo-Minus	Capsule		
N7	Slim effect 24h	Powder sachet		
N8	Slim fast	Capsule		
N9	Slimula	Capsule		
N10	Cacti-Nea	Tablet		
N11	Fruta bio	Capsule		
N12	Instant slim	Capsule		
N13	La Jiao Shou Shen	Capsule		
N14	Lipo Bomb	Capsule		
N15	Natural Max Slimming	Capsule		
N16	PAI YOU GUO	Capsule		
N17	Pure fat 3 days	Capsule		
N18	Sleep and slim	Capsule		
N19	Slimming essence	Capsule		
P1	1 day diet	Capsule		×
P2	Majestic Slimming capsule	Capsule		×
P3	Royal Slim	Capsule		×
P4	Citrus'Fit	Capsule		×
P5	Xiushentang	Capsule		×
P6	Xiyoujiqingzhi capsule	Capsule		×
P7	Body beauty	Coffee bag		×
P8	Green coffee 1000	Coffee bag		×
P9	Japan hokkaido slimming pills ^b	Capsule		×
S1	A-Slim	Capsule	×	
S2	Best Shown coffee	Coffee bag	×	
S3	Dr Mao slimming capsules	Capsule	×	
S4	Fruit & plant slimming capsule	Capsule	×	
S5	Fruta bio	Capsule	×	
S6	Herbal Flos Ionicerae	Capsule	×	
S7	Lingzhi cleansed slim tea	Tea bag	×	
S8	Lipo 8 burn Slim	Capsule	×	
S9	Shoufsy	Capsule	×	
S10	Slim-vie	Capsule	×	
S11	SLIMXTREM	Capsule	×	
S12	Via Slim	Capsule	×	
PS1	7 Days herbal Slim	Capsule	×	×
PS2	Fruta planta (grape)	Capsule	×	×
PS3	Fruta planta (grape)	Capsule	×	×

PS4	Pineapple plus	Capsule	×	×
PS5	Seven super color Slim & Health	Capsule	×	×
PS6	Seven super color Slim & Health	Capsule	×	×
PS7	Seven super color Slim & Health	Capsule	×	×
PS8	Shape	Capsule	×	×
PS9	Super Slim	Capsule	×	×
PS10	Li Da	Capsule	×	×
PS11	St Nirvana	Capsule	×	×
T1	7 days herbal slim	Capsule		
T2	Extra slim	Capsule		x
T3	Slim Xtreme Gold	Capsule		
T4	Lida daidaihua	Capsule		
T5	Apidessum	Capsule		
T6	Super slim	Capsule		×
T7	Figure-up	Capsule		×
T8	SlimBio Capsules	Capsule		
T9	Nuozitai	Capsule	×	
T10	Daidaihua	Capsule		
T11	NuoMeiRin	Capsule		
T12	Lipo 9	Capsule	×	
T13	St nirvana herbal slimming	Capsule	×	
X1	Açaí boost	Capsule		Raspberry ketone
X2	Perfect Slim	Capsule		Fluoxetine

^a N, non-adulterated (natural) group; P, phenolphthalein-adulterated group; S, sibutramine-adulterated group; PS, both sibutramine and phenolphthalein-adulterated group); T, test samples, newly purchased slimming dietary supplements; X, atypical samples.

^b This dietary supplement was erroneously reported as containing sibutramine in the paper of our group [4]. It actually contains phenolphthalein as the adulterant.

For the 13 newly purchased samples (T1–T13), HF ^1H NMR qualitative analysis was first explored to rapid overview of products and the results could be used as references for further LF NMR analysis. Fig III - 1 shows the HF ^1H NMR spectra of 3 typical samples. In sample T2, phenolphthalein was detected by the signals of its aromatic protons with chemical shifts ranging between 6.80 and 7.91 ppm. The presence of sibutramine in sample T9 was identified by its aromatic protons at 7.44 ppm and methyl protons in the low-frequency zone. Contrastly, sample T8 exhibited only weak signals of the natural matrix, which can be distributed as a natural sample. The rapid screening by HF ^1H NMR was also supported by previous publication [4].

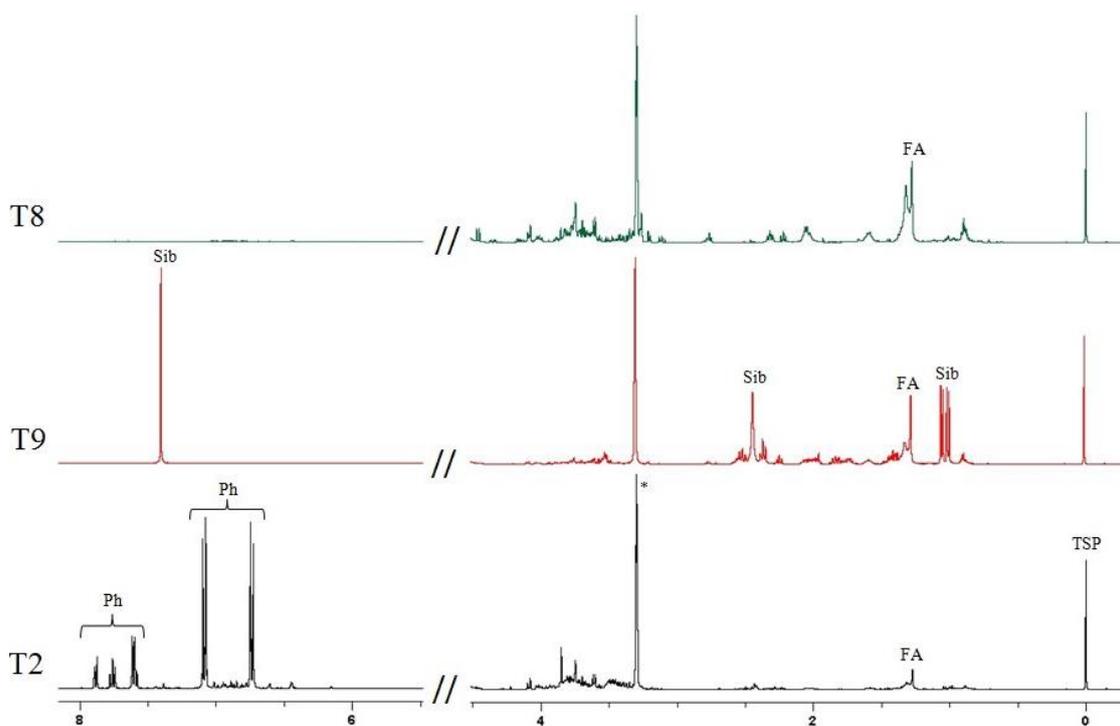


Fig III - 1. Typical spectra of newly added samples recorded at HF ^1H NMR (400 MHz). Ph: Phenolphthalein; Sib: Sibutramine; FA: Fatty acids; TSP: Internal reference; *: CD_2HOD .

3.2 LF ^1H NMR analysis

All slimming DS were analyzed in duplicate by LF ^1H NMR in deuterated methanol. The recording time of each spectrum was 15.5 min, and the profiles of typical samples are illustrated in Fig III - 2.

Although LF ^1H NMR spectra are rather poorly resolved, the main characteristic signals of sibutramine and phenolphthalein, the two most common adulterants of slimming DS, are easily detected alone or in combination. As it can be seen in Fig III - 2, sibutramine is identified in samples S5 and PS2 by the signals of its aromatic protons at 7.41 ppm and of its methyl groups at 2.49 (CH_3 12 and 13) and 1.02 (CH_3 16 and 17) ppm. Likewise, aromatic protons of phenolphthalein give a characteristic pattern (6.5-8.0 ppm) that can be observed in DS P1 and PS2. Sample N5 is a DS without adulterant and, except for the reference and solvent signals, only the signal of some CH_2 protons of fatty acids from plant extracts is readily detected at 1.27 ppm. Minor signals corresponding to aromatic protons of natural polyphenols or other natural compounds are also detected in a few samples.

The LF ^1H NMR profile well corresponds to HF ^1H NMR, with signals more spread and overlap in the spectra. For instance, phenolphthalein exhibits a broad multiplet between 7.4 and 8.0 ppm which corresponds to three separated signals on HF ^1H NMR (7.91 ppm, m, H-7; 7.78 ppm, td, H-5; 7.63 ppm, m, H-4 & H-6). Another significant difference about phenolphthalein is that the AAX'X' system of two doublets on HF ^1H NMR (7.14 ppm, H-10, H-15, H-17, H-21; 6.80 ppm, H-11, H-14, H-18, H-20), transforms to an AA'BB' system centered at 6.94 ppm on LF ^1H NMR [4, 7]. For sibutramine, comparing with HF NMR, two singlets with the broad shape at 7.43 and 2.49 ppm are detected on LF ^1H NMR spectra; and two doublets at 1.03 and 1.06 ppm are significantly overlapped.

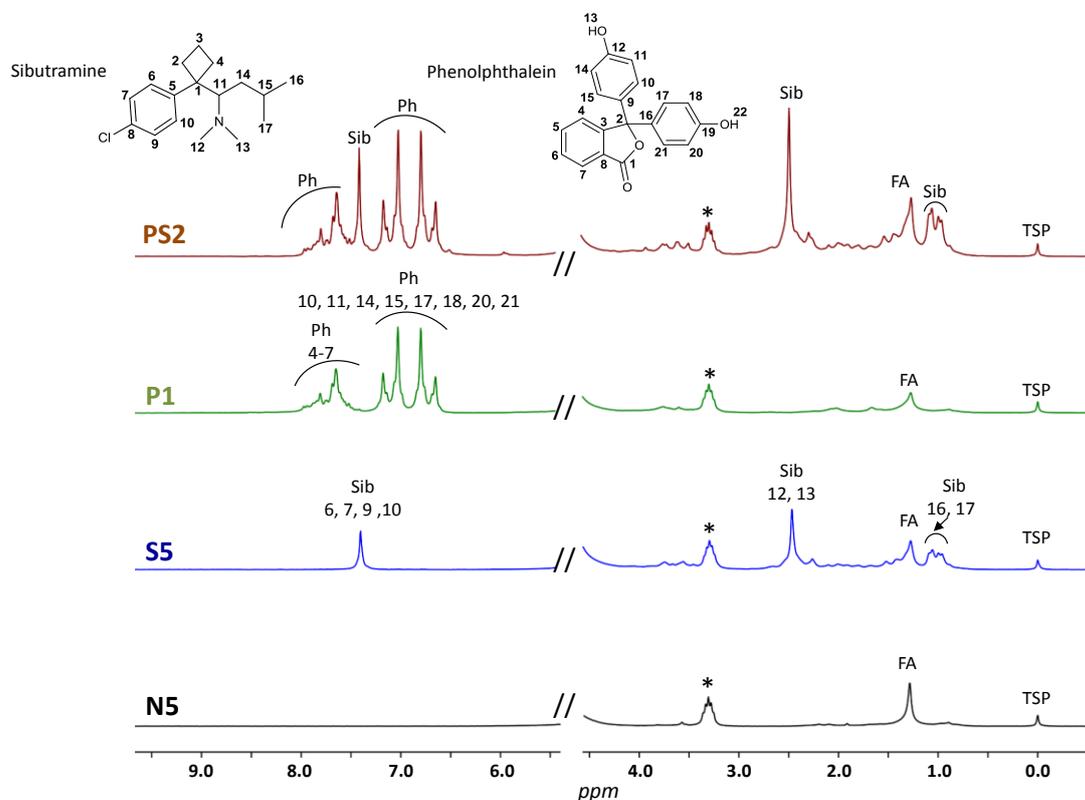


Fig III - 2. Typical LF ^1H NMR spectra of weight-loss dietary supplements recorded at 60 MHz (N, non-adulterated (natural) group; S, sibutramine-adulterated group; P, phenolphthalein-adulterated group; PS, both sibutramine and phenolphthalein-adulterated group). Ph: Phenolphthalein; Sib: Sibutramine; FA: Fatty acids; TSP: Internal reference; *: CD₂HOD.

3.3 Chemometrics Analysis of LF ^1H NMR data

To start the chemometric analysis, a statistical model was built by performing a two-class comparison: DS without adulterant (natural: N, $n = 19$) were compared to DS containing either sibutramine (S, $n = 12$) or phenolphthalein (P, $n = 9$), samples (S) and (P) being considered together ($n = 21$) as “adulterated samples”. After spectra processing (6–8 ppm region, see experimental part), bucketing and normalization of the data, the Partial Least Squares-Discriminant Analysis (PLS-DA) led to a predictive model with two principal PLS components and good validation criteria ($Q^2 = 0.61$, $R^2Y = 0.76$, $CV\text{-ANOVA} = 2.3 \times 10^{-18}$). All Q^2 and R^2 values were lower in the permutation test than in the model, confirming its goodness. The classification of all samples was then obtained from the two-class model based on the predicted Y-values (Y_{predPS} , which is the Y value predicted by the model based upon the X block variables (resonance intensities at given ppm)) indicating the probability that a sample belongs to one class of the model (adulterated or non-adulterated).

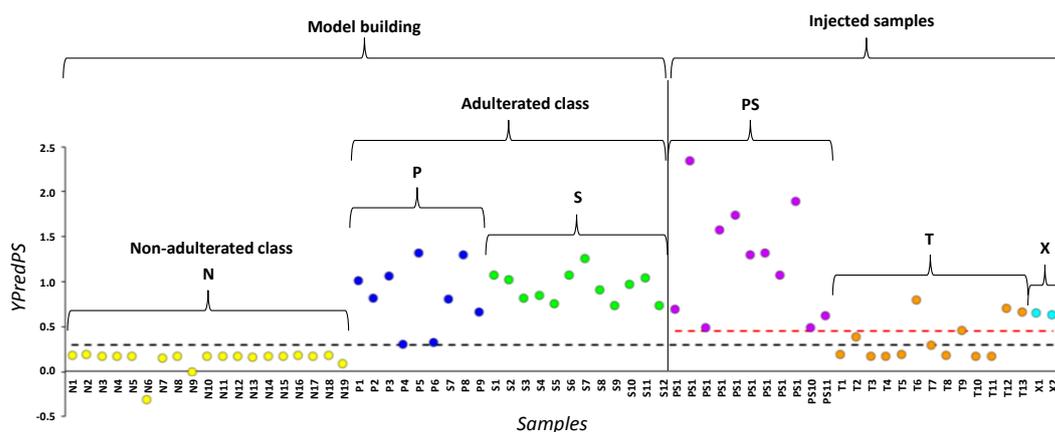


Fig III - 3. Predicted Y-values (Y_{predPS}) obtained for the 66 DS analyzed based on the two-class PLS-DA model comparing natural samples (N) to adulterated samples (samples (P) and (S) considered together as a single class of adulterated samples). Samples above the red dashed line ($Y_{\text{predPS}} = 0.45$) are defined as adulterated and those below the black dashed line ($Y_{\text{predPS}} = 0.30$) as natural. PS, both sibutramine and phenolphthalein-adulterated group; T: test samples, newly purchased DS; X: atypical DS.

YPredPS values for the 66 DS analyzed in this study are reported in Fig III - 3. Samples (N), (S) and (P) ($n = 40$), whose content was previously known [4], were considered for the definition of a Y-value threshold between adulterated and non-adulterated DS. An YPredPS value close or superior to 1 would indicate that the sample is likely to belong to the adulterated class while an YPredPS value close to 0 would indicate that the sample is likely to be natural. Conventionally, a threshold of 0.65 was defined for samples belonging to a defined class and a 0.65–0.35 range for samples borderline to the defined class [8]. In our study, it appears that the adulterated samples (P) and (S) have YPredPS > 0.65 , except P4 and P6 whose YPredPS are 0.30 and 0.32 respectively, whereas the highest YPredPS for (N) samples is 0.18. Based on the knowledge of the content of these two DS, we thus defined the lowest limit of the threshold at 0.30 (black dashed line in Fig III - 3). Samples (PS) previously shown as adulterated by both sibutramine and phenolphthalein were then injected into the model. Their YPredPS values being > 0.47 , the upper limit of the threshold was set at 0.45 (red dashed line in Fig III - 3). So, DS with YPredPS values > 0.45 were considered as belonging to the adulterated class, those with YPredPS values < 0.30 to the non-adulterated class, and samples with YPredPS values between 0.30 and 0.45 were considered as borderline.

If we apply these criteria to the newly purchased DS (T, test samples), the classification shows that samples T6, T9, T12, and T13 are predicted adulterated, and samples T2 and T7 borderline, whereas other T samples are predicted natural with Y values ≤ 0.18 (Table III - 1). Two atypical DS, X1 (YPredPS = 0.65) and X2 (YPredPS = 0.63), which will be discussed later, are predicted adulterated by the two-class model. In conclusion, this preliminary rapid analysis with the two-class PLS-DA model can be considered as the first screening of adulterated slimming DS leading to classification between natural, adulterated or possibly adulterated (borderline) samples.

Table III - 1. Classification list showing predicted Y-values (YPredPS) for test samples (T) based on the two-class PLS-DA model built with LF ¹H NMR data and completed by the visual observation of the projection of the samples on the three-class PLS-DA model shown in Fig III - 4A.

Identification	Predictive Y-value classification from the two-class PLS-DA		Projection on the three-class PLS-DA model shown in Fig III - 3A	
	YPredPS	Classification	Class membership	Adulterant
T1	0.18	natural	N	- ^a
T2	0.37	borderline	P	phenolphthalein
T3	0.16	natural	N	-
T4	0.17	natural	N	-
T5	0.18	natural	N	-
T6	0.79	adulterated	P	phenolphthalein
T7	0.30	borderline	P	phenolphthalein
T8	0.17	natural	N	-
T9	0.45	adulterated	S	sibutramine
T10	0.17	natural	N	-
T11	0.17	natural	N	-
T12	0.69	adulterated	S	sibutramine
T13	0.65	adulterated	S	sibutramine

^a Sample without adulterant.

To go further in the classification of the DS, a new PLS-DA analysis was carried out in which samples (N), (P) and (S) were considered as three distinct groups. A good predictive model was obtained with two principal PLS components ($Q^2 = 0.66$, $R^2Y = 0.74$), a p-value of the CV-ANOVA of 3.4×10^{-21} , and a permutation test successfully performed. The score plot of this three-class PLS-DA shows clear discrimination between the three categories of DS (Fig III - 4A). Samples (P) (dark blue) and (S) (green) appear more spread out than samples (N) (yellow) because of the variable amount of adulterant in each sample ranging from 8 to 16 mg per unit for sibutramine in samples (S) and from 5 to 55 mg per unit for phenolphthalein in samples (P) [4].

(PS) samples (purple) projected in this three-class PLS-DA model are located closer to (P) than to (S) samples (Fig III - 4B). This observation is in agreement with higher amounts of phenolphthalein compared to sibutramine contained in most samples [4]. The score plot of the projection of test samples (T) in the model confirms the classification proposed in Table III - 1 but affords a more precise analysis (Fig III - 4C). Indeed, samples T1, T3–5, T8, T10, and T11 overlap with (N) samples and can thus be considered as natural. Samples T9, T12, and T13 contain the adulterant sibutramine whereas samples T2, T6, and T7 contain phenolphthalein. It can be noticed that none

of the (T) samples belong to the (PS) class, *i.e.* contain a mixture of phenolphthalein and sibutramine. The statistical analysis of the (T) samples was done blindly, *i.e.* without a thorough examination of their LF (and HF) ¹H NMR spectra, these findings were confirmed by the visual analysis of these spectra.

The fact that the two samples T2 and T7 were considered as borderline in the classification established from the predicted Y-values of the previous two-class model (Table III - 1) but are now better characterized by the three-class model (Fig III - 4C) can also be explained by the visual observation of their LF ¹H NMR spectra. Indeed, as reported in Fig III - 5, signals of phenolphthalein are detected in samples T2 and T7 but with a lower signal-to-noise ratio than in the P1 spectrum due to the low amount of adulterant in these DS. We mentioned above that signals corresponding to aromatic protons of natural polyphenols or other natural compounds were detected in a few (N) samples (as an illustration, the LF ¹H NMR spectrum of sample N6 is shown in Fig III - 5). Their chemical shifts and intensities close to those observed for phenolphthalein in T2 and T7 samples led to the classification of these DS as borderline in the first approach (Table III - 1).

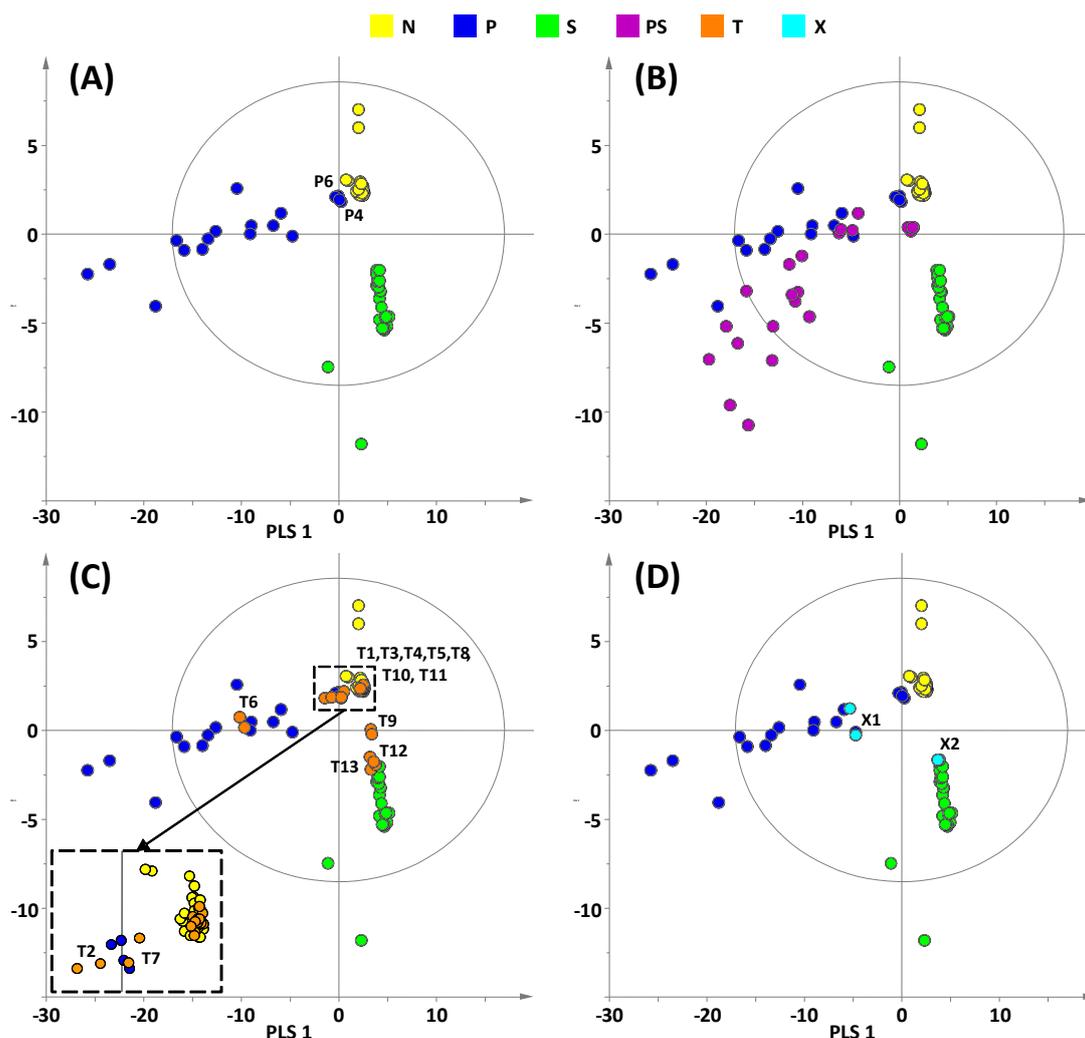


Fig III - 4. (A) Score plot of the PLS-DA three-class model built from LF ^1H NMR spectra of samples N (non-adulterated), S (adulterated with sibutramine), and P (adulterated with phenolphthalein). Score plots (B), (C) and (D) show the projection of samples PS (adulterated with both sibutramine and phenolphthalein), T (test samples) and X (atypical samples, see text) respectively on the built model (A).

A limitation of the present work is illustrated by the examples of the two DS X1 and X2. These samples appear as adulterated when considering their predicted Y-values (0.65 for X1 and 0.63 for X2) (Fig III - 3). Moreover, the projection of their LF ^1H NMR spectra in the PLS-DA three-class model indicates that the adulterants are phenolphthalein for X1 and sibutramine for X2 (Fig III - 4D). In fact, we demonstrated in a previous HF ^1H NMR study [4] that these two samples contain respectively raspberry ketone, a natural phenolic compound (probably intentionally added due to its high concentration in this particular DS), and fluoxetine, an antidepressant drug

illegally added. As the model was built with only the LF ^1H NMR data of DS (N), (S) and (P), the chemometric analysis leads to the misclassification of X1 and X2. The reason can be found in their LF ^1H NMR spectra (Fig III - 5). Indeed, the main aromatic signal of fluoxetine has a chemical shift (7.37 ppm) close to that of sibutramine (7.41 ppm) and the large aromatic multiplet of raspberry ketone overlaps with the resonances of phenolphthalein. Although chemical shifts of these chemicals are slightly different from those of sibutramine or phenolphthalein, the multi-alignment procedure applied before the statistical treatment results in their misclassification. Nevertheless, even if the adulterant statistically identified in X2 is not the good one (*i.e.* sibutramine instead of fluoxetine), this DS remains unsafe for the consumer and the goal of the statistical screening for detecting dubious samples is thus achieved.

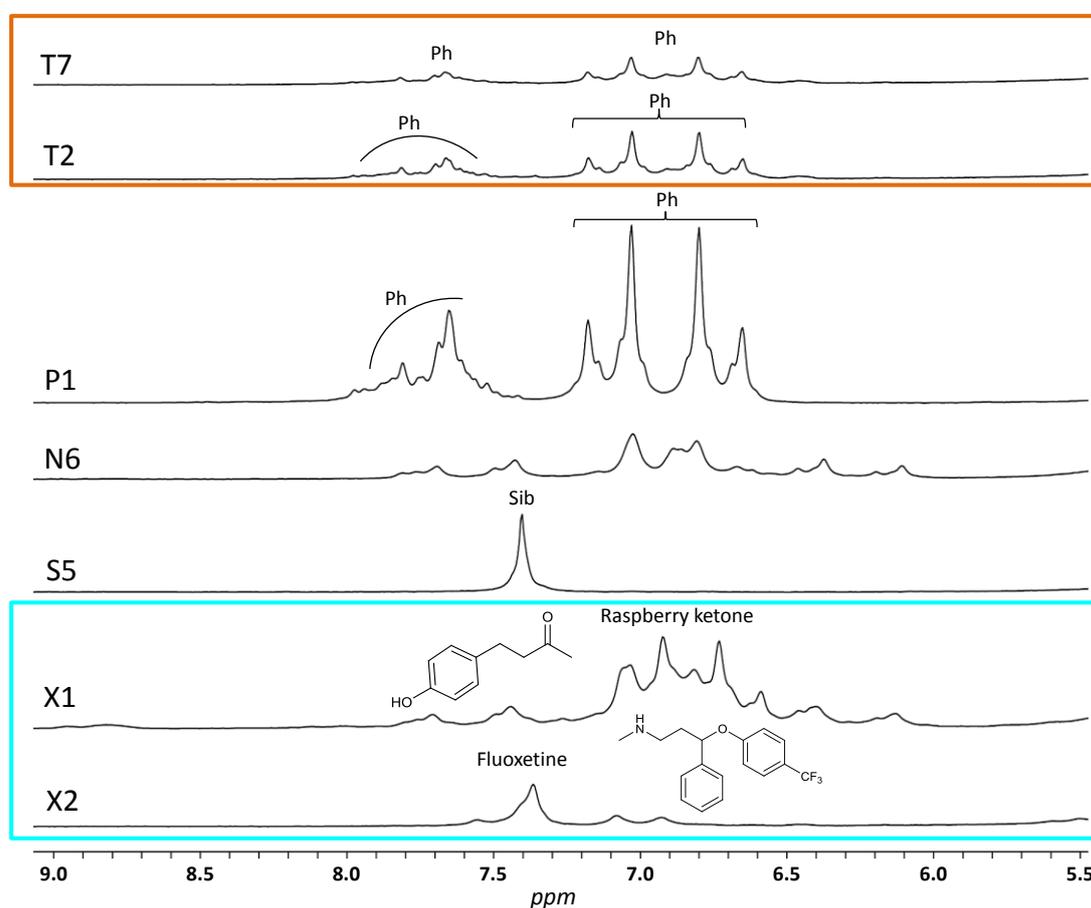


Fig III - 5. LF ^1H NMR spectra of some weight-loss dietary supplements recorded at 60 MHz. Ph: phenolphthalein; Sib: sibutramine.

In conclusion, the three-class PLS-DA works well as it enables a correct prediction of the nature of the adulterant sibutramine or phenolphthalein, the two banned drugs most commonly added to weight-loss DS to improve their effectiveness. The lowest limit of phenolphthalein concentration detected by the model is 3 mg per 100 mg of powder, which corresponds to ≈ 6 mg per unit if a mean capsule content weight of 200 mg is considered. The lowest limit of sibutramine concentration could not be reached because all the analyzed DS had YPredPS values > 0.7 , very far from the 0.3 value that characterizes the limit between adulterated and non-adulterated DS (Fig III - 3). A source of classification error is nevertheless possible if an adulterant or a natural compound has a structure leading to ¹H NMR signals in the resonance frequency areas considered to build the model. For example, the characteristic signal of the methyl protons 16 and 17 of sibutramine at 1.02 ppm could not be used to create the model because it was often overlapped with the resonance of fatty acids.

4. Conclusion

Our study shows that applying a chemometric treatment to LF ¹H NMR data is a means of widening the field of application of the technique, in particular for the analysis of complex mixtures. This approach has been successfully proposed in agri-food applications for the analysis and authentication of edible oils [9, 10] and meat [11]. Very recently, a similar approach was used for the analysis of substandard and falsified medicines [12]. Our study expands the field to the adulteration of DS, a problem at the crossroads between agri-food and health products. In the case of slimming DS adulteration, the analytical process proposed can be useful for the first-line detection of samples liable to be adulterated without resorting to expert analysis of the ¹H NMR spectra. Sample preparation is simple and fast, and LF ¹H NMR acquisition is easy, user-friendly and does not require specific NMR knowledge. The perspective of this study would be to automate the whole process to propose a turnkey method that could be implemented in quality control labs.

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Chapter IV Analysis of Synthetic Cannabinoids in E-liquids: A Proton and Fluorine NMR Study from a Conventional Spectrometer to a Compact One

1. Introduction

E-liquid products are spread worldwide with the popularity of e-cigarettes, especially among younger groups, but the psychoactive ingredients in these products have become an issue for the health risks [1]. Synthetic cannabinoids (SCs) are one group of new psychoactive substances adulterated in smoking agents including e-liquid that have been reviewed in *chapter I* (section 5.1 and 5.3). Moreover, the chemical structures of SCs are quite diverse and a large number of new psychoactive SCs have appeared on sale, which makes it even more difficult to distinguish them [2]. Until now, there are only a few publications concerning the characterization of SCs in e-liquid products (*chapter I*, section 5.4.2).

The goal of this study was to evaluate the ability of both high and low-field NMR for the characterization of e-liquids content. Proton and fluorine NMR analyses were implemented with both high- (400 MHz) and low-field (60 MHz) spectrometers. SCs and other flavouring compounds were identified. GC-MS was used as a confirmatory and reference method for the identification of SCs and flavouring agents. To the best of our knowledge, this study is the first using compact LF NMR for characterization of SCs compounds and flavouring agents in e-liquids.

2. Materials and experiments

2.1 Materials

13 e-liquid samples from customs seizures were provided by SCL laboratory (French Customs). The identification of SCs within each sample was provided by the SCL based on a routine GC-MS analysis and was further verified in this study. Standard SCs JWH-210, 5F-AKB48 and 5F-MDMB-PICA were obtained from Cayman Chemical (Ann Arbor, MI, USA). TSP, TFT, vanillin, ethyl maltol, limonene, methoxybenzyl alcohol, raspberry ketone and Cr(acac)₃ were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 GC-MS analysis

Each sample was stirred by vortex for 30 s before aliquoting. Then 250 μ l of each e-liquid sample were mixed with 500 μ l CHCl_3 . Analyses were performed using an Agilent 6890 N Gas Chromatograph coupled with a GCT Premier CAB109 TOF mass spectrometry (Waters). An HP-5MS capillary column (30 m \times 0.25 mm, 0.25 μ m film thicknesses) was used to perform chromatography separation. Helium as carrier gas was delivered at a constant flow of 1.0 ml/min. The temperature program was used as follows: the initial temperature was 70 $^\circ\text{C}$ with 2 min holding time, increased to 200 $^\circ\text{C}$ at 5 $^\circ\text{C}/\text{min}$, continue rising to 280 $^\circ\text{C}$ with a rate of 10 $^\circ\text{C}/\text{min}$, 24 min holding time at 280 $^\circ\text{C}$, the total run time was 60 min. The injection volume was 10 μ l including 4 samples with split injection modes (1:20). Mass spectra were recorded in the range m/z 30–650. The compounds were identified using SWGDRUG 3.3 (www.swgdrug.org) and NIST 08 databases.

2.3 NMR analysis

2.3.1 Sample preparation for NMR

200 μ l of each e-liquid were diluted with 400 μ l of MeOD, and mixed with 30 μ l of TSP (10 mM, MeOD) as reference for ^1H NMR experiments. ^{19}F NMR experiments were run for the 10 e-liquid samples (**4-13**) containing fluorinated SCs 5F-MDMB-PICA, 5F-ADB, 5F-AKB48 and ADB-FUBINACA. For these ^{19}F experiments, 200 μ l of each sample were diluted with 400 μ l of MeOD and then mixed with 9.1 μ l of TFT (1M in MeOD, final concentration 15 mM). 2 mg of $\text{Cr}(\text{acac})_3$ were added inside the NMR tube as relaxation reagent. HF and LF ^{19}F NMR experiments were performed by analysis of the same NMR tubes. All quantitative experiments were done in triplicate.

2.3.2 HF NMR analysis

HF ^1H NMR experiments were performed on a Bruker Avance 400 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) equipped with a 5 mm Triple resonance probe (TXO) with ^{19}F direct detection. Typical acquisition parameters optimized for quantitative experiments were as follows: number of scans (NS) 128, pulse width 4.52 μ s (flip angle 30 $^\circ$), acquisition time 3.40 s, spectral width 12 ppm, 32 K data points, and an additional relaxation delay of 10 s; the recording time was thus *ca.* 29 min. The signal of TSP set at 0 ppm was used as an internal reference for chemical shift measurement. Three samples with lower concentration of SCs (**1-**

3) were recorded by increasing the NS to 1024 for obtaining appropriate S/N. HF ^1H NMR data were processed by using Bruker TopSpin 2.1 software with one level of zero-filling and Fourier transformation after multiplying FIDs by an exponential line-broadening function (LB) of 0.3 Hz.

HF ^{19}F NMR experiments were performed on the same spectrometer and probe at 376 MHz. First, a basic zg sequence allowed to acquire fluorine-coupled proton spectra, then quantitative experiments were carried out using inverse gated decoupling sequence with following parameters: number of scans (NS) 32, pulse width 4.57 μs (flip angle 30°), acquisition time 1.57 s, spectral width 221.36 ppm, 256 K data points, LB at 2.0 Hz and an additional relaxation delay of 3 s for complete longitudinal relaxation; the recording time was thus less than 3 min. The signal of TFT set at -63.72 ppm was used as an internal reference for chemical shift measurement and quantitative analysis.

2.3.3 LF NMR analysis

Spectra were acquired on a PulsarTM benchtop NMR spectrometer (Oxford Instruments, Abingdon, UK) operating at a frequency of 59.7 MHz for ^1H and 56.17 MHz for ^{19}F . The temperature inside the spectrometer was 310 K. The acquisition was performed with the SpinFlow 1.2.0.1 software (Oxford Instruments) and the processing with MNova 11.0 (Mestrelab Research, Santiago de Compostela, Spain).

^1H NMR spectra FIDs were recorded with a flip angle of 90° (12.5 μs), a spectral width of 5000 Hz, and 8 K complex points (acquisition time of 1.64 s). The relaxation delay was set at 2 s, and 1024 transients were recorded leading to a total acquisition time of 1 h 2min. For data processing, the FIDs with one level of zero-filling were apodized with an exponential (0.3 Hz) filter and automatic Whittaker smoother baseline correction was applied.

For LF ^{19}F NMR experiments, FIDs were recorded with a flip angle of 90° , a spectral width of 20833 Hz, and 32 K complex points (acquisition time of 1.57 s). The relaxation delay was set at 1 s, and 2048 transients were recorded leading to a total acquisition time of 1 h 27 min. For the data processing, the FIDs were apodized with an exponential (3.0 Hz) filter; an automatic baseline correction has been applied.

2.4 NMR quantification

The concentrations were measured by comparing the signal areas of convenient nucleus (written in bold in Table IV - 2) of targeted compounds with the signal of reference, the area of each NMR peak being directly proportional to the number of nuclei giving rise to it. After phasing and baseline correction of the spectra, the targeted signals were integrated. The amount of each compound expressed in mg/ml of e-liquid sample was determined using the following general equation:

$$\text{Amount (mg / ml)} = \frac{A_{SCs}}{N_{SCs}} \times [REF] \times \frac{N_{REF}}{A_{REF}} \times V_{total} \times \frac{Mw_{SCs}}{V_{e-liquid}} \times 10^{-3}$$

[REF] (mM): Concentration of reference (TSP or TFT).

A_{SCs} and A_{REF} : Integrated signal areas of SCs and reference respectively.

N_{SCs} and N_{REF} : Number of nuclei giving rise to the signal considered.

V_{total} (μ L): Total volume of solution in NMR tube.

Mw_{SCs} (g/mol): Molecular weight of SCs.

$V_{e-liquid}$ (μ L): Volume of e-liquid used for the NMR assay.

2.5 Statistical Analysis

Excel was used for calculation of statistical parameters (mean, standard deviation and relative standard deviation), regression analysis and two-way ANOVA analysis.

3. Results and discussion

3.1 Qualitative analysis of e-liquids

In the first step of this study, 13 e-liquid samples were screened by HF 1 H NMR in order to characterize their SCs content and other chemicals present in the mixtures. In the same time, GC-MS was used as a gold standard method to confirm the identification of SCs as well as flavouring components.

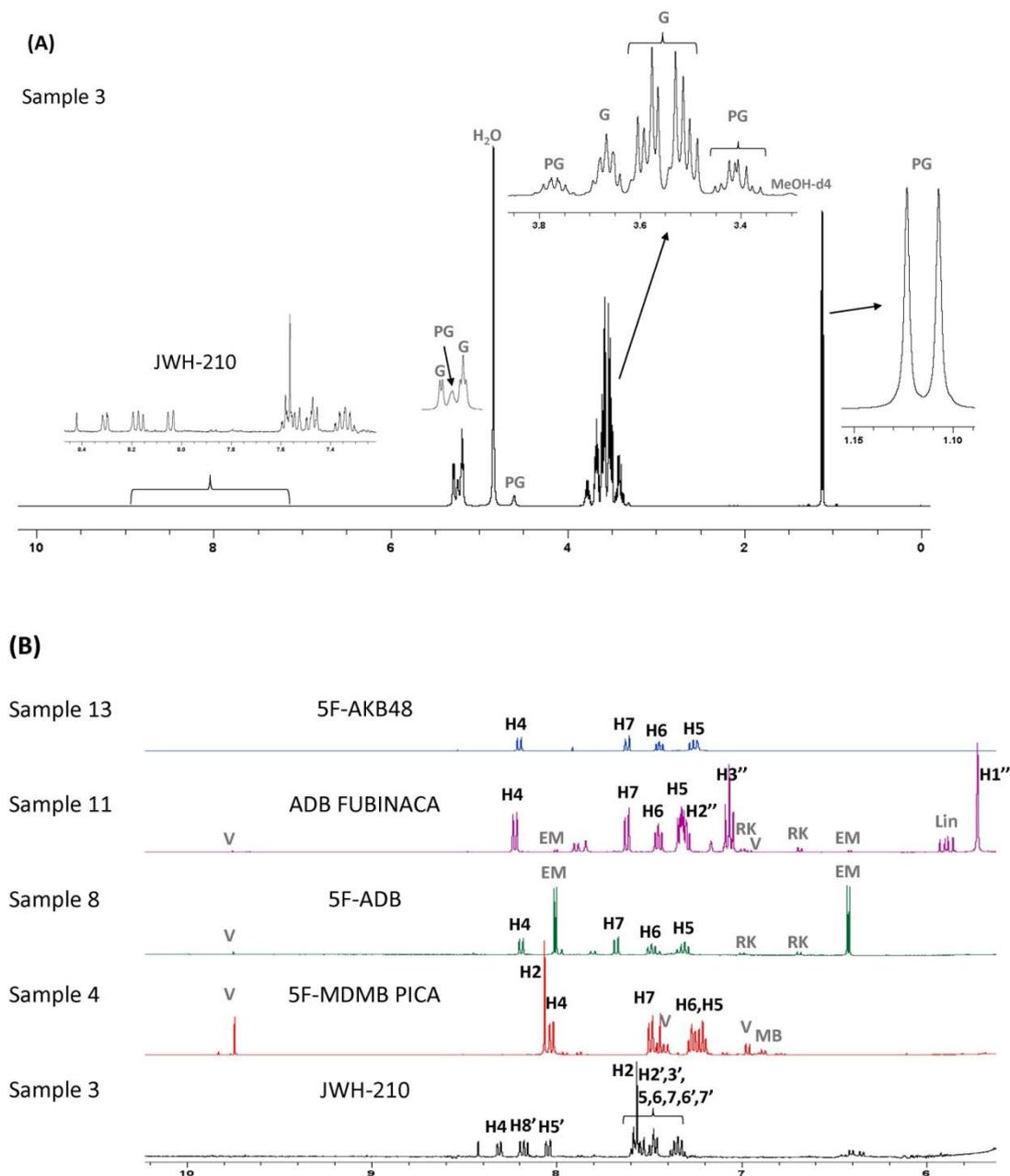


Fig IV - 1. Proton NMR spectra of e-liquids (400 MHz, in methanol-d₄) (A) Whole NMR spectrum of sample 3 (B) Overlap of 5 spectra of e-liquids containing different SCs (5.7-10.2 ppm). EM: Ethyl maltol, V: vanillin, RK: Raspberry ketone, Lin: Linalool.

The HF ¹H NMR spectra of all 13 samples allow the detection of major signals of propylene glycol (PG) and glycerol which constitute the matrix of e-liquids. As illustration, Fig IV - 1 (A) shows the complete HF ¹H NMR spectrum of one e-liquid sample 3. Matrix signals of PG and glycerol have the most significant signals at upfield area. Doublet of the methyl of

PG was detected at 1.11 ppm (6.4 Hz), multiplets are detected at 3.77 (<CH) and 3.41 (-CH₂) ppm as well as broad signals for hydroxyl protons at 4.60 and 5.24 ppm. Glycerol (CH and CH₂) lead to multiplets in the range of 3.47-3.70 ppm whereas hydroxyl groups resonate as doublet and triplet at 5.29 and 5.19 ppm respectively, depending on the rate of hydroxyl proton exchange, these signals are more or less board. Moreover, ethylene glycol and/or polyethylene glycol are detected in samples **9-12** from the characteristic intense broad signals around 3.6 ppm. At last, ethanol was detected in samples **6, 8, 10** and **11** by its methyl signal at 1.2 ppm whereas quadruplet of methylene overlaps with signal of glycerol at 3.59 ppm. The presence of ethanol in e-liquid increases the solubility of SCs and flavours and can significantly lower the viscosity of glycerol, which improves the formation of the aerosol for inhalation. Peace and co-workers previously evaluated alcohol content in e-liquid by headspace gas chromatography with flame ionization detector (HS-GC-FID) [3].

In addition to matrix, signals of psychoactive SCs are detected in the NMR spectra. Besides NMR analysis, GC-MS was performed for verification of SCs identification which was previously given by the Customs Laboratory. GC-MS results were obtained by comparison with the SWGDRUG library. Data are reported in Table IV - 1. Five SCs were identified: JWH-210 was detected in samples **1-3**, 5F-MDMB-PICA in samples **4-6**, 5F-ADB in samples **7-9**, ADB-FUBINACA in samples **10-12** and 5F-AKB48 in sample **13**. Structures of these SCs are based on indole or indazole rings with differently substituted carboxamide and alkyl side chains, four SCs being fluorinated.

The HF ¹H NMR profile (5.7-10.2 ppm) of samples **3, 4, 8, 11** and **13** are reported in Fig IV - 1 (B) and the assignments of SCs signals are given in Table IV - 2. Characteristic signals with close chemical shifts and multiplicity are observed for the indazole protons (H4, H5, H6 and H7) of 5F-ADB, ADB FUBINACA and 5F-AKB48 whereas signals of the indole ring (H2, H4, H5, H6, H7) in JWH-210 and 5F-MDMB PICA have a different spectral signature. For SCs bearing fluorine on the alkyl chain, doublet of triplet is observed for H5" due to both ²J coupling with fluorine nucleus and ³J_{HH} with neighbouring methylene. Protons from methylenic groups H1" adjacent to the nitrogen atom of indazole ring give characteristic unshielded triplets (≈ 4.3-4.5 ppm). All signals are not reported in Table IV - 2 due to overlap with matrix signals, for instance, singlet of methyl ester (5F-ADB or 5F-MDMB PICA) overlaps with the signal of propylene glycol around 3.7-3.8 ppm. NMR data characteristics for SCs are corresponding to previously published data [4-7].

Table IV - 1: SCs detected in e-liquids by GC/MS analysis.

Compounds	Formula	Retention time (min)	Theoretical exact mass (m/z)*	Acquired accurate mass (m/z)	Mass accuracy error (ppm)	Nominal fragments (m/z)	CAS
Synthetic cannabinoids (SCs)							
5F-ADB	C ₂₀ H ₂₈ FN ₃ O ₃	36.04	377.2109	377.2106	0.8	233, 289, 321	1838134-16-9
5F-MDMB PICA	C ₂₁ H ₂₉ FN ₂ O ₃	37.76	376.2157	376.2158	0.3	144, 212, 232, 288	1971007-88-1
ADB-FUBINACA	C ₂₁ H ₂₃ FN ₄ O ₂	40.50	382.1800	382.1801	0.3	109, 254, 309, 338	1445583-51-6
5F-AKB48	C ₂₃ H ₃₀ FN ₃ O	43.72	383.2367	383.2368	0.3	145, 233, 294, 355	1400742-13-3
JWH-210	C ₂₆ H ₂₇ NO	45.88	369.2087	369.2094	1.9	214, 312, 352	824959-81-1

* Theoretical exact mass is calculated by the mass of molecular ion (M⁺·) with losing one electron.

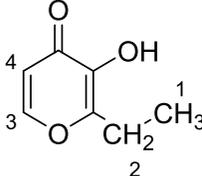
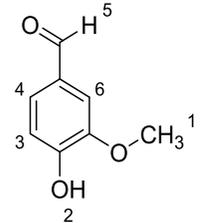
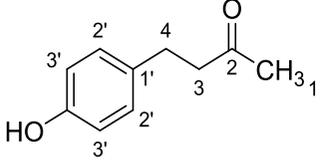
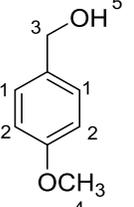
TableIV - 2. The high-field ¹H NMR (400 MHz) characteristics signals of synthetic cannabinoids detected in e-liquid samples in MeOD. Signals in bold were used for quantification. Proton signals mentioned as overlap or missing in the table were not readily detected due to overlap with other signals.

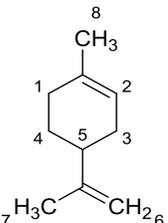
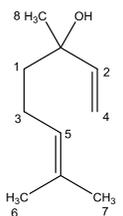
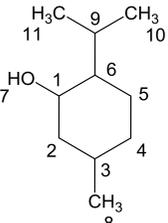
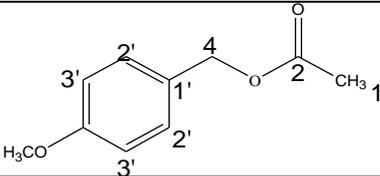
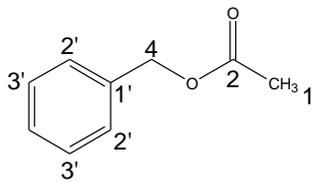
¹ H label	JWH-210			5F-MDMB PICA			5F -ADB			ADB FUBINACA			5F-AKB48		
	δ (ppm)	M ^a	J(Hz)	δ (ppm)	M	J(Hz)	δ (ppm)	M	J(Hz)	δ (ppm)	M	J(Hz)	δ (ppm)	M	J(Hz)
2	7.56	s		8.06	s										
4	8.31	dd	7.0; 1.3	8.02	d	7.8	8.20	d	8.2	8.22	d	8.2	8.20	d	8.2
5	7.30-7.60	m		7.21	m		7.31	m		7.31	m		7.26	m	
6	7.30-7.60	m		7.27	m		7.48	m		7.44	m		7.44	m	
7	7.30-7.60	m		7.49	d	8.2	7.68	d	8.6	7.61	d	8.5	7.62	d	8.5
1"	Overlap			4.26	t	7.1	4.54	t	7.0	5.72	s		4.48	t	7.0
2"	1.78	qt	7.5	1.92	qt	7.5	2.01	qt	7.5	7.31	m		1.98	qt	7.5
3"	Overlap			1.42	m		1.43	m		7.06	appt	8.8	1.42	m	
4"	Overlap			1.69	m		1.72	m					1.72	m	
5"	0.82	t	7.0	4.40	dt	47.5/6.0	4.39	dt	47.5/6.0				4.39	dt	47.5/6.0
2'	7.44-7.60	m		Overlap			Overlap			Overlap			2.20	m	
3'	7.44-7.60	m											2.13	bs	
4'													1.78	bs	
5'	8.04	d	8.4												
6'	7.30-7.60	m													
7'	7.30-7.60	m													
8'	8.18	d	8.6												
CH ₃	1.42	t	7.5												
(CH ₃) ₃				1.09	s		1.08	s		1.09	s				

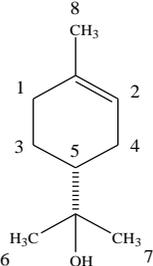
^a M: multiplicity; s: singlet; d: doublet; dd: doublet of doublet; t: triplet; dt: doublet of triplet; q: quadruplet; qt: quintuplet; m: multiplet.

In addition to matrix and SCs signals, other chemicals were detected in the HF ^1H NMR spectra of samples, flavouring compounds like limonene, vanillin, ethyl maltol, raspberry ketone, methoxybenzyl alcohol, linalool and menthol were successfully detected. Indeed, flavouring ingredients are widely used in e-liquids to give fruit aromas or other pleasant scents. ^1H NMR assignments of flavouring compounds detected in all samples are reported in Table IV - 3, identification of flavouring compounds in e-liquids were confirmed by spiking with standard compounds. Vanillin, the most common flavour agent, was detected in 8 out of 13 samples. The more significant signal is that of the aldehyde proton which has the higher chemical shift with a singlet detected at 9.74ppm (Fig IV - 1B), protons on the aromatic ring resonate in the range 6.9-7.5 ppm whereas singlet of methoxy group can be detected at 3.92 ppm. Ethyl maltol which has sweet caramelized sugar smell was detected in six samples. It has two characteristic doublets at 8.00 and 6.42 ppm corresponding to aromatic protons, whereas protons from ethyl group are detected at 2.74 ppm (q, H2) and 1.22ppm (t, H1). Characteristic signals of raspberry ketone were detected in three samples with doublets of para-substituted aromatic ring at 7.00 and 6.68 ppm and singlet of the methyl group at 2.11 ppm. Linalool was detected in sample **11**, characteristic doublet of doublet at 5.89 ppm is shown in Fig IV - 1B. The characteristic up-field signals of menthol allow its detection in sample **13**. The identification of flavouring compounds is generally supported by previous GC-MS characterization of e-liquids [8, 9].

Table IV - 3. High-field ^1H NMR and GC-MS data of flavouring compounds detected. NMR data were obtained from e-liquid samples spectra in MeOD, only signal detected are reported.

Compound	Structure	^1H NMR	GC-MS			Detected in samples
		δ (ppm), multiplicity ^b (J, Hz), attribution	Retention time	Formula Measured accurate m/z Value (mass error in ppm)	m/z of major characteristic fragment ions observed	
Ethyl maltol		8.00, d (5.5), H3 6.42, d (5.5), H4 2.74, q (7.6), CH ₂ 1.22, t (7.6), CH ₃	9.89	C ₇ H ₈ O ₃ 140.0467 (0.7)	55, 69, 71, 97, 139	7-12
Vanillin		9.74, s, H5 7.46, m, H4 7.44, s; H6 6.96, d (8.5), H3 3.92, s, -CH ₃	15.00	C ₈ H ₈ O ₃ 152.0462 (3.9)	81, 151, 109, 123	4-9, 11,12
Raspberry ketone		7.00, d (8.5), H2' 6.69, d (8.5), H3' 2.11, s, H1	19.18	C ₁₀ H ₁₂ O ₂ 164.0834 (1.2)	107, 131, 149	8, 10, 11
Methoxybenzyl alcohol		6.88, d (8.7), H2 4.52, s, H3	12.04	C ₈ H ₁₀ O ₂ 138.0676 (0.7)	77, 109, 121	4

<p>Limonene</p> 	<p>4.68, m, H6 1.62, broad singlet, H8</p>	5.50	<p>$C_{10}H_{16}$ 136.1245 (1.5)</p>	68, 79, 93	6
<p>Linalool</p> 	<p>5.89, dd, (17.4;10.8), H2 5.01, dd (10.8, 1.6), H4(1H) 1.65, broad s, CH3 7 1.58, broad s, CH3 6 1.23, broad s, CH3 8</p>	7.19	<p>$C_{10}H_{18}O$ (3.9)</p>	71, 93, 121	10,11
<p>Menthol</p> 	<p>1.61, m, H5 (1H) 0.92, d (7.1), CH3 10 0.91, d (6.6), CH3 8 0.84, m, H5 (1H) 0.79, d (6.9), CH3 11</p>	- ^c	-	-	13
<p>Anisyl acetate</p> 	ND ^a	15.39	<p>$C_{10}H_{12}O_3$ (1.1)</p>	77,91,121	4
<p>Benzyl acetate</p> 	ND	8.77	<p>$C_9H_{10}O_2$ (2.0)</p>	79, 91, 108	4

α -Terpineol		Overlap ^b	9.62	C ₁₀ H ₁₈ O (2.6)	59, 93, 121, 136	6
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^a No signals detected in ¹H NMR spectra for low concentrations.

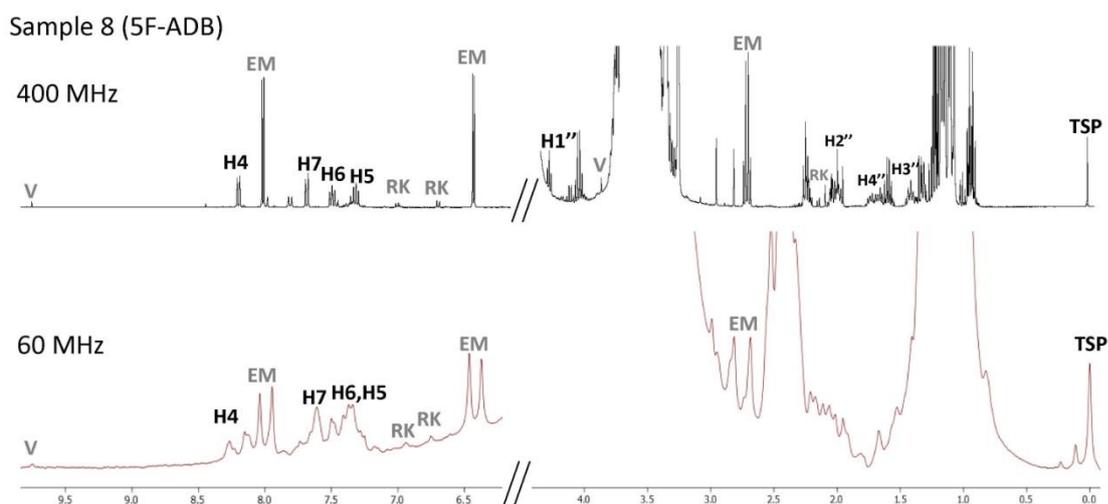
^b Signals overlap with matrix in ¹H NMR.

^c Menthol was not detected in GC-MS analysis.

3.2 Low-Field ^1H NMR profiling

All samples were then analyzed by using the benchtop 60 MHz ^1H NMR spectrometer with the same NMR tubes and solutions as HF. The ^1H NMR spectra of sample **8** analyzed on both HF and LF spectrometers are shown in Fig IV - 2 (A). Compared to HF spectra, LF signals are broader with more signals overlapping due to the lower resolution. Signals are especially crowded in the low frequency region (0.5-4.5 ppm) where targeted signals are erased by intense matrix signals, but some hints still can be gotten in ethylenic/aromatic region (*i.e.* above 5.6 ppm region). Fig IV - 2 (B) shows the five typical LF profiles of samples **3**, **4**, **8**, **11** and **13**. The reported zoom is the same as in Fig IV - 1(B) in HF spectrum. For sample **3**, the SC JWH-210 being at low concentration, it cannot be detected under the chosen experimental conditions. For the four other samples, even if signals are broad and similarities appear, the LF profile of each sample differs from the others. For instance, signal of H2 from 5F-MDMB PICA in sample **4** provides a spectral signature different from H1" of ADB FUBINACA in sample **11**. The SCs profile of 5F-ADB and 5F-AKB48 in samples **8** and **13** are closer, even if the multiplet around 7.2 ppm is more spread out in sample **13**. Concerning flavouring compounds content, the singlet of vanillin is observed at 9.74 ppm in samples **4** and **8**, likewise for the two characteristic doublets of ethyl maltol in sample **8**.

(A)



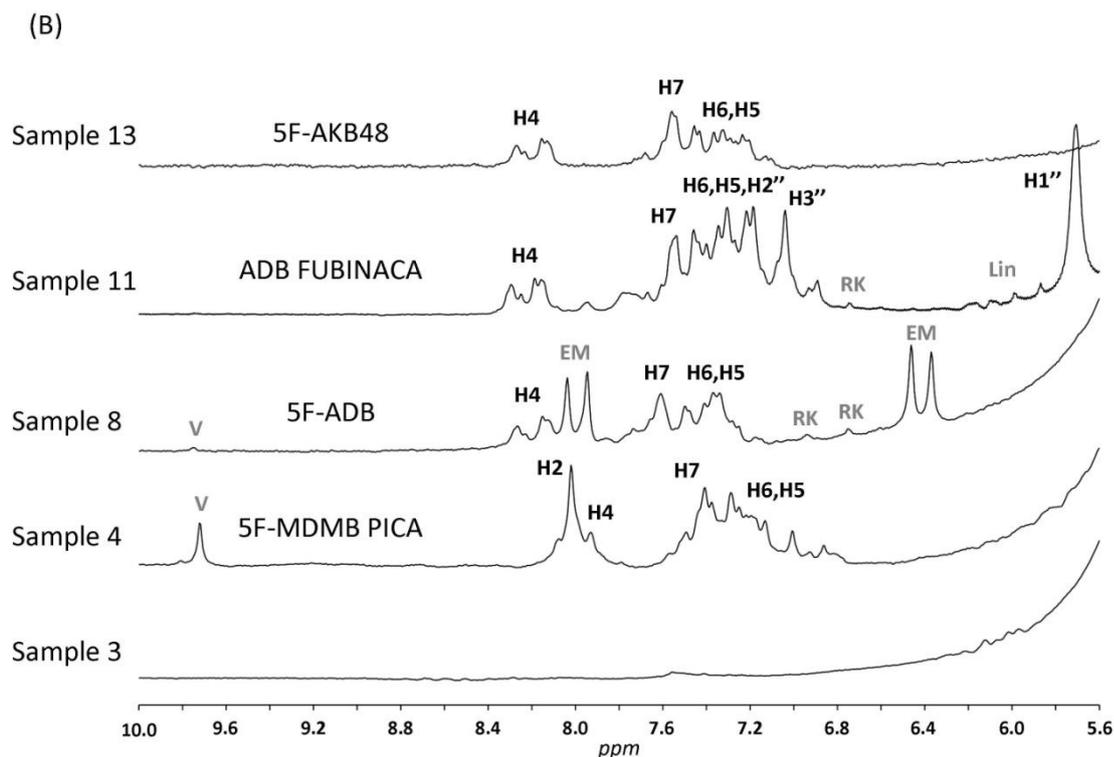


Fig IV - 2. LF (60 MHz) ^1H NMR spectra. (A) Comparison of the ^1H NMR spectra of sample **8** recorded in CD_3OD on both HF (400 MHz) and LF (60 MHz) spectrometers; (B) 5 superimposed spectra (60 MHz) with different SCs (5.7-10.2 ppm). EM: Ethyl maltol, V: vanillin, RK: Raspberry ketone, Lin: Linalool

According to LF proton analyses of all samples, it appears that despite intense matrix signals, the detection of characteristic signals of SCs can be performed if their concentration is not too low. These ^1H LF NMR experiments constitute a proof of concept that direct profiling of e-liquids containing SCs could be relevant and should be applied on a larger number of samples before chemometrics treatment as recently proposed in Agri-food and pharmaceutical applications [10-12]. However, in this study, we do not further investigate the proton nucleus NMR experiments on the compact spectrometer because the method is not suitable for quantitative purpose.

3.3 ^{19}F NMR analysis

It was reported during the last decades an increasing number and rate of emergence of fluorinated SCs, this fact is explained by an increased CB1 receptor potency of fluorinated analogues [13]. Insofar the ^{19}F nucleus has favourable NMR characteristics (nuclear spin of 1/2, relatively narrow lines, 100% natural abundance, good sensitivity (83% that of proton) and

large chemical shift range), the fluorine NMR analysis should be of great interest for these fluorinated cannabinoids as reported recently by Naqi *et al* [14].

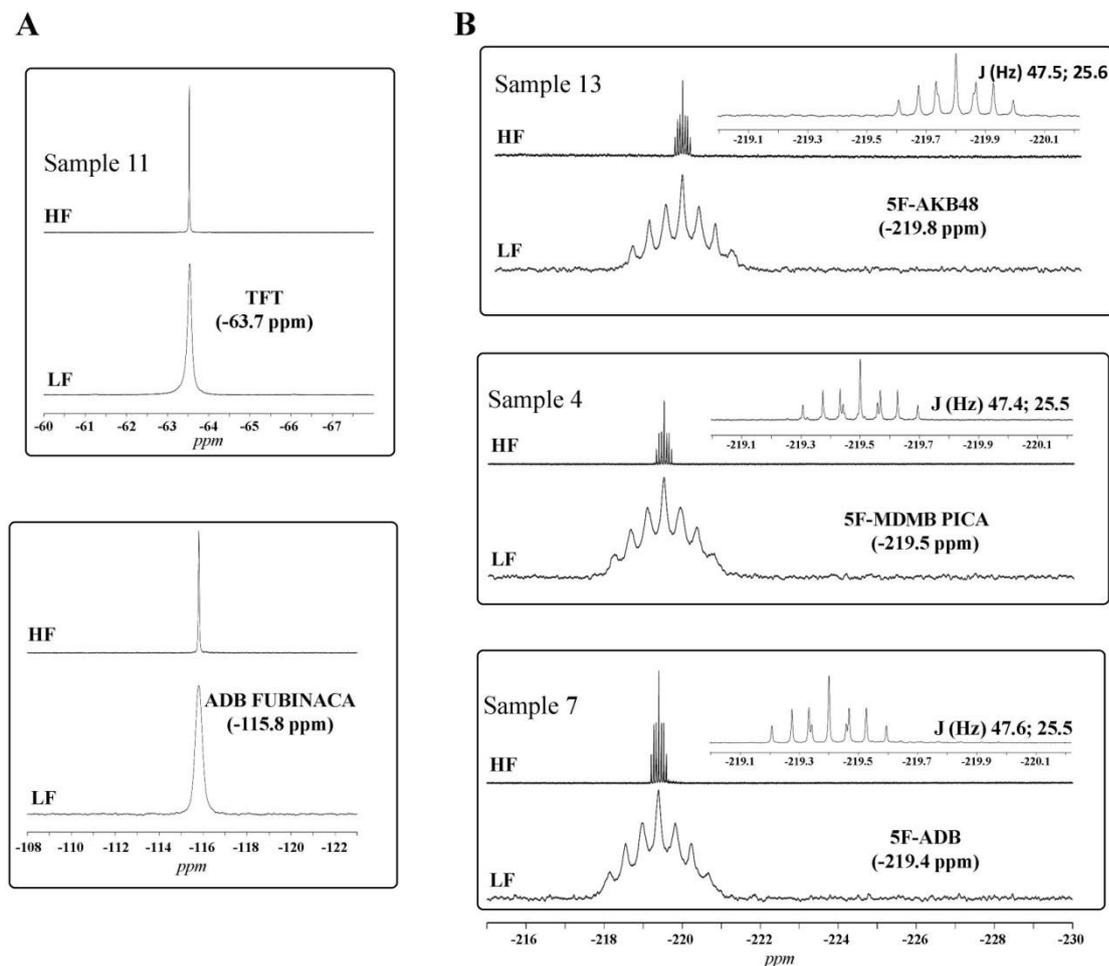


Fig IV - 3. ^{19}F NMR signals of TFT and SCs in e-liquids samples recorded at 376 MHz (HF) and 56.17 MHz (LF). (A) Signals of TFT and ADB FUBINACA in sample **11**; (B) Signals of 5F-AKB48, 5F-MDMB PICA and 5F-ADB in sample **13**, **4** and **7** respectively.

The fluorinated SCs detected in this study can be analyzed using ^{19}F NMR. Spectra were recorded for all samples at both HF and LF NMR (376 and 56.2 MHz respectively). Typical spectra without proton decoupling are presented in Fig IV - 3, with HF and LF spectra stacked. Trifluorotoluene (TFT) was added in all samples as reference for chemical shift (-63.7 ppm) and quantification. Fluorine of ADB FUBINACA linked to the aromatic ring lead to a singlet at -115.8 ppm (Fig IV - 3A). Fluorine nuclei in the end position of the alkyl chains for 5F-AKB48, 5F-MDMB, 5F-ADB give signals around -220 ppm (Fig IV - 3B). The two closer resonances are those of 5F-ADB and 5F-MDM-PICA with a $\Delta(\text{Hz})$ of 37.8 Hz at HF and 6 Hz

at LF. In the HF spectra, they resonate as triplet of triplets due to coupling with methylene H5" ($^2J_{\text{HF}}=47.5 \pm 0.1$ Hz) and H4" ($^3J_{\text{HF}}=25.5 \pm 0.2$ Hz). For the same samples, LF signals are more spread as coupling constant values are independent with magnetic fields and their multiplicity is close to an apparent septuplet ($^3J_{\text{HF}}\approx 23.7 \pm 0.4$ Hz).

Unlike LF proton NMR analyses of e-liquid that present limitations for quantitative purpose and cannot be implemented because of strong signals overlap, ^{19}F NMR opens the possibility of setting up quantitative analyzes.

3.4 Quantitative analysis

3.4.1 Setting up of quantitative conditions

The quantitative study aimed to consider proton and fluorine HF qNMR as reference quantitative methods to evaluate the ability of quantification of LF NMR using fluorine-19. Indeed, HF qNMR is recognized as a powerful method for quantification of compounds in various mixture samples for both ^1H [4, 15-18] and ^{19}F [17-19]. Recently, HF ^{19}F NMR was used for quantification of fluorinated SCs in herbal blends [14]. In this study, in order to accurately quantify SCs in e-liquids, MeOH-d₄ was chosen as solvent because of good miscibility with e-liquids. First, T₁ relaxation time was determined by the inversion-recovery pulse sequence method to set acquisition parameters of quantitative NMR experiments for both proton and fluorine nuclei.

HF T₁ values of protons selected for quantification and internal reference were measured in samples **3**, **4**, **7**, **11** and **13**. The longest T₁ was that of reference signal TSP measured at 3.6 s in standard solutions and T₁ of protons of SCs and TSP measured in samples of e-liquids were always shorter. T₁ values of targeted signals for quantification were measured in the range of 0.4-1.80 s in samples. All ^1H resonances of SCs were thus considered as fully relaxed since more than 99% of the signal intensity of the proton with the longest T₁ was recovered in the recording conditions used (flip angle of 30° and repetition time of 15.1 s).

For fluorine HF NMR quantifications, the relaxation agent Cr(acac)₃ was added inside the NMR tube to reduce the relaxation time of ^{19}F nuclei. The T₁ of 4 fluorinated SCs in e-liquids were decreased to lower than 200 ms, TFT has the longest T₁ but it remains below 300 ms. In the same way on the LF spectrometer with the same tubes, T₁ of SCs were less than 300 ms and that of TFT was below 350 ms. Spectra recording conditions were chosen to reach a full

relaxation of signals. Indeed, at HF, we used 4.57 s of repetition time for a flip angle of 30°, so all ¹⁹F nuclei were completely relaxed since more than 100% of the ¹⁹F signals were recovered. In LF experiments, with a flip angle of 90° and 2.57 s of repetition time, more than 99.9% of the ¹⁹F signals were recovered. Experiment time was set at 2.5 min (32 scans) and 90 min (2048 scans) for HF ¹⁹F [12] and LF ¹⁹F NMR experiments leading to a S/N of 200 and 50 respectively. In the LF analysis, the limit of detection (LOD) and quantitation (LOQ) were estimated by analyzing the diluted solutions of standard SCs (5F-AKB48 and 5F-MDMB PICA) with known concentrations. The LOD of 5F-MDMB PICA on LF ¹⁹F qNMR was evaluated at 0.33 mg/ml, and LOQ was 0.92 mg/ml for the same signal. The LOD and LOQ values are 0.25 mg/ml and 1.0 mg/ml for 5F-AKB48 respectively. The LOQ determined on the standards are significantly lower than the concentration of four species of fluorinated SCs in e-liquids (Table IV - 4). Moreover, the LOQ of ADB FUBINACA is better due to the multiplicity of the signal (singlet).

3.4.2 Quantitative analyses of SCs in e-liquids

Both ¹H and ¹⁹F qNMR experiments were successfully implemented, the quantitative results of SCs in each sample are reported in Table IV - 4. For ¹H NMR quantification, although some SCs signals overlap, at least one characteristic signal can be integrated for each SC. Concerning the ¹⁹F qNMR experiments, signals of SCs and reference (TFT) are clearly identified and can be easily integrated in both HF and LF experiments.

HF proton and fluorine NMR are both recognized as quantitative methods [14, 15]. Results reported in Table IV - 4 showed that, even if a matrix effect could bias the quantification in ¹H NMR, data obtained by both techniques are in good agreement. Indeed, the linear regression equation of the HF ¹H and ¹⁹F values displayed a slope of 0.990 ± 0.028 , a y-intercept of -0.338 ± 0.455 and a correlation coefficient of 0.980. Then, by comparing LF ¹⁹F with HF ¹⁹F quantifications, we demonstrated the correct agreement. Indeed, the linear regression equation of these data set displayed a slope of 1.085 ± 0.035 , a y-intercept of -0.942 ± 0.543 and a correlation coefficient of 0.975. These results demonstrated that NMR is a useful method to quantify SCs in complex e-liquid samples, and that the ¹⁹F NMR quantification of fluorinated SCs can be achieved by using a compact LF NMR spectrometer without specific standard compounds nor purification of targeted compounds.

Table IV - 4. Quantification of SCs in 13 e-liquids measured by using HF ^1H , ^{19}F NMR and LF ^{19}F NMR. Results are expressed as mg/ml of SC \pm SD. All experiments were performed in triplicate.

Amount(mg/ml) Sample ^b	Cannabinoids	HF NMR		LF NMR
		^1H NMR	^{19}F NMR	^{19}F NMR
1	JWH-210	0.85 \pm 0.03	- ^a	-
2		0.89 \pm 0.04	-	-
3		1.12 \pm 0.04	-	-
4	5F-MDMB PICA	18.38 \pm 0.27	19.33 \pm 0.27	18.41 \pm 0.25
5		17.23 \pm 0.21	17.98 \pm 0.11	17.31 \pm 0.26
6		15.23 \pm 0.51	14.88 \pm 0.36	14.28 \pm 1.22
7	5F-ADB	15.59 \pm 0.21	15.60 \pm 0.24	16.04 \pm 0.61
8		7.34 \pm 0.07	7.73 \pm 0.25	7.98 \pm 0.17
9		10.85 \pm 0.36	11.71 \pm 0.43	11.78 \pm 0.51
10	ADB FUBINACA	15.12 \pm 0.22	15.36 \pm 0.06	15.20 \pm 0.48
11		16.56 \pm 0.31	17.83 \pm 0.41	17.53 \pm 0.44
12		15.67 \pm 0.25	16.29 \pm 0.21	14.95 \pm 0.13
13	5F-AKB48	24.09 \pm 0.03	24.05 \pm 0.06	22.69 \pm 0.48

^a JWH-210 without fluorine nuclei on the structure.

^b Sample numbering follows the list of Table IV - 3.

As shown in Table IV - 4, thirteen e-liquid products contained various concentrations of SCs, sample **1-3** contained a significantly lower dose JWH-210 around 1 mg/mL, whereas sample **13** contained more than 20 mg/ml of 5F-AKB48. The contents of other fluorinated SCs including 5F-MDMB-PICA, 5F-ADB and ADB-FUBINACA were ranged between 7 to 20 mg/ml in samples **4-12**. Quantitative results of each sample by different approaches were verified with acceptable precision (RSD < 10 %). Furthermore, concerning the difference of qNMR results from three approaches (HF ^1H NMR, HF ^{19}F NMR and LF ^{19}F), the two-way ANOVA analysis revealed no statistically significant differences ($p > 0.05$). The noticeable point is that JWH-210 as the second generation of SCs adulterated in smoking agents has been banned after 2010 in many countries, and the contents are obviously lower in samples **1-3** involved in this research [20]. Whereas the newly emerged 3rd generation SCs of 5F-ADB [21], 5F-AKB48 [22], 5F-MDMB PICA [23] and ADB FUBINACA [24] were detected to have a high order of content in illegal products. Such a higher content of newer SCs adulterated in e-liquid products is alarming. Additionally, these 3rd generations SCs have a significantly higher CB1/CB2 binding potent than JWH-210 and predecessor THC (Table IV - 5), and 5F-AKB48 also binds with nanomolar affinity to these receptors [25], which can pose a great health danger

to consumers. In 2018, 5F-ADB, ADB-FUBINACA and 5F-MDMB PICA were the first, third and fifth-most common synthetic cannabinoid identified in drugs seized by US-DEA, and 5F-AKB48 was also noted in this list [26]. Therefore, the quality control of e-liquids containing illegal SCs becomes really important and urgent.

Table IV - 5. EC₅₀ values of different SCs

Drug	CB1 EC ₅₀ (nM)	CB2 EC ₅₀ (nM)
JWH-210 [27]	25.3	17.5
5F-MDMB PICA [28]	0.45	7.4
5F-ADB [28]	0.59	7.5
ADB FUBINACA [29]	1.2	3.5
THC [29]	172	- ^a

^a THC no CB2 agonist activity in the central nervous system.

In this study, NMR as a powerful analytical method can bring both qualitative and quantitative information for quality control of SCs in e-liquid. A phenomenon is that the novel SCs usually have fluorinated structures, the reason is that the introduction of ¹⁹F into SCs can enhance the psychoactive potency, and also to evade the legal control imposed on specified SCs [21]. Thus, the increase of 3rd generation SCs including a large part of fluorinated SCs, allows proposing ¹⁹F NMR for screening samples liable to be adulterated with SCs. Moreover, concerning LF NMR application, even if LF ¹H NMR quantitative analysis is difficult to implement in complex mixtures, LF ¹⁹F qNMR as an easy and robust quantitative method constitutes a relevant alternative for fluorinated SCs analysis. Thus, we think that LF NMR is a "low-cost" NMR method that could act an important role in the forensic lab.

4. Conclusion

In this study, 13 commercial e-liquid formulations were screened by both ¹H and ¹⁹F NMR. HF ¹H NMR was used for a holistic analysis of SCs and flavouring compounds in e-liquids, data were also confirmed by GC-MS. Moreover, the ability of benchtop LF NMR spectroscopy for both ¹H and ¹⁹F nuclei was evaluated. To the best of our knowledge, this is the first study dealing with analysis of e-liquid containing SCs by using LF NMR. Even though ¹H LF NMR has shown limits for quantitative applications particularly due to (i) huge gap of concentration between matrix and active compounds and (ii) signals overlaps, some typical signals can however be distinguished. The interesting novelty of the study is that LF ¹⁹F NMR can easily reach the quantification of fluorinated SCs without complex sample preparation and the need

of specific standard compounds. Thanks to the detection specificity of LF fluorine NMR, this study can open the way to routine detection of illicit fluorinated SCs in various matrices such as e-liquids, herbal blends or bath salts.

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Conclusion and Perspectives

This doctoral thesis aimed to explore the capabilities and limitations of benchtop LF NMR spectroscopy for quality control and fraud detection of complex mixture samples.

In the first study dedicated to cinnamon samples analysis, we have shown that even though LF ^1H NMR is less sensitive and spectra much less resolved than HF NMR, the specific signals of key compounds (E)-cinnamaldehyde and coumarin still can be assigned. Then, the combination of LF NMR data with multivariate PCA analysis has proven to be an interesting way for classification of cinnamon species and determines a quantification result of coumarin and (E)-cinnamaldehyde. For cinnamon dietary supplements, PCA was further used to identify samples with a relatively high content of coumarin. We have thus demonstrated that LF NMR can provide valuable clues on the quality control of cinnamon species.

The second study focused on the quality control of herbal slimming dietary supplements. We have shown that LF NMR in combination with chemometric methods allowed detecting adulteration with pharmaceutical substances. According to the PLS-DA model built, samples containing sibutramine and/or phenolphthalein or non-adulterated samples were successfully classified. At last, two atypical samples were analyzed in order to discuss the limitation of the approach due to the poor spectral resolution.

In the third study, LF NMR was applied to the analysis of synthetic cannabinoids in a complex matrix of e-liquid products. In that case, proton LF NMR allows detection of typical signals of synthetic cannabinoids and signals of flavouring compounds. The huge signal intensity difference between the matrix and target compounds was one major issue and the quantitative analysis of synthetic cannabinoids with LF ^1H NMR couldn't be reached. However, the interest of this study was to focus on the fluorine nucleus that allows detection and quantification of the fluorinated cannabinoids.

Overall, in this thesis, benchtop LF NMR was explored as a simple analytical method for quality control of herbal products and e-liquids. Our study broadens the applications of LF NMR. Indeed, despite the low sensitivity of the technique and the complexity of mixtures analyzed, useful hints for chemical analysis can be obtained from LF NMR. In the case of herbal species discrimination and adulterants identification in commercial products, the proposed

analytical process and statistical approach could be useful for the first-line detection of products liable to be adulterated.

We have shown that LF NMR spectroscopy can be applied for botanical species discrimination and adulterants detection in herbal products, there are still perspectives of this field.

- Recent technological or sequence developments can be valuable in the field of LF NMR and help improve its potentials. For instance, pulsed field gradients implemented in some commercial LF NMR instruments are useful for solvent suppression or two-dimensional NMR sequences. Moreover, ultrafast 2D NMR recently implemented enables the acquisition of a whole 2D spectrum, providing structural information within a single scan. A novel method to gain on the sensitivity of observed signals that can be applied to LF is to use hyperpolarization of nuclear spins. Indeed, the signal amplification by re-versible exchange (SABRE) is considered as a rapid and efficient method and can be applied to liquid samples under standard conditions with relatively cheap equipment. Finally, to increase the resolution of spectra, pure-shift method offers significant advantages to collapse a multiplet to a singlet in ^1H NMR spectra.
- The combination of LF NMR data with chemometric treatment is an effective and efficient way to overcome the relatively low sensitivity and the signals overlap. It allows chemists to classify complex samples and detect fake samples. Significant progress in this field will require implementation of share databases, as is done in HF NMR-based metabolomics. Then chemometrics or machine learning tools could eventually be used for recognition of spectral pattern of a targeted specie or of mixtures.
- In analytical chemistry, the complementarity of techniques and their chemometric treatment in an “omic” manner is of course of great interest and has developed considerably over the last ten years. Recently, like compact NMR spectrometers, benchtop or portable equipment for GC-MS or LC-MS has appeared on the market. It can be envisaged that in future years, an efficient integrated analytical process with “low-cost” benchtop equipments may be proposed.

Résumé

Introduction générale

La Résonance Magnétique Nucléaire (RMN) est, outre une puissante méthode de détermination structurale, une technique analytique non sélective, qui peut se montrer particulièrement adaptée à des applications de contrôle qualité comme par exemple pour l'analyse de produits naturels. Comparée à des méthodes chromatographiques de routine, la RMN peut fournir à la fois des informations structurales et des résultats quantitatifs. De plus, c'est une méthode robuste et non destructrice qui nécessite une préparation d'échantillon relativement simple, ce qui la rend bien adaptée à l'analyse directe de milieux complexes tels que des produits alimentaires, des extraits de produits naturels, des médicaments ou d'autres formulations [1-3].

Cependant, le coût élevé des spectromètres RMN à haut champ et de leur maintenance cryogénique limite leur utilisation pour l'analyse chimique de routine. La commercialisation récente de spectromètre RMN de paillasse à bas champ (LF) pourrait être une alternative intéressante pour pallier à ces inconvénients [4, 5]. En effet, bien que les spectres soient moins bien résolus, la RMN LF, avec un appareillage nécessitant peu de maintenance et une facilité d'utilisation, peut offrir aux analystes des performances suffisantes pour résoudre certaines problématiques d'analyse. Elle ouvre ainsi de nombreuses perspectives d'application, en particulier comme méthode peu onéreuse de screening rapide. Pour les échantillons les plus complexes, des analyses statistiques multivariées des données spectroscopiques peuvent être mise en œuvre pour extraire des informations pertinentes [4-6].

Les travaux réalisés au cours de cette thèse portent sur l'exploration de la RMN LF de paillasse comme outil d'analyse pour le contrôle qualité de matrices complexes, comme des compléments alimentaires ou des mélanges contenant des molécules psychoactives. Le but de ces recherches est d'identifier et de quantifier toutes les substances actives, naturelles ou synthétiques pouvant être présentes dans ces milieux.

Les préparations ou compléments alimentaires à base de plantes sont devenus incontournables en raison des préoccupations grandissantes des consommateurs pour leur santé et de l'image positive des produits naturels. Cependant, il est courant, sur ce marché très lucratif,

de trouver des produits falsifiés avec, par exemple, des informations d'étiquetage erronées sur leur origine ou sur les ingrédients présent.

La première étude porte sur la cannelle qui est une épice très appréciée pour sa saveur aromatique et ses possibles bienfaits sur la santé [7, 8]. Il existe principalement deux espèces de cannelle : la vraie cannelle de Ceylan ou *Cinnamomum verum* (*C. verum*) et la Cannelle Casse ou *Cinnamomum cassia* (*C. cassia*). Les deux espèces sont largement commercialisées, avec une très large prédominance sur le marché de la cannelle Casse à cause de son prix plus bas lié aux nombreuses plantations de cette espèce à travers le monde [9]. Elle remplace fréquemment la *C. verum* lors de la conception des produits à base de cannelle sans que le consommateur en soit informé. La *C. cassia*, contient une teneur plus élevée en coumarine que la *C. verum* dans laquelle elle est présente sous forme de trace (0.004% m). Une consommation excessive de cette molécule peut entraîner l'apparition d'une hépatotoxicité [7]. Le contrôle et l'authentification de la cannelle commercialisée sous forme d'épice ou de complément alimentaire, est donc très importante pour la santé du consommateur et nous nous sommes intéressés à ces deux catégories d'échantillons.

La deuxième étude est axée sur un exemple plus sérieux de falsification. En effet, dans certains cas, des fabricants peu scrupuleux ajoutent frauduleusement des molécules synthétiques pharmacologiquement actives pour améliorer l'efficacité d'un complément alimentaire [5, 10]. Un complément alimentaire est défini comme une denrée alimentaire, constituée de produits naturels dont le but est de compléter un régime alimentaire normal, mais l'adultération des compléments alimentaires est devenue ces dernières années un problème de santé publique [11]. Le contrôle de la qualité de ces produits à base de plantes est donc d'un intérêt majeur. Dans ce contexte nous avons utilisé la RMN LF comme outil pour la détection de produit frauduleux pour une catégorie de compléments alimentaires dit de « confort », les amaigrissants.

Le dernier projet porte sur l'analyse des produits utilisés pour le vapotage, les e-liquides, qui se vaporisent sous forme d'aérosol à inhaler lorsque la cigarette électronique est activée [12, 13]. Le marché des cigarettes électroniques étant mal contrôlé dans de nombreux pays, il est fréquent de trouver en plus de la composition de base en propylène glycol, glycérol et eau, des molécules actives ajoutées, comme par exemple des cannabinoïdes de synthèse (SCs). Ce sont ces substances, qui solubilisées dans un e-liquide, vont produire des effets psychoactif sur le consommateur. De nombreux cas ont déjà été reporté sur l'utilisation de ces SCs [14-16]. Nous

avons réalisé une étude par RMN LF pour identifier et quantifier les SCs dans une optique de contrôle ou recherche de produits illicites dans ces e-liquides. Lors de cette étude, certaines limites de la RMN LF ^1H ont été atteintes mais la RMN LF ^{19}F a montré un fort potentiel pour l'analyse de SCs fluorés.

Ce mémoire débute par une introduction générale. Il se poursuit par le **Chapitre 1**, une partie bibliographique qui présente l'application de la RMN LF pour le contrôle de la qualité des produits à base de plantes et la détection des fraudes. Ce chapitre décrit la classification et la composition des espèces de cannelle, introduit le problème des adultérations dans les compléments alimentaires amaigrissants et présente la problématique des cannabinoïdes de synthèse détectés dans des produits récréatifs. Tous ces sujets sont présentés en décrivant les méthodes analytiques couramment utilisées. Les **Chapitre 2 et 3** présentent respectivement le contrôle de la qualité des produits commerciaux à base de cannelle et des compléments alimentaire amincissants à base de plantes. Le **Chapitre 4** décrit la détection de cannabinoïdes de synthèse dans des e-liquides en utilisant la RMN du proton et du fluor, sur des spectromètres à haut et bas champ. Enfin, une conclusion générale avec des perspectives termine ce manuscrit.

Chapitre I Etude bibliographique

1.1 RMN à bas champ pour le contrôle qualité

Les spectromètres de paillasse modernes sont commercialisés avec des intensités de champ magnétique faibles correspondant à des fréquences de résonance de 40 à 100 MHz. Grâce à un ensemble d'évolutions techniques (aimants, électronique, séquence), les spectromètres RMN LF offrent actuellement aux analystes une facilité d'utilisation et des performances satisfaisantes. Ils permettent des contrôles de routine, sur un appareillage peu encombrant, pour un cout relativement bas et sans maintenance cryogénique [4-6]. En effet, de nombreuses applications récentes de RMN LF ont décrit le contrôle qualité de différentes matrices dont des huiles alimentaires [17], de la viande [18], des médicaments [19] ou des compléments alimentaires [5].

Bien sûr, la RMN LF a une résolution spectrale inférieure à la RMN conventionnelle et l'attribution des signaux ainsi que l'identification des composés peut devenir délicate dans des mélanges complexes ou des extraits de plantes. Cependant, il est souvent possible de repérer un ou plusieurs signaux caractéristiques et ainsi d'obtenir des informations pertinentes. Un traitement des données RMN LF par des analyses chimiométrique supervisées ou non supervisées, comme par exemple l'analyse en composantes principales, va permettre d'identifier les similitudes et les différences entre les échantillons malgré la complexité de ces mélanges [18, 20, 21].

1.2 Cannelle: de la composition à l'authentification

Comme décrit précédemment, il existe principalement deux espèces de cannelle (Fig I-1) utilisées dans le monde. La cannelle Casse (*C. cassia*) est l'espèce la plus largement répandue et donc la moins chère. Toutefois, elle contient une teneur significativement plus élevée en coumarine hépatotoxique que la vraie cannelle de Ceylan (*C. verum*) et la question de l'authenticité de la cannelle est donc un point important pour contrôle de la qualité des produits à base de cannelle [22].



Fig I-1. Deux espèces de cannelle, *C. verum* (à gauche) et *C. cassia* (à droite).

La cannelle contient divers composés aromatiques tels que le (E)-cinnamaldéhyde, l'eugénol, l'acide cinnamique et le cinnamate, l'ensemble des composés contribuent à l'arôme et au goût épicé spécifique de la cannelle [23]. Durant les vingt dernières années, différents bienfaits ou propriétés thérapeutiques ont été rapportés pour le cinnamaldéhyde et les autres composants de la cannelle comme des effets antioxydants [24], antidiabétiques [25], anti-inflammatoires [26], antibactériens [27], antifongiques [28] et même anticancéreux [29].

Les chromatographies liquides (HPLC) et gazeuses (GC) couplées ou non avec la spectrométrie de masse ou d'autres méthodes de détection sont les méthodes qui ont été principalement utilisées pour le contrôle de la qualité des produits à base de cannelle. Les résultats ont montré que les échantillons de *C. verum* ne contenaient que des traces de coumarine (<0.004%) alors que les *C. cassia* en contenaient des quantités substantielles (≈0.1-12%). Seules quelques études ont proposé d'utiliser la RMN comme méthode d'analyse de la cannelle. *Killday et al* ont quantifié les principaux composés aromatiques présents dans des échantillons de cannelle en poudre et dans des compléments alimentaires par RMN du proton à 600 MHz [8]. Dans une autre étude, les analyses chimiométriques des données RMN ¹H ont été effectuées pour distinguer les échantillons d'écorce de *C. verum* et de *C. cassia*. Une analyse supervisée OPLS-DA des signaux RMN a montré que l'eugénol était un marqueur de *C. verum* et que les acides gras étaient présents en plus grande quantité dans les échantillons de *C. cassia* [1].

1.3 Compléments amincissants à base de plantes et adultération

Les compléments alimentaires amincissants à base de plantes sont souvent annoncés comme 100% naturels, efficaces pour la perte de poids et sont généralement considérés par les consommateurs comme sans danger pour la santé [5, 30]. Cependant, de nombreux cas d'adultération avec des substances actives ont été rapportés. Ces ajouts sont réalisés de manière intentionnelle dans le but d'améliorer l'efficacité du produit commercialisé. Des adultérants de

diverse nature ont déjà été identifiés comme des anorexiques, des anxiolytiques, des antidépresseurs, des laxatifs ou des diurétiques présents seuls ou parfois en mélange [31, 32].

La sibutramine est un médicament anorexique qui est couramment détecté dans les compléments alimentaires amaigrissants. Il a été retiré du marché en 2010 à cause du risque d'accident cardiaque lié à ses effets secondaires comme la forte augmentation de la tension artérielle et de la fréquence cardiaque chez les patients ayant consommé régulièrement cet molécule [33]. La phénolphtaléine est la seule substance laxative identifiée dans les formulations amincissantes. Elle est souvent associée à la sibutramine pour induire un effet amincissant significatif à court terme [34]. Les effets de la phénolphtaléine peuvent être graves pour la santé avec notamment des risques de cancers [35-37]. D'autres adultérants ont également été signalés dans les compléments alimentaires amincissants comme la fluoxétine [30], l'orlistat [10, 30], la caféine [11, 35], la fenfluramine [34, 35], le sildénafil [30, 34] ou la lorcaserine [38].

Outre le couplage chromatographie-spectrométrie de masse (LC/MS ou LC-MS/MS), la RMN et en particulier la RMN ^1H HF, peut être utilisée pour la détection et la caractérisation structurale des adultérants dans les compléments alimentaires amincissants [30]. Une première étude réalisée par *Pagès et al* a évalué les capacités d'une RMN de paillasse à 60 MHz pour identifier les adultérants pharmaceutiques dans des compléments alimentaires. La sibutramine et la phénolphtaléine ont été facilement détectées après une préparation simple et rapide des échantillons [5].

1.4 Cannabinoïdes synthétiques et ajouts illicites dans les produits commerciaux

En raison d'un cadre strict de régulation interdisant dans de nombreux pays la vente et la consommation de cannabis, des cannabinoïdes synthétiques (SCs) utilisés comme produits récréatifs psychoactifs ont fait leur apparition ces dernières années dans les produits pour fumeurs ou dans d'autres formulations [39-42]. La Fig I-2 présente des mélanges de plantes et d'e-liquides à vapoter contenant des SCs. Certaines préparations contiennent des SCs ajoutés par imprégnation dans des mélanges d'herbes ou les mélangent directement avec la matrice dans le cas des e-liquides. Les SCs peuvent avoir des structures proches des cannabinoïdes naturels comme le THC ou des structures totalement différentes. Ils ont cependant tous des activités pharmacologiques similaires et une forte affinité pour les récepteurs CB1 et / ou CB2 [14].

Certain SCs peuvent être plus dangereux que la THC, comme par exemple le SC HU-210 avec une action 100 à 800 fois plus puissante que le THC [43]. Afin d'échapper à la réglementation, de nombreux SCs ont été synthétisés. Il existe à l'heure actuelle, trois générations de composés et plus de sept familles structurales [39, 41, 44].



Fig I-2. Exemple de produits commerciaux contenant des SCs: à gauche, mélanges à base de plantes [45]; à droite, e-liquides [46].

En raison de la diversité et de l'évolution rapide des structures des SCs ainsi que de l'absence de données publiées, la détection de ces composés est devenue un challenge pour les analystes. La RMN est une technique de choix, puisqu'elle fournit à la fois des informations structurales et quantitatives. Elle peut être utilisée en complément ou être une alternative aux méthodes chromatographiques couplées ou non à la spectrométrie de masse. Si la RMN HF a déjà été largement utilisée pour la caractérisation structurales de SCs, peu d'étude ont à notre connaissance été réalisées par RMN LF [4, 47].

Chapitre II Évaluation de la spectroscopie RMN du proton à bas champ pour le contrôle de la qualité d'échantillons de cannelle

Dans cette étude, 28 échantillons, 14 épices et 14 compléments alimentaires ont été analysés par RMN HF (400 MHz) et LF (60 MHz). La RMN HF a été utilisée comme méthode de référence à la fois pour réaliser une analyse qualitative et quantitative des principaux constituants. Ensuite, le potentiel de la RMN LF comme outil de contrôle pour la qualité des échantillons de cannelle a été évalué.

2.1 Profilage RMN des échantillons

Après des essais préliminaires de préparation des échantillons et d'analyse, le méthanol (MeOH-d₄) a été choisi comme solvant d'analyse car il permettait d'obtenir un très bon taux d'extraction avec une préparation simple des échantillons. Tous les échantillons ont été analysés par RMN HF à partir de prises d'essais de 15 et 100 mg de poudre. La prise d'essai de 15 mg permet de réaliser une extraction complète des molécules les plus abondantes ($\approx 97\%$) et celle de 100mg permet de quantifier les molécules les moins abondantes ($\approx 0.1-1$ mg/g de poudre). Pour les analyses par RMN LF, seule la pesée à 100 mg a été considérée en raison de la sensibilité plus faible de l'appareil.

La Fig II-1(A) présente le spectre RMN HF du proton d'un échantillon de cannelle présentant les attributions des principaux signaux, en particulier celles des composés aromatiques : le (E)-cinnamaldéhyde avec le H1 de la fonction aldéhyde à 9,66 ppm, la coumarine avec les protons éthyléniques H3 et H4 à 6,42 et 7,94 ppm, l'acide (E)-cinnamique, le o-méthoxy-cinnamaldéhyde et l'alcool cinnamylique. D'après ces profils RMN, quatre compléments alimentaires ne contenaient pas de cannelle (ou à l'état de trace) contrairement à ce qui était annoncé sur la notice. L'eugénol qui est un marqueur de *C. verum* [1, 48], a été identifié et quantifié dans 5 échantillons, tandis qu'aucun signal de la coumarine n'a été détecté dans ces mêmes échantillons, permettant de le classer dans la famille de la vraie cannelle *C. verum*. Les échantillons contenant un niveau détectable et quantifiable de coumarine (16 au total) ont été considérés comme appartenant à la famille des *C. cassia* sans classification plus précise selon l'origine géographique ou l'espèce. Enfin 3 échantillons n'ont pu être classés à partir des données RMN, dans la mesure où seul le (E)-cinnamaldéhyde a été détecté. La Fig II-1(B) présente le spectre RMN LF du même échantillon de cannelle. Les signaux des protons

H1 et H2 du (E)-cinnamaldéhyde ainsi que les protons H3 et H4 de la coumarine ont pu être facilement identifiés.

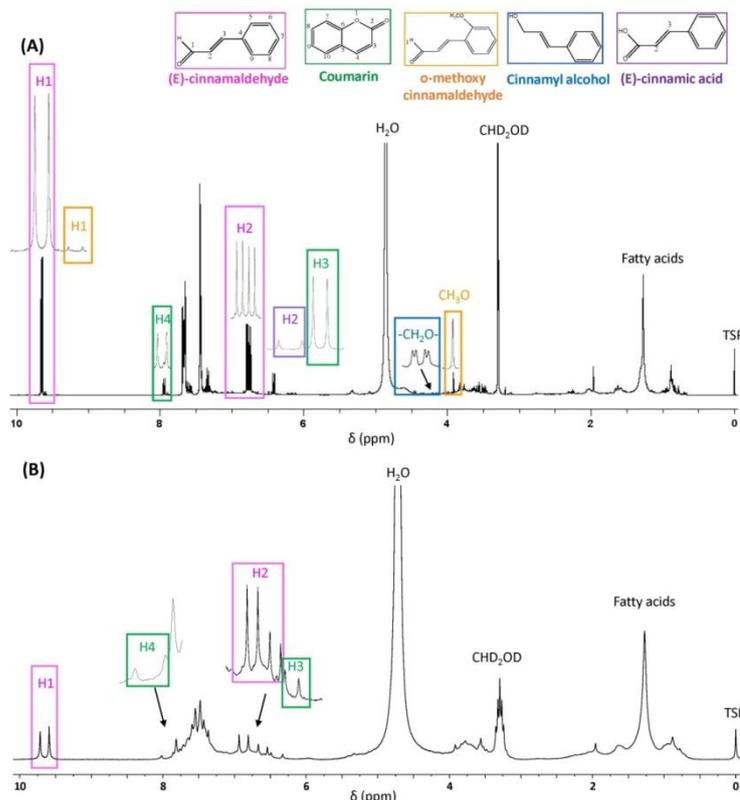


Fig II-1. Spectres RMN ^1H HF (A) et LF (B) d'un échantillon de cannelle mettant en évidence les principaux signaux des composés aromatiques. La durée d'enregistrement était de 8 min à HF et de 2 h à LF.

L'analyse quantitative n'a pu être réalisée avec précision en RMN LF. En effet, la perte de sensibilité par rapport à la RMN HF, nous oblige, pour rester dans des temps d'analyse corrects, de travailler avec des quantités plus importantes de poudre (100 mg). La perte de résolution va également entraîner des difficultés pour l'intégration des signaux RMN, puisque en plus des superpositions de signaux plus fréquentes qu'à HF, certains signaux d'intérêt, comme les protons H3 et H4 de la coumarine, résonnent sous forme d'un système AB. Enfin, lors du traitement des spectres, la correction de la ligne de base est délicate et aura un impact plus important sur les résultats à cause du plus faible rapport signal sur bruit des signaux. Nous avons néanmoins choisi d'enregistrer les profils RMN LF de tous les échantillons dans des conditions expérimentales non quantitatives puis d'appliquer un traitement statistique pour proposer une pseudo-quantification des 2 composés principaux.

2.2 Classification des échantillons de cannelle par RMN LF ^1H combinée à la chimiométrie

Dans un premier temps, une ACP a été générée à partir de la région des protons oléfiniques et aromatiques (5.7-11 ppm) des spectres RMN LF de tous les échantillons commerciaux (Fig II-2A). Sur la base de l'analyse quantitative HF, trois classes d'échantillons ont été colorées sur le score plot de l'ACP: en rouge, les échantillons qui ne contiennent ni (E)-cinnamaldéhyde ni coumarine, en bleu les échantillons classés comme *C. verum*, c'est-à-dire sans coumarine mais contenant de l'eugénol et enfin en vert, tous les autres échantillons. On peut remarquer une très bonne séparation de ces 3 classes avec le regroupement des échantillons de *C. verum* et une très grande dispersion des échantillons verts, liée aux proportions très variables de coumarine et de (E)-cinnamaldéhyde.

Le deuxième score plot (Fig II-2B) est uniquement basé sur l'analyse statistique d'une partie des doublets H3 et H4 de la coumarine. On distingue le regroupement des échantillons bleus et rouges dans lesquels la coumarine n'a pas été détectée. Dans le groupe vert, on distingue 2 échantillons, dans la partie inférieure droite du score plot le long du premier axe, qui ont une quantité plus élevée de coumarine ce qui est en accord avec la quantification à HF (5.4 et 6. mg/g) et 2 autres échantillons atypiques situés dans la partie supérieure droite du score plot, le long de l'axe 2, en raison de la quantité importante d'acide cinnamique (3.74 et 4.10 mg/g) dont le signal du proton H2 se superpose avec celui du proton H3 de la coumarine.

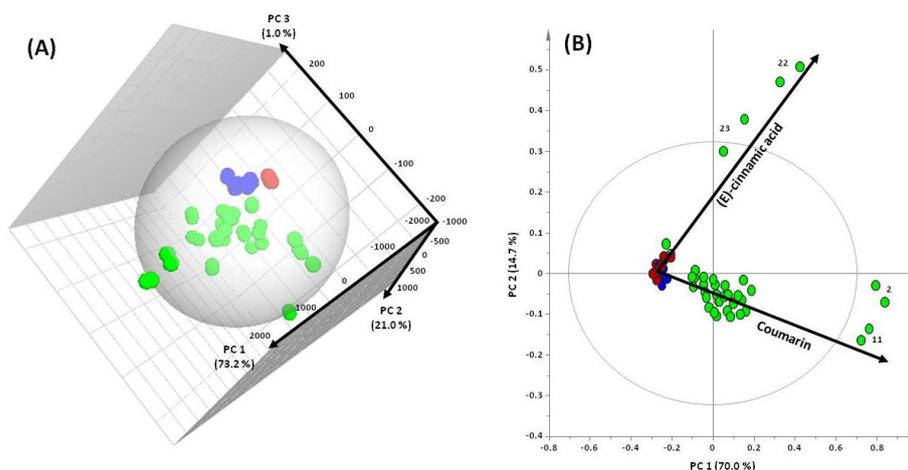


Fig II-2. (A) Score plot 3D de l'ACP générée avec les signaux de la région 5.7 et 11 ppm pour tous les échantillons. (B) Score plot de l'ACP générée avec une partie des signaux de la coumarine.

Par la suite, nous avons proposé de créer une gamme de quantification, focalisée sur des signaux spécifique du (E)-cinnamaldéhyde (H1) ou de la coumarine (composante gauche du H4). Pour cela, nous avons fait l'enregistrement des spectres LF de solutions standards de ces 2 composés à des concentrations correspondant à des quantités réelles dans les échantillons commerciaux. Nous avons généré une ACP à partir de ces données (appelées données d'entraînement) puis projeté les spectres des échantillons commerciaux (appelés données tests) sur le score plot de cet ACP. Les résultats sont présentés sur la Fig II-3. Les score plots (A) et (B) montrent l'organisation des échantillons standards du (E)-cinnamaldéhyde et de la coumarine partant des plus faibles concentrations (à gauche du score plot) aux concentrations les plus élevées (à droite du score plot). Ainsi une gamme de concentration a pu être créée, de 0 à 30 mg/g pour le (E)-cinnamaldéhyde et de 0 à 6 mg/g pour la coumarine. Le score plot (C) et (D) de la Fig II-3 représente la projection des données tests, sur le score plot précédent, permettant ainsi le classement des échantillons commerciaux en fonction de leur teneur en (E)-cinnamaldéhyde ou en coumarine. Ils vont se positionner dans la gamme de concentration établie, ce qui permet d'atteindre une pseudo-quantification des composés ciblés.

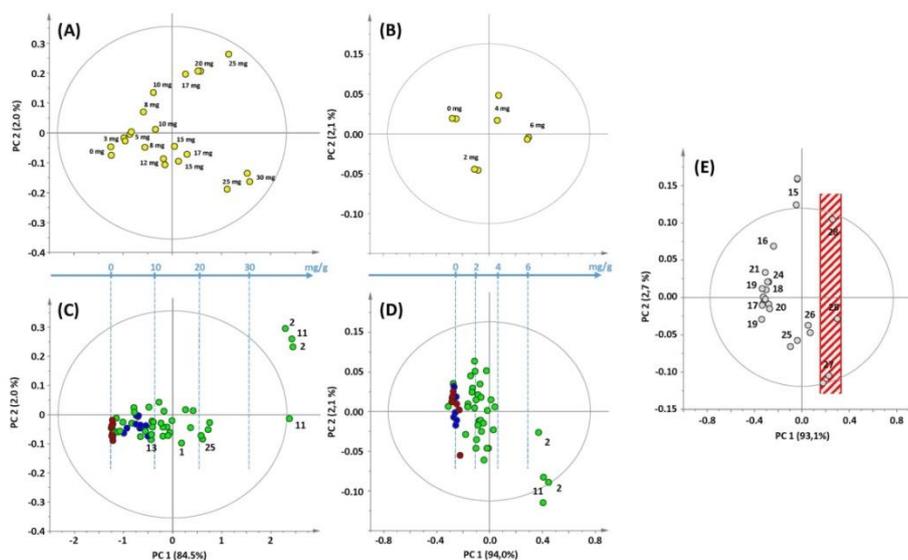


Fig II-3. Classification des échantillons à partir des données RMN LF. Les score plots des ACP ont été construits à partir des spectres RMN LF de solutions de standard de (E)-cinnamaldéhyde (A) et de coumarine (B). Les score plots (C) et (D) montrent la projection des échantillons commerciaux sur les score plots générés en (A) et (B) respectivement. (E) projection des compléments alimentaires sur le score plot généré en (B) selon la posologie recommandée. Les limites hépatotoxiques (0.1 mg / kg) pour un poids corporel de 60 à 80 kg sont indiquées en rouge.

Les résultats observés sont en bonne adéquation avec les quantifications obtenues par RMN HF. Par exemple, les deux échantillons **2** et **11** qui contiennent le plus de (E)-cinnamaldéhyde (36,1 et 32,5 mg/g respectivement) se localisent au-delà des 30 mg/g sur le score plot (Fig II-3C). Les échantillons **13**, **1** et **25** contenant 10.8, 15.8 et 20.0 mg/g se retrouvent dans la classe 10-20 mg/g. Enfin les échantillons en rouge qui ne contiennent pas de (E)-cinnamaldéhyde, se retrouvent au niveau de la limite de 0 mg/g sur le score plot. La même approche a été réalisée sur la coumarine et les résultats sont reportés sur la Fig II-3D. Tous les échantillons dépourvus de coumarine (rouge et bleu) se trouvent sur la limite 0 mg/g. La quasi-totalité des échantillons commerciaux contiennent moins de 4 mg/g de coumarine, à l'exception des deux échantillons **2** et **11** qui ont des concentrations proches à 6 mg/g. Ces résultats sont en accord avec les valeurs de quantification obtenues à HF.

Enfin, la figure Fig II-3E propose une classification limitée aux compléments alimentaires et prenant en compte la consommation journalière recommandée sur les notices. Les deux échantillons mis en évidence peuvent présenter des risques pour le consommateur (**27** et **28**) car la dose ingérée de coumarine par jour (7,7 et 8,4 mg) se situe au-delà de la limite hépatotoxique (0.1 mg/kg par jour) en prenant un poids corporel de référence de 70 kg (7 mg par jour).

Conclusion

Dans ce projet, nous avons utilisé la RMN LF comme technique de contrôle de la teneur en (E)-cinnamaldéhyde, composé majoritaire de la cannelle et surtout de la coumarine, composé à risque, permettant ainsi de contrôler la qualité de l'échantillon. Malgré l'identification de signaux caractéristiques pour ces 2 composés, la quantification s'est avérée trop délicate dans des conditions raisonnables d'enregistrement des expériences. Nous avons alors utilisé l'approche chimométrique pour créer une gamme de quantification et classer nos échantillons. Ces résultats ont permis de mettre en évidence le potentiel du couplage de la RMN LF et des analyses statistiques comme outils de contrôle peu onéreux et relativement simple d'utilisation pour des mélanges complexes à base de plantes.

Chapitre III RMN à bas champ et analyse chimométriques pour détecter l'adultération de compléments alimentaires amincissants

Dans cette étude, après une préparation simple et rapide des échantillons, 66 compléments alimentaires amincissants ont été analysés par RMN LF ^1H . L'analyse des données spectrales RMN LF ^1H par une approche chimométrique a été mise en œuvre pour détecter la présence d'adultérants.

3.1 Analyse RMN LF ^1H

Dans un premier temps, les spectres RMN ^1H de tous les compléments alimentaires ont été enregistrés dans du méthanol deutéré. Même si la résolution des spectres RMN LF ^1H est relativement faible, les principaux signaux, caractéristiques de la sibutramine et de la phénolphtaléine, ont été facilement détectés dans les groupe S (adultéré par de la sibutramine), P (adultéré par de la phénolphtaléine) et PS, ce dernier contenant à la fois de la sibutramine et de la phénolphtaléine. La Fig III-1 montre les spectres typiques des quatre classes de compléments alimentaires analysés. La sibutramine a été identifiée dans les échantillons S5 et PS2 par les signaux de ses protons aromatiques à 7.41 ppm et de ses groupes méthyles à 2.49 et 1.02 ppm. De même, les protons aromatiques de la phénolphtaléine donnent un motif caractéristique entre 6.5 et 8.0 ppm qui a été observé dans les échantillons P1 et PS2. Enfin, l'échantillon N5 est un complément alimentaire sans adultérant et, à l'exception des signaux de la référence et du solvant, seul les protons des d'acides gras d'extraits de plantes ont été facilement détectés, comme les CH_2 à 1.27 ppm. Des signaux mineurs de protons aromatiques de polyphénols ou d'autres composés naturels ont également été détectés dans certains échantillons.

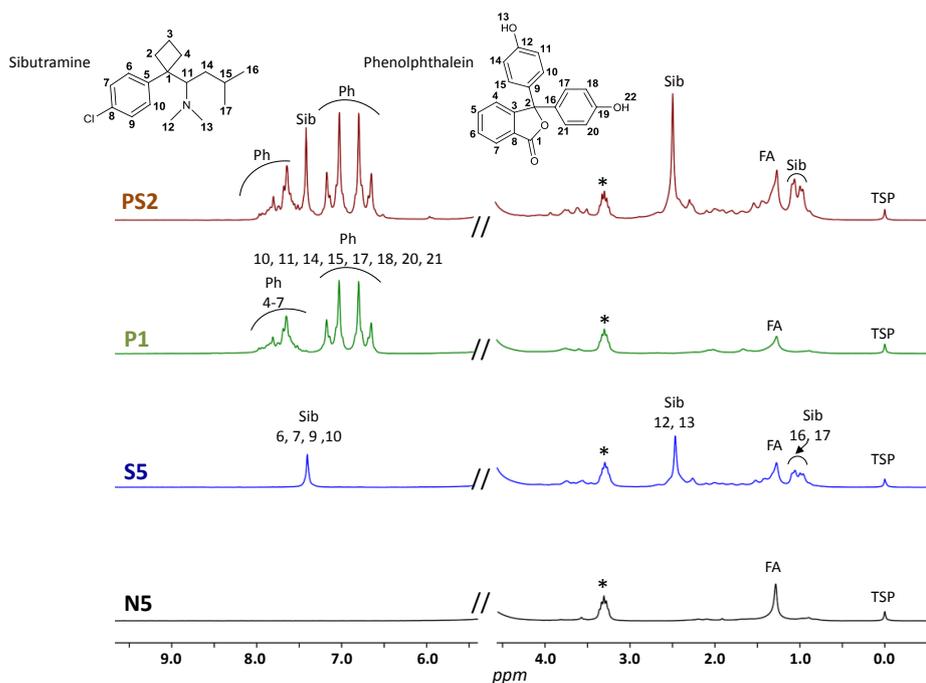


Fig III-1. Spectres RMN LF ^1H typiques de compléments alimentaires amaigrissants enregistrés à 60 MHz (N, groupe non adultéré (naturel); S, groupe adultéré à la sibutramine; P, groupe adultéré à la phénolphtaléine; PS, groupe adultéré à la fois par la sibutramine et par la phénolphtaléine). Ph: Phénolphtaléine; Sib: Sibutramine; FA: acides gras; TSP: référence interne; *: CD_2HOD .

3.2 Analyse chimiométrique

Des analyses statiques supervisées ont permis de construire un modèle valide de PLS-DA à partir des données issues des spectres RMN LF ^1H de tous les échantillons. Les valeurs Y_{predPS} , qui indiquent la probabilité d'un échantillon d'appartenir à une classe, sont reportées sur la Fig III-2. Les échantillons (N), (S) et (P) ($n = 40$), dont le contenu était précédemment connu [30], ont été pris en compte pour générer le modèle et pour définir le seuil de valeur Y entre les compléments alimentaires adultérés et non adultérés. Les échantillons PS et treize échantillons (T) nouvellement achetés ont été testés en nous basant sur ce modèle. Nous avons pu classer l'ensemble des échantillons avec succès dans les groupes des « naturels », $Y_{\text{predPS}} > 0,45$, « adultérés », $Y_{\text{predPS}} < 0,30$ ou « limites » avec des valeurs Y_{predPS} entre 0,30 et 0,45. Tous les échantillons PS qui contiennent un mélange sibutramine et phénolphtaléine ont tous été classés comme « adultérés ». Pour les nouveaux échantillons testés, 4 sont classés comme adultérés, 2 limites et tous les autres sont naturels ($Y_{\text{predPS}} < 0,18$). L'analyse des spectres a permis de confirmer ces résultats.

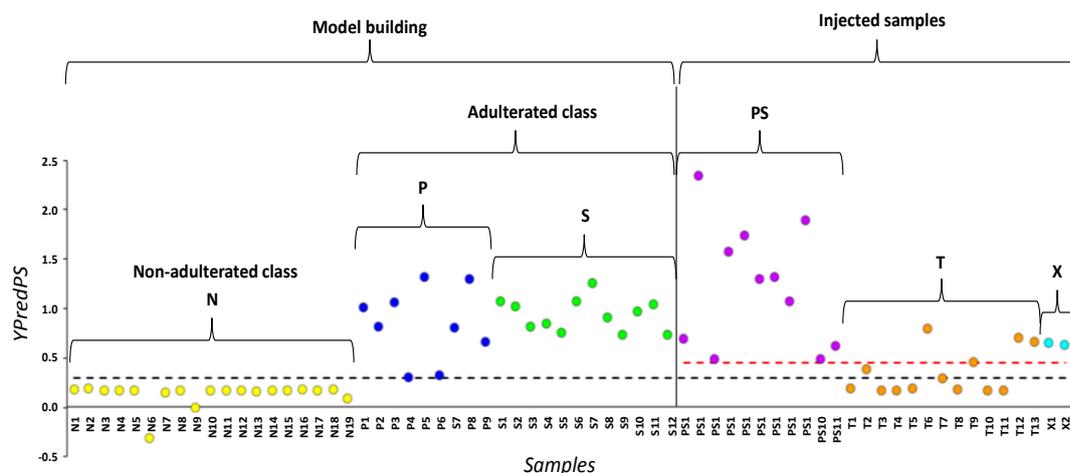


Fig III-2. Valeurs-Y prédites (YpredPS) obtenues pour les 66 compléments alimentaires analysés sur la base du modèle PLS-DA à deux classes comparant des échantillons naturels (N) à des échantillons adultérés (échantillons (P) et (S) considérés ensemble comme une seule classe d'échantillons adultérés). PS, groupe sibutramine et phénolphtaléine adultéré; T: échantillons test (nouvellement achetés) ; X: échantillons atypiques.

Cette classification a ensuite été affinée avec un nouveau modèle construit à partir des mêmes 40 compléments alimentaires mais en considérant cette fois trois classes distinctes (non adultérés, adultérés par de la sibutramine et adultérés par de la phénolphtaléine). Le score plot de cette PLS-DA à trois classes (Fig III-3A) montre une discrimination claire entre les trois familles, les non adultérés sont tous regroupés alors que ceux de la classe P et S sont plus éparpillés en raison de leur concentrations très variables en sibutramine et phénolphtaléine. Par la suite les trois autres familles ont été projetées sur ce modèle. L'ensemble des PS se localisent entre le groupe P et S sur le score plot (Fig III-3B) indiquant clairement la présence de sibutramine et de phénolphtaléine dans ces échantillons. Pour les échantillons T, les 7 précédemment classés comme naturel, se localise bien dans le groupe N (Fig III-3C). Pour les 4 adultérés, 3 contiennent de la sibutramine et un de la phénolphtaléine. Pour les 2 échantillons classés « limite », ils ont pu être classés dans la famille des adultérés avec la présence de phénolphtaléine. On peut noter cependant une limite de cette approche illustrée par les exemples des deux compléments alimentaires atypiques X1 et X2. Ces échantillons apparaissent comme adultérés lorsque l'on considère leurs valeurs YpredPS (0.65 pour X1 et 0.63 pour X2) (Fig III-2). De plus, la projection de leurs spectres RMN LF ^1H dans le deuxième modèle de PLS-DA indique que ces échantillons sont adultérés, par de la phénolphtaléine pour X1 et de la

sibutramine pour X2 (Fig III-3D). En fait, chacun de ces échantillons contenait respectivement de la fluoxétine et de la "cétone de framboise". Les signaux de ces composés sont partiellement superposés avec les principaux signaux aromatiques de la sibutramine et de la phénolphtaléine dans les spectres LF, les classant par erreur dans les groupes S et P. Cependant, au regard des spectres, les profils de ces deux molécules étaient différents de ceux des adultérants analysés. Dans ce cas, le screening à l'aveugle mène à une mauvaise classification des échantillons, mais l'examen attentif des spectres permet de corriger cette erreur.

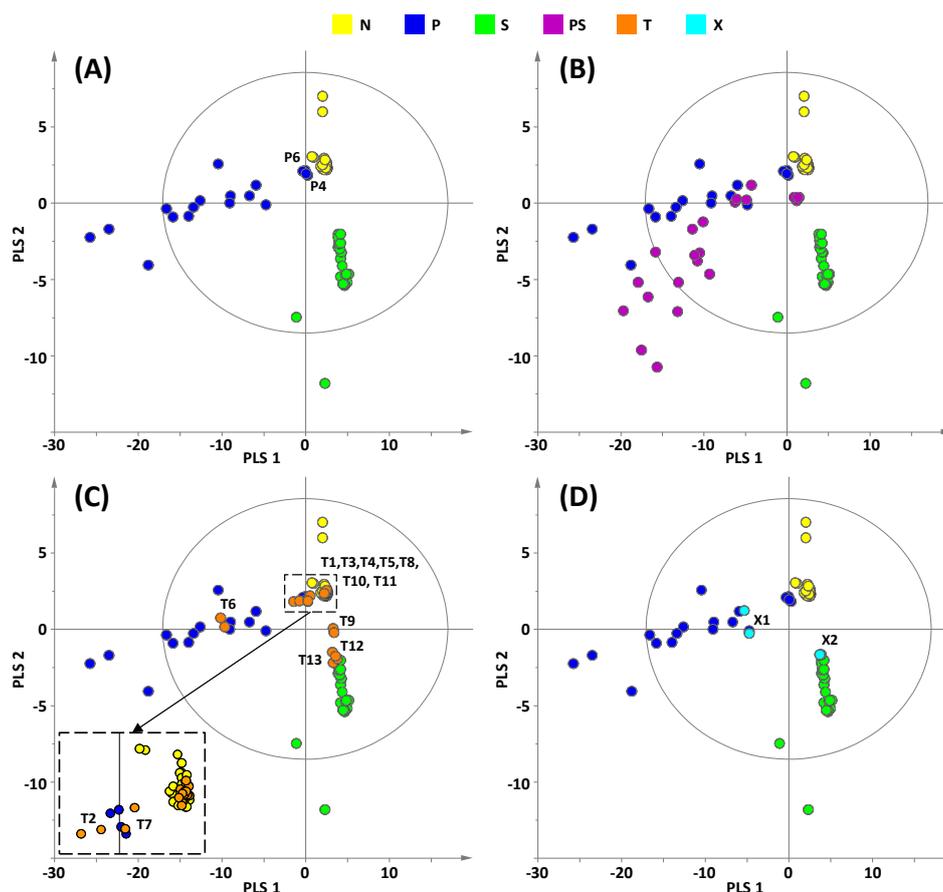


Fig III-3. (A) Score plot du modèle PLS-DA (Q^2 0,66, R^{2Y} 0,74) à trois classes construit à partir des spectres RMN LF ^1H des échantillons N (non adultéré), S (adultérés avec de la sibutramine) et P (adultérés avec de la phénolphtaléine). Les score plots (B), (C) et (D) montrent la projection des échantillons PS (adultérés à la fois avec la sibutramine et la phénolphtaléine), T (échantillons tests) et X (échantillons atypiques) respectivement sur le modèle construit (A).

Conclusion

Dans ce projet, nous avons montré que malgré les limites de la RMN LF nous avons pu, avec succès, appliquer la RMN LF pour le contrôle de la qualité des compléments alimentaires amaigrissants. En effet, les concentrations importantes en adultérants permettent de pallier au problème de sensibilité alors que le manque de résolution spectrale est compensé par l'utilisation de méthodes chimiométriques. Un screening rapide (facilité de préparation des échantillons, acquisition inférieure à 20 min) par RMN LF pourrait être considéré comme une technique de première ligne pour l'analyse de formulations amincissantes. Des analyses chimiométriques permettront de classer rapidement ces échantillons comme propres à la consommation, frauduleux ou nécessitant des analyses plus approfondies.

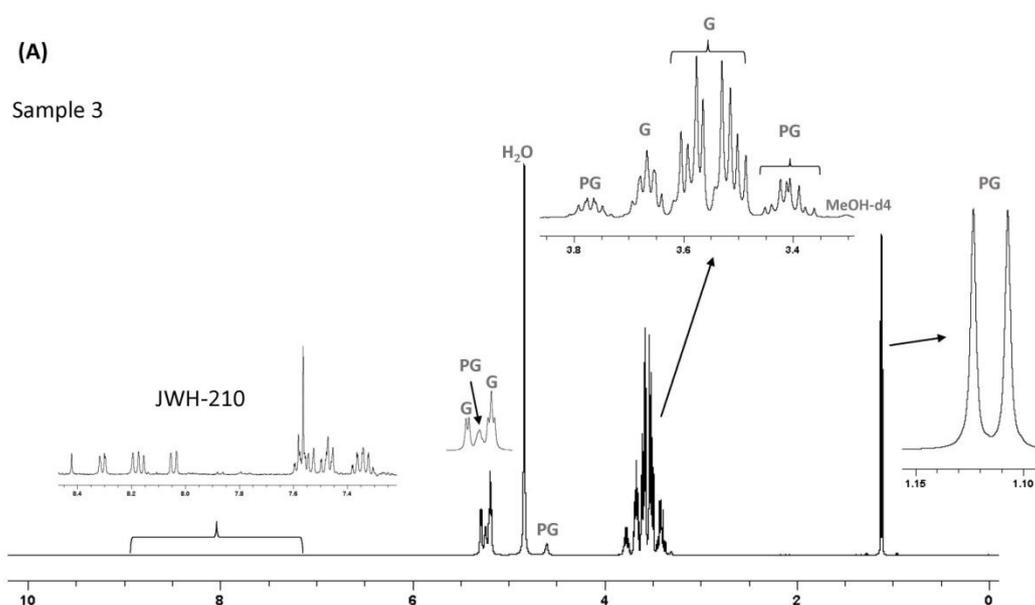
Chapitre IV Analyse de cannabinoïdes de synthèse dans les e-liquides par RMN ^1H et ^{19}F .

Le but de cette étude était d'évaluer la capacité de la RMN à bas champ pour l'analyse de cannabinoïdes dans les e-liquides, à la fois pour leur détection et leur quantification. Probablement du fait de la complexité de la matrice, il n'est à notre connaissance, pas reporté à ce jour d'analyses quantitatives de cannabinoïdes dans des e-liquides, ni par RMN ni par d'autres méthodes. Cette étude a porté sur 13 échantillons fournis par le laboratoire SCL de Massy des douanes. Nous avons mis en œuvre des analyses RMN haut champ et bas champ complétées par des analyses GC-MS.

4.1 Analyse qualitative des e-liquides

4.1.1 Analyse par RMN ^1H HF

Les spectres RMN HF ^1H des 13 échantillons ont permis de détecter en première approche les signaux intenses de la matrice. La Fig IV-IA montre le spectre RMN HF ^1H complet d'un échantillon d'e-liquide (3) avec l'attribution des signaux majoritaires du propylène glycol et du glycérol. Les signaux d'éthylène glycol, de polyéthylène glycol ou encore d'éthanol ont également été détectés dans la matrice, pour certain échantillons. La présence d'éthanol dans les e-liquides augmente la solubilité des SC et des arômes dans la matrice et peut considérablement réduire la viscosité du glycérol, améliorant la formation de l'aérosol pour l'inhalation [46].



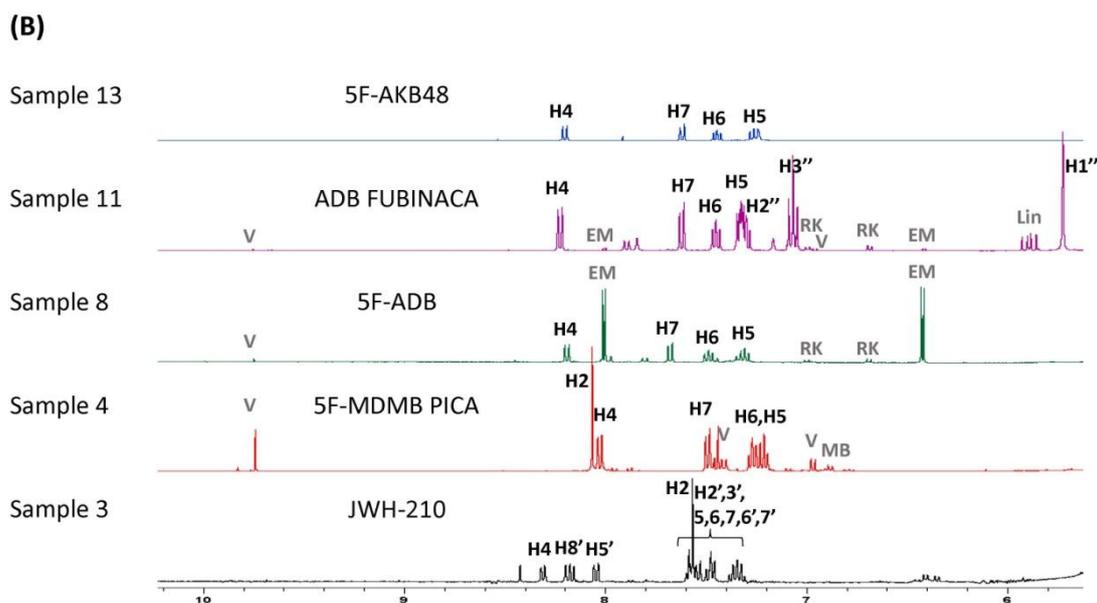


Fig IV-I. Spectres RMN ^1H de e-liquides (400 MHz, méthanol- d_4) (A) Spectre complet de l'échantillon **3** (B) Partie éthylénique aromatique (5.7-10.2 ppm) de 5 spectres de e-liquides contenant différents SCs. EM: Ethyl maltol, V: vanilline, RK: Raspberry ketone (cétone de Framboise), Lin: Linalol.

L'analyse des spectres RMN a permis de mettre en évidence les signaux des SCs psychoactifs. Cinq SCs dont les structures sont présentées dans la figure Fig IV-1 ont été détectés dans ces échantillons: JWH-210 (échantillons **1-3**), 5F-MDMB-PICA (échantillons **4-6**), 5F-ADB (échantillons **7-9**), ADB-FUBINACA (échantillons **10-12**) et 5F-AKB48 (échantillon **13**). Ils possèdent des éléments structuraux cycliques de type indole ou indazole et divers substituants comme les groupes carboxamide, chaînes alkyles etc. On peut remarquer que quatre de ces cannabinoïdes sont fluorés.

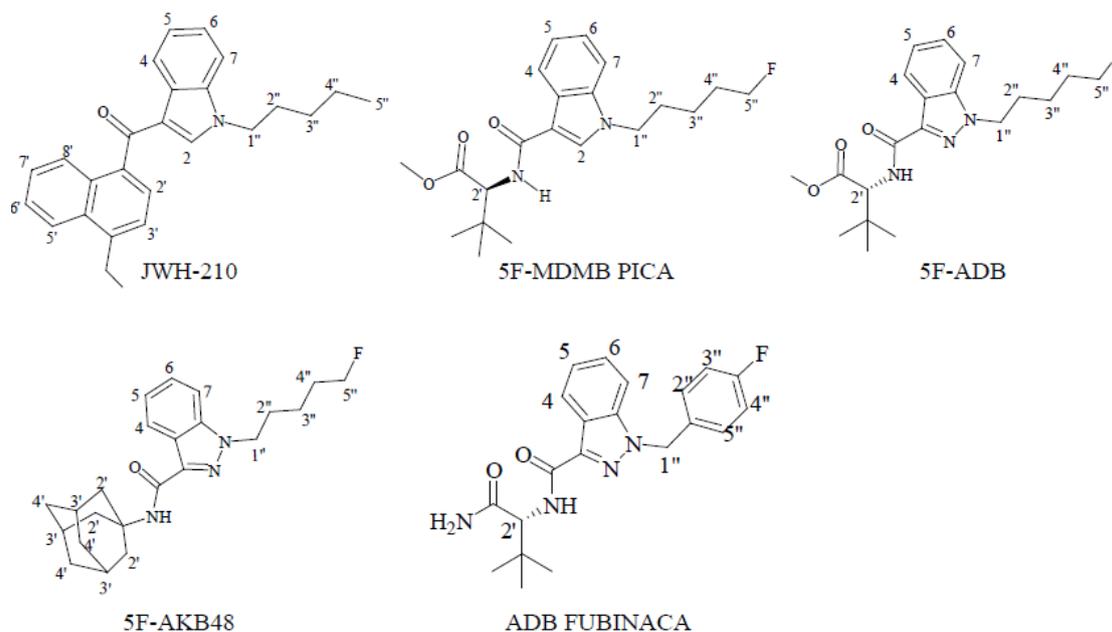


Fig IV-1. Structure chimique des cannabinoïdes de synthèse (SCs) détectés dans les e-liquides.

Des signaux caractéristiques avec des déplacements chimiques et des multiplicités proches sont observés pour les protons indazole (H4, H5, H6 et H7) du 5F-ADB, ADB FUBINACA et 5F-AKB48 tandis que les signaux du cycle indole (H2, H4, H5, H6 et H7) dans JWH-210 et 5F-MDMB PICA ont une signature spectrale différente. Pour les SCs portant un fluor sur la chaîne alkyle, un triplet dédoublé est observé pour le H5" dû aux couplages avec le noyau fluor ($^2J_{HF}$) et avec le méthylène voisin ($^3J_{HH}$). Les caractéristiques RMN observés pour ces SCs étaient en adéquation avec celles récemment publiées [19, 49-51].

Outre la matrice et les SCs, les autres signaux RMN détectables dans ces échantillons de e-liquides sont ceux d'ingrédients utilisés pour aromatiser ces préparations. Nous avons ainsi pu détecter et identifier des signaux du limonène, de la vanilline, de l'éthyl maltol, de la cétone de framboise, de l'alcool méthyloxybenzylique, du linalol ou encore du menthol.

Nous ne présenterons pas ici les résultats de GC-MS qui complètent nos analyses qualitatives par RMN et confirment l'identification des composés.

4.1.2 Analyse par RMN 1H LF

L'analyse par RMN 1H LF à 60 MHz, montre qu'une partie des signaux d'intérêts sont altérés par les signaux intenses de la matrice, en particulier dans la région des basses fréquences (0.5-4.5 ppm). La comparaison des spectres à haut et bas champs pour un même échantillon est présentée dans la Fig IV-2 ci-dessous. Même si certains signaux ou profils caractéristiques

peuvent être identifiés aussi bien pour les SCs que pour des composés de types arômes, nous avons jugé que dans cette application pour laquelle nous visions une analyse quantitative, la RMN ^1H à bas champ ne permettait pas d'atteindre cet objectif et nous n'avons pas poursuivi nos investigations.

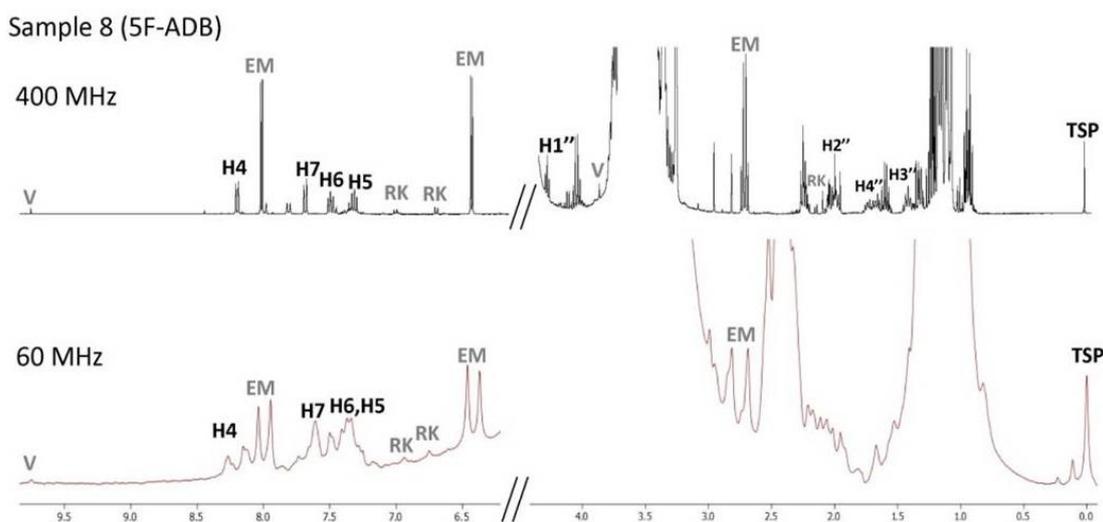


Fig IV-2. Comparaison d'un spectre RMN ^1H à 400 MHz et à 60 MHz s pour l'échantillon 8 dans CD_3OD . EM: ethyl maltol, V: vanilline, RK: raspberry ketone (Cétone de Framboise), Lin: Linalol.

4.1.3 Analyse par RMN du fluor-19

Les SCs fluorés détectés dans les e-liquides peuvent être analysés par RMN ^{19}F . Les spectres ont été enregistrés pour tous les échantillons sur les spectromètres HF et LF à respectivement 376 et 56,2 MHz. Les spectres sans découplage du proton sont présentés dans la Fig IV-3. Du trifluorotoluène a été ajouté dans tous les échantillons comme référence de déplacement chimique (-63,7 ppm) et de quantification. Le fluor d'ADB FUBINACA lié au cycle aromatique conduit à un singulet à -115,8 ppm. Les noyaux de fluor en position terminale des chaînes alkyle pour 5F-AKB48, 5F-MDMB PICA, 5F-ADB donnent des multiplets autour de -220 ppm. Dans les spectres HF, ils résonnent sous forme de triplet de triplets en raison du couplage avec le méthylène $\text{H5}''$ ($^2J_{\text{FH}}$) et $\text{H4}''$ ($^3J_{\text{FH}}$). Pour les mêmes échantillons, les signaux LF sont plus étalés car les valeurs des constantes de couplage sont indépendantes des champs magnétiques et leur multiplicité est proche d'un septuplet apparent.

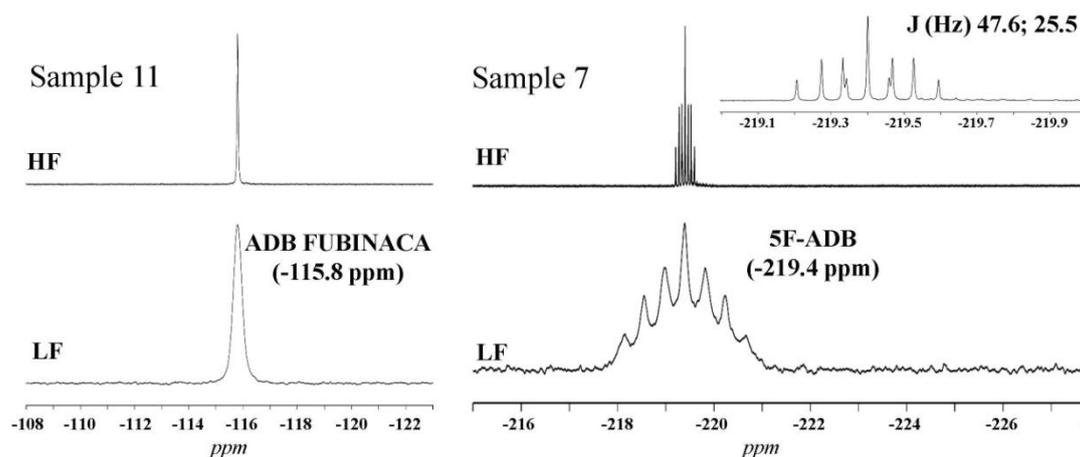


Fig IV-3. Signaux caractéristiques en ^{19}F NMR de SCs dans des e-liquids. Les spectres ont été enregistrés à 376 MHz (HF) et 56.17 MHz (LF). ADB FUBINACA dans l'échantillon **11**; 5F-ADB dans l'échantillon **7**.

Contrairement à la RMN du proton LF des e-liquides, pour laquelle le chevauchement des signaux empêche la mise en œuvre d'analyses quantitatives, la RMN ^{19}F ouvre cette possibilité à haut et bas champs.

4.2 Analyse quantitative

La mise au point des conditions d'analyses quantitatives a été faite en RMN HF pour le proton et le fluor et en RMN bas champ pour le fluor. Nous nous sommes placés dans des conditions de relaxation totale des signaux, grâce à la mesure des temps de relaxation T1 et pour la RMN du fluor, un agent de relaxation, l'acétyl acétonate de chrome III a été ajouté dans les solutions. Les durées des expériences sont de 33 min pour la RMN ^1H HF, 3 min pour la RMN ^{19}F HF et 1 h 30 pour la RMN ^{19}F LF. En RMN du proton, un signal caractéristique a été utilisé pour la quantification pour chaque cannabinoïde.

Les résultats sont présentés dans le graphe ci-dessous pour les échantillons contenant les SCs fluorés. La RMN HF aussi bien fluor que proton sont des méthodes quantitatives reconnues [1, 52-55]. Elles sont tout à fait applicables aux e-liquides malgré les signaux intenses de la matrice dans le cas de la RMN du proton. Les applications quantitatives en RMN bas champ sont encore peu développées et nous montrons ici que la RMN du fluor à bas champ est tout à fait pertinente et adaptée à cette quantification. Pour valider ces résultats quantitatifs, nous avons vérifié statistiquement la concordance des données ^1H HF versus ^{19}F HF et ^{19}F LF versus ^{19}F HF.

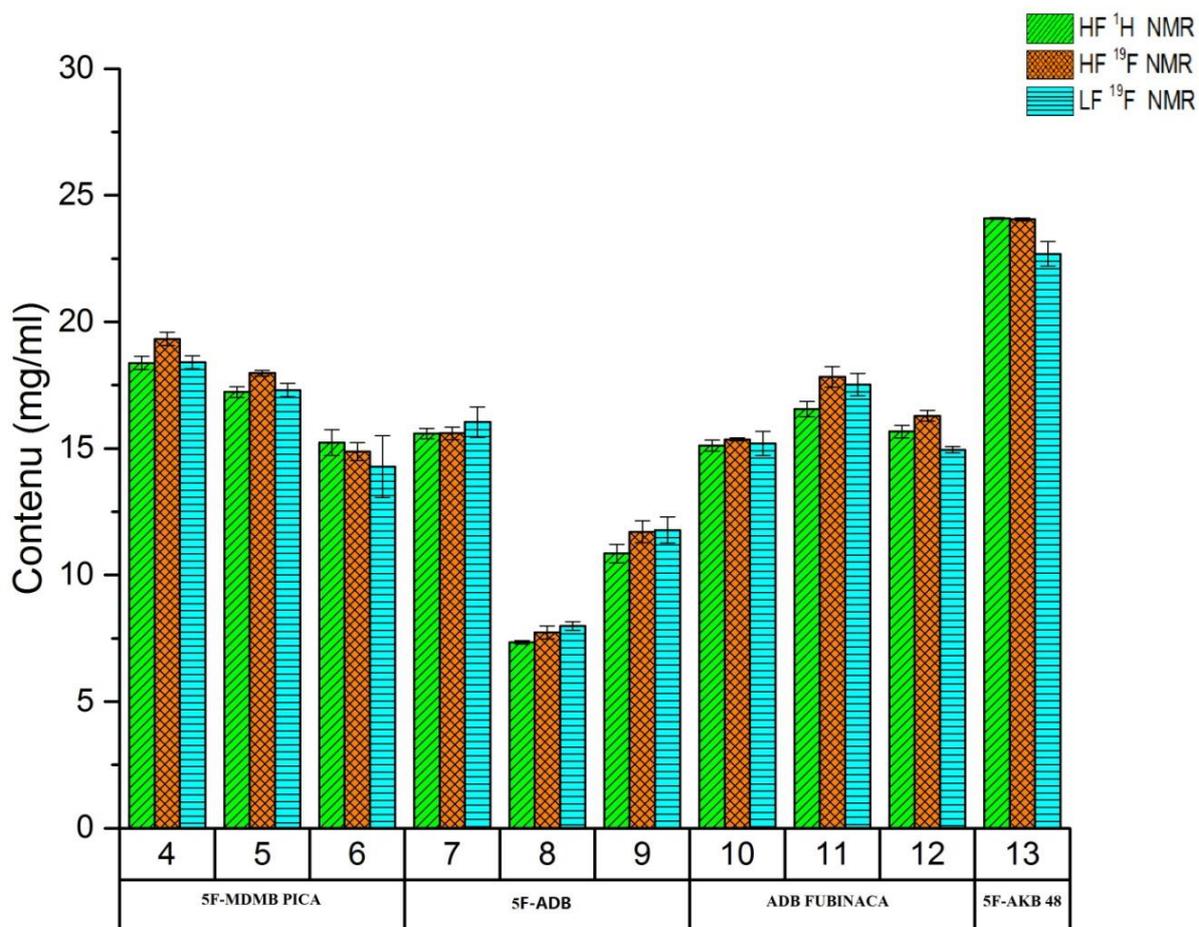


Fig IV-4. Histogramme présentant les résultats des analyses quantitatives de SCs dans des e-liquides.

Il n'existe pas, à notre connaissance, de données sur les concentrations de SCs dans les e-liquides. On constate ici que les concentrations de ces composés fluorés se situent entre 7 et 24 mg/mL. On peut supposer que ces concentrations sont "adaptées" par les fabricants de ces produits illicites en tenant compte des effets psychoactifs associés et donc des affinités avec les récepteurs CB1 et CB2 des cannabinoïdes.

Conclusion

Cette étude, est à notre connaissance la première étude portant sur l'analyse de SCs dans des e-liquides par RMN à bas champ. La RMN ^1H LF a montré des limites pour les applications quantitatives, en particulier en raison d'un énorme écart de concentration entre la matrice et les composés actifs et les chevauchements de signaux. Le point fort de cette étude est que la RMN du LF ^{19}F peut facilement permettre la quantification des SCs fluorés sans préparation d'échantillon complexe et sans besoin d'un composé standard spécifique. Grâce à la spécificité de détection de la RMN du fluor LF, cette étude peut ouvrir la voie à un screening de routine pour la détection de SCs fluorés illicites dans diverses matrices telles que les e-liquides, les mélanges à base de plantes ou les "sels de bain".

Conclusion générales

Dans cette thèse, la RMN LF de paille a été explorée en tant que méthode analytique simple à mettre en œuvre dans une optique de contrôle de la qualité ou la détection de fraudes. Nos études élargissent le champ d'application de la RMN LF. En effet, malgré la faible sensibilité de la technique et la complexité des mélanges analysés, des informations structurales et quantitatives sur les constituants peuvent être obtenus. Dans le cas de l'analyse de produits naturels et de l'identification des adultérants dans les produits commerciaux, le processus analytique incluant une approche statistique pourrait être utile pour la réalisation de premiers screening de produits susceptibles d'être falsifiés.

Il est vraisemblable que dans les années à venir la RMN à bas champ, ne nécessitant pas de cryogénie et donc réduisant la consommation de ressources fossiles par rapport à la RMN conventionnelle, va encore évoluer et ses champs d'application se diversifier. Ces progrès vont s'appuyer sur les développements méthodologiques récents allant des possibilités diverses ouvertes par les gradients de champs équipant maintenant les spectromètres bas champ commerciaux jusqu'au techniques d'hyperpolarisation qui permettront de significativement améliorer les limites de détection des spectromètres bas champ.

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Publications

1. Nao Wu, Stéphane Balayssac, Saïda Danoun, Myriam Malet-Martino, and Véronique Gilard. "Chemometric analysis of low-field ^1H NMR spectra for unveiling adulteration of slimming dietary supplements by pharmaceutical compounds." *Molecules* 25, no. 5 (2020): 1193.
2. Nao Wu, Stéphane Balayssac, Gaëtan Assemat, Saïda Danoun, Sébastien Déjean, Myriam Malet-Martino, and Véronique Gilard. " Evaluation of low-field versus high-field proton NMR spectroscopy for quality control of cinnamon samples." *Journal of Food Composition and Analysis* (Under Review).
3. Quality control of cinnamon samples using High-Field and Low Field proton NMR spectroscopy, Nao Wu, Gaëtan Assemat, Stéphane Balayssac, Myriam Malet-Martino, Véronique Gilard, APA2018 - Advances in Pharmaceutical Analysis, 12-13 July 2018, Lille, France. (Poster).
4. High-Field and Low-Field proton NMR spectroscopy for the analysis of cinnamon samples marketed as food or dietary supplements, Nao Wu, Gaëtan Assemat, Stéphane Balayssac, Véronique Gilard, Myriam Malet-Martino, DA-PBA 2018, joint meeting 11th International Symposium on Drug Analysis and 29th International Symposium on Pharmaceutical and Biomedical Analysis, September 9-12, 2018, Leuven, Belgium. (Poster).
5. Evaluation of low-field proton NMR spectroscopy for quality control of cinnamon sample, Nao Wu, Stéphane Balayssac, Saïda Danoun, Myriam Malet-Martino, Véronique Gilard, Plants with health benefits and biomolecules of interest. From plantlets to tablets, 2nd Indian - French Symposium Hyderabad, 27th - 29th November 2019, Bordeaux. (Poster).
6. Analysis of cinnamon samples using high-field and low-field proton NMR spectroscopy; Nao Wu, Gaëtan Assemat, Stéphane Balayssac, Myriam Malet-Martino, Véronique Gilard; 10^{ème} Conférence Internationale de Chimie Toulouse-Kiev, June 3~5th 2019, Toulouse France. (Conference presentation).

RMN à bas champ pour le contrôle qualité et la détection de fraudes: applications à l'analyse de compléments alimentaires et de e-liquides

L'objectif de cette thèse était d'explorer la capacité et les limites de la RMN à bas champ (LF) de paillasse pour le contrôle de la qualité et la détection de fraude dans des produits commerciaux. Les études ont porté sur trois applications.

Premièrement, la RMN LF a été utilisée pour classer les espèces de cannelle dans des produits commerciaux culinaires ainsi que dans des compléments alimentaires. Les spectres de RMN LF permettent la détection de signaux typiques et le traitement des données de RMN LF par une analyse chimiométrique a été exploré pour classer les espèces de cannelle et prédire la teneur en coumarine hépatotoxique dans les produits.

Deuxièmement, nous avons appliqué la RMN LF à l'analyse d'un lot de compléments alimentaires amincissants, les signaux des substances synthétiques ont pu être détectés efficacement et une analyse PLS-DA a permis la discrimination rapide des échantillons contenant des adultérants.

La dernière recherche a évalué l'efficacité de la RMN LF pour le contrôle de la qualité des e-liquides. La RMN du proton a été explorée pour détecter les signaux typiques des cannabinoïdes synthétiques (SC). Enfin, la RMN du fluor-19, une méthode d'analyse sélective a été développée pour identifier et quantifier les SC fluorés dans les e-liquides.

Mots clés: RMN, RMN bas champ, contrôle qualité, détection de fraude, produits à base de plantes, e-liquides, adulteration.

Low-field NMR for quality control and fraud detection: application to the analysis of dietary supplements and e-liquids

The purpose of this thesis was to explore the ability and limitation of benchtop low-field (LF) NMR for quality control and fraud detection in commercial products. The studies focused on three applications.

First, LF NMR was used to classify the cinnamon species in cinnamon culinary products and dietary supplements. LF NMR spectra allow the typical signals detection and the LF NMR data combined with chemometric analysis was further explored to classify cinnamon species and predict the content of hepatotoxic coumarin in products.

Second, we applied LF NMR to screen batch of slimming dietary supplements, signals of synthetic adulterants can be effectively detected and the application of PLS-DA allowed the rapid discrimination of samples with adulterants.

The last research evaluated the efficiency of LF NMR for quality control of smoking e-liquids. Proton NMR was explored to detect typical signals of synthetic cannabinoids (SC). Finally, fluorine-19 NMR, a selective analytical method was developed to identify and further quantify fluorinated SC in e-liquid.

Keywords: NMR, low-field NMR, quality control, fraud detection, herbal products, e-liquids, adulteration.