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DESENVOLVIMENTO DE TÉCNICAS MODERNAS DE ESPECTROMETRIA DE MASSAS PARA A ANÁLISE DE COMPOSTOS BIOATIVOS BENÉFICOS E TÓXICOS EM ALIMENTOS

THE DEVELOPMENT OF MODERN MASS SPECTROMETRY TECHNIQUES FOR THE ANALYSIS OF BENEFICIAL AND TOXIC BIOACTIVE COMPOUNDS IN FOODS

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Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciências.

Thesis presented to the School of Medical Sciences from the University of Campinas as part of the requisites to obtain the title of Ph.D. in Sciences

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vocês são tantos e tão incríveis,
dedico este trabalho.

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RESUMO

A necessidade de métodos analíticos mais rápidos e com alta eficiência é o que move a pesquisa aplicada às áreas de ciência e análise de alimentos. A metabolômica é uma plataforma extremamente versátil, que integra dados estatísticos e experimentais na produção de resultados analíticos de alta confiabilidade. Aliado às técnicas modernas de espectrometria de massas, isso torna este segmento extremamente fértil para a realização de melhorias e desenvolvimento. Este projeto teve como escopo a análise direta de amostras de diversos alimentos com características funcionais, como azeites, amendoim e chocolates, com mínimo preparo de amostra, através de espectrometria de massas convencional (MS) e por imagem (MSI). Foram avaliadas características químicas de sua composição em casos de adulterações, degradações ou contaminações. Para tal finalidade, fontes de inonização como a dessorção a laser assistida por matriz (MALDI) e o spray de elétrons (ESI) foram amplamente exploradas. Toda a elucidação estrutural teve o respaldo de reações de fragmentação e análises por espectrometria de massas de alta resolução (ESI-Orbitrap). Os compostos (marcadores) foram tanto previamente definidos (*target analysis*) quanto identificados pós-análise (*metabolic fingerprinting*).

Palavras chave: biomarcadores; análise de alimentos; espectrometria de massas.

ABSTRACT

The urge for faster and highly efficient analytical methods is the drive force in food research and analysis. Metabolomics is a versatile platform that integrates both statistical and experimental data, providing highly reliable analytical results. Along with the modern mass spectrometric techniques, there is a very prominent scenario for the development of new and improved approaches. This project aimed at developing direct analysis methods for samples of various functional food products, such as olive oil, peanuts and chocolate through both conventional mass spectrometry (MS) and mass spectrometry imaging (MSI), with little sample preparation. Chemical characterization, degradation and adulteration processes were abundantly monitored within this scope. Ionization sources, such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) were largely explored tools in this context. Structural elucidations were performed with the assistance of high-resolution mass spectrometry (ESI-Orbitrap). Chemical markers were either previously elected for a targeted analysis, or identified after the analytical process, as in metabolic fingerprinting.

Keywords: biomarkers; food analysis; mass spectrometry

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LISTA DE ABREVIATURAS

ACN	Acetonitrila (*Acetonitrile*)
AFB1	Aflatoxina B1
AFB2	Aflatoxina B2
AFG1	Aflatoxina G1
AFG2	Aflatoxina G2
AUC	Área sob a curva (*Area under the curve*)
CE	Eletroforese capilar (*Capillary electrophoresis*)
CHCA	Ácido alfa-ciano hidróxicinâmico
CID	Dissociação induzida por colisão (*Collision-induced dissociation*)
CP	Cloropropanol (*Chloropropanol*)
DART	Análise direta em tempo real (*Direct analysis in real time*)
DESI	Ionização de dessorção por elétron-spray (*Desorption electrospray ionization*)
DSC	Calorimetria diferencial de varredura (*Differential scanning calorimetry*)
EASI	Ionização em ambiente por *spray* sônico (*Easy ambient sonic-spray ionization*)
ESI	Ionização por elétron-spray (*Electrospray ionization*)
EVOO	Azeite de oliva extravirgem (*Extra virgin olive oil*)
FTIR	Espectroscopia em infravermelho por transformada de Fourier (*Fourier transform infrared spectroscopy*)
GC	Cromatografia gasosa (*Gas chromatography*)
HO	Óleo de avelã (*Hazelnut oil*)
HPLC	Cromatografia líquida de alta eficiência (*High-performance liquid chromatography*)
HRMS	Espectrometria de massas de alta resolução (*High-resolution mass spectrometry*)
HSM	Microscopia com câmara de aquecimento (*Hot-stage microscopy*)
LC	Cromatografia líquida (*Liquid chromatography*)

LDI	Ionização/dessorção a laser (*Laser desorption/ionization*)
LVOO	Azeite de oliva virgem Lampante (*Lampante virgin olive oil*)
MALDI	Ionização/dessorção a laser assistida por matriz (*Matrix-assisted laser desorption/ionization*)
MeOH	Metanol (*Methanol*)
MS	Espectrometria de massas (*Mass spectrometry*)
MSI	Espectrometria de massas por imagem (*Mass spectrometry imaging*)
MS/MS	Espectrometria de massas em *tandem*
OO	Azeite de oliva (*Olive oil*)
PCA	Análise de componentes principais (*Principal component analysis*)
PCAH	Hidrocarbonetos aromáticos policlorados (*Polychlorinated aromatic hydrocarbons*)
PDMS	Polidimetilsiloxano
SFC	Cromatografia por fluido supercrítico (*Supercritical fluid chromatography*)
SO	Óleo de soja (*Soybean oil*)
STE	Extração por tira sortiva (*Sorptive tape extraction*)
SPILDI	Ionização/dessorção a laser por *imprint* em placa de sílica (*Silica plate imprinting laser desorption/ionization*)
STELDI	Ionização por dessorção a laser com extração semelhante a tira sortiva (*Sorptive tape-like extraction laser desorption ionization*)
TGA	Análise termogravimétrica (*Thermogravimetric analysis*)
TLC	Cromatografia por camada delgada (*Thin-layer chromatography*)
TOF	Tempo de voo (*Time-of-flight*)
VOO	Azeite de oliva virgem (*Virgin olive oil*)

SUMÁRIO

INTRODUÇÃO GERAL

Nos últimos anos, tem-se criado uma grande tendência no investimento em pesquisas capazes de realizar análises essenciais para garantir a qualidade e a segurança de alimentos. A avaliação e o desenvolvimento de métodos analíticos adequados para as necessidades de um grande país produtor como o Brasil, bem como a natureza dos seus produtos, demandam a geração dados confiáveis e assertivos acerca dos alimentos comercializados ou potencialmente comercializáveis. O desenvolvimento de uma infraestrutura analítica eficiente garante à população alimentos de agregado valor nutritivo, com bom nível de segurança quanto a contaminantes e, portanto, de alta qualidade. Além disso, é possível também evitar a rejeição de produtos com potencial para exportação, respeitando os mais altos padrões de qualidade internacionais. Dessa forma, o uso de técnicas instrumentais rápidas, com alta precisão, exatidão, versatilidade e simplicidade operacional torna-se extremamente desejável dentro do escopo de análise de alimentos. [1, 2] Nesse segmento, figuram com grande destaque as técnicas baseadas em espectrometria de massas (MS). O desenvolvimento de novas fontes de ionização, as melhorias em analisadores (como o aumento da sensibilidade e especificidade) e a criação de sistemas de aquisição de dados com grande rapidez qualitativa e quantitativa contribuíram em grande escala para a aplicação da MS em análise de moléculas e ativos encontrados em alimentos. [3, 4]

Tendo em vista esse promissor cenário, a espectrometria de massas tornou-se a principal ferramenta em metabolômica de alimentos. Com início no

final dos anos 90, a análise do metaboloma e a impressão digital metabólica (*metabolic fingerprinting* – MF) conquistaram grande aplicação em rotinas de análises de alimentos. [5, 6] Rapidez, fácil interpretação de dados e métodos simplificados na elucidação do perfil químico das amostras são características que trouxeram forte interesse na última década, tanto por parte da indústria e academia, para incorporar e desenvolver essas metodologias em laboratórios especializados. Some-se isso ao fato de o grande mercado econômico de produtos com alto valor nutricional e com garantida qualidade exigir estratégias analíticas altamente refinadas plataforma metabolômica estará em nível privilegiado, inclusive, nas próximas décadas. [5, 7]

Metabolômica em Alimentos

A definição mais aceita de Metabolômica é a de que esse ramo da ciência trata da pesquisa e análise qualitativa e/ou quantitativa de todos os metabolitos em um sistema ou processo bioquímico. [8] Esses metabólitos são intrínsecos ao nível de atividade bioquímica (ou fisiopatológica) desse organismo, via metabólica ou processo, facilitando sua correlação com o fenótipo ou resultado desenvolvido. A expansão da utilização de estratégias metabolômicas é salientada em estudos de diferentes áreas do conhecimento, como doenças humanas [9], toxicologia [10], análise de plantas [11], nutrição humana [12] e, mais recentemente, controle de qualidade e processo. [13, 14]

De forma geral, a metabolômica é didaticamente dividida em duas estratégias: análise de moléculas-alvo (“*target analysis*”) ou análise de identidade amostral (“*metabolic fingerprinting*”). Como os próprios nomes sugerem, na primeira deseja-se obter dados sobre composição e/ou função

molecular de uma via metabólica ou molécula em particular, onde métodos bastante específicos são desenvolvidos para essa finalidade. Sua contrapartida, por sua vez, tem por finalidade estabelecer uma “impressão digital” da amostra, baseando-se em sinais característicos de um grupo particular de moléculas/classes de compostos presentes na mesma, estabelecendo um “padrão químico”, muitas vezes mesmo sem a necessidade de quantificação e/ou elucidação estrutural. [15] Os compostos identificados através destas estratégias metabolômicas podem ser validados e, posteriormente, utilizados como biomarcardores, também chamados de “compostos identificadores”, que conferem à amostra características importantes do ponto de vista de processamento, controle de qualidade, segurança e autenticidade de alimentos, sempre mediante a sua presença ou ausência. [16]

Diferentes abordagens metabolômicas podem ser efetivamente empregadas na análise de contaminantes químicos e microbiológicos de alimentos. Diferentes fungos e bactérias contaminantes alimentares podem ser identificados através das técnicas utilizadas em metabolômica. Elas podem ser significativamente mais rápidas, baratas, eficazes e especificas do que algumas técnicas bioquímicas tradicionalmente empregadas; um bom exemplo é a utilização de MALDI-MS na rápida identificação de microrganismos. [17] As toxinas produzidas por microrganismos podem também ser determinadas e quantificadas através da plataforma metabolômica, como no exemplo de algumas classes de micotoxinas. [18] As análises de contaminantes vão além daqueles oriundos de fontes microbiológicas; os contaminantes químicos, como poluentes orgânicos persistentes (POPs), agrotóxicos geralmente empregados

como praguicidas no cultivo de matérias primas alimentares podem ser detectados por essas técnicas, num desafio ainda pouco explorado em metabolômica de alimentos [6, 19, 20]

Técnicas de Espectrometria de Massas para Determinação de Substâncias em Alimentos utilizando a Plataforma Metabolômica.

Apesar de a consolidação da espectrometria de massas como técnica de referência no âmbito de química analítica quali e quantitativa ter ocorrido já desde as décadas de 60 e 70, sua utilização em alimentos foi, por muito tempo, restrita por impedimentos tecnológicos. Estes foram contornados através de melhorias e desenvolvimento de duas das mais largamente distribuídas técnicas de ionização. Essa consequente popularização da ionização por spray de elétrons (ESI) [3] e da ionização por dessorção a laser assistida por matriz (MALDI) [4] ampliou enormemente a gama de aplicações de MS ao final dos anos 80 e início da década seguinte. Ademais, o desenvolvimento de novos analisadores e as melhorias nas técnicas e controle de reações de fragmentação molecular (MS^n) permitiram a aplicação da espectrometria de massas altamente específica na elucidação estrutural de compostos, ampliando ainda mais seu leque na área de alimentos. Assim, segmentos como segurança alimentar e toxicologia de alimentos são desafios potencialmente auxiliados por meio desse desenvolvimento tecnológico.

MALDI

Essa técnica de ionização preconiza essencialmente a utilização de uma matriz, composta de moléculas orgânicas de caráter ácido e baixo peso

molecular, para o recobrimento da amostra. Um feixe de laser (infravermelho ou ultravioleta) incide e vaporiza a amostra recoberta com matriz e esta, por suas características químicas, auxilia a ionização dos analitos presentes na amostra através da doação ou retirada de íons H^+, podendo gerar íons positivos do tipo $[M + H]^+$ ou negativos do tipo $[M – H]^-$. As moléculas ionizadas e no estado gasoso são analisadas e, posteriormente, detectadas. [4] Em 1997, Caprioli *et al.* introduziram a técnica de MALDI acoplada à espectromeria de massas por imagem (MSI), na qual imagens químicas são geradas a partir de espectros de massas adquiridos ponto-a-ponto (*pixels*) em uma superfície de amostra qualquer. Essa introdução da MSI possibilitou a identificação e colocalização de compostos *in situ* em virtualmente qualquer tipo de amostra, num *plot* que pode ser em duas ou três dimensões. [21, 22].

A aplicação dessa técnica em alimentos se deu recentemente, quando pesquisadores japoneses caracterizaram antocianinas presentes no pericarpo de arroz negro de forma direta [23]. Esse trabalho elucida de maneira excelente o potencial da MSI em alimentos, pois fornece dados de distribuição espacial, tanto em relação às substâncias benéficas quanto à presença e localização de contaminantes químicos e/ou microbiológicos.

ESI-MS

O processo de ionização por spray de elétrons forma moléculas tanto positivamente quanto negativamente carregadas (cátions e ânions, respectivamente). Isso depende do tipo de solução na qual a amostra se encontra: soluções ácidas favorecem a formação de cátions e soluções negativas, ânions. Em ambos os casos, os íons são formados em solução, que é submetida a um spray eletrolítico que oxida os íons negativos, deixando as

gotas produzidas no spray com excesso de íons positivos (ESI positivo), ou alternativamente através da redução dos íons positivos, com excesso de íons negativos (ESI negativo). O feixe de íons é colimado, formando o “cone de Taylor” e, através de contra-corrente de nitrogênio, o solvente começa a ser evaporado e o volume das gotas começa a ser gradativamente reduzido.

Ocorre então repulsão crescente entre íons de mesma carga, com possível subdivisão (decomposição) das gotas, e estes íons são eventualmente transferidos para a fase gasosa em um processo brando e eficaz (dessolvatação). A partir desta etapa, operam os processos normais de análise, seleção e detecção de íons na fase gasosa por MS [3].

Espectrometria de Massas de Alta Resolução

A alta resolução é dada pela capacidade de um instrumento de massas em identificar um composto com alto grau de certeza, baseado na relação massa/carga (*m/*z) do mesmo, utilizando como parâmetro a massa exata de cada isótopo dos elementos que o compõem. Essa capacidade de resolver sinais distintos é dada na unidade de medidas FWHM, onde quanto maior seu valor, maior a resolução do instrumento. Os erros de massas, desvios existentes entre o valor medido e o valor real da massa exata, são dados partes por milhão (ppm) sendo desejável, portanto, um resultado com o menor erro possível expresso em ppm. Além da alta resolução e exatidão, essa técnica destaca-se por fornecer informações que atuam na elucidação estrutural de moléculas desconhecidas, sendo possível atribuir fórmulas moleculares ($C_cH_hN_nO_oS_s$) inequivocamente para medidas de baixa massa molar (< 450 u.m.a.) utilizando o princípio de massas exatas e defeitos de

massas. [24] Devido à complexidade das matrizes alimentares, instrumentos analíticos de resolução de massa limitada podem levar a resultados imprecisos. Espectrômetros de massas com analisadores do tipo *Orbitrap* comercializados já alcançam experimentalmente resoluções de até 500.000 FWHM com erros de massas inferiores a 1 ppm tendo, portanto, excelente aplicabilidade na área de alimentos.

Reações de Fragmentação (MSn)

Outra ferramenta auxiliar na elucidação estrutural são as reações de fragmentação de íons, também chamadas de MS/MS ou espectrometria de massas em *tandem*. Diversos instrumentos suportam essa função, especialmente os híbridos, com dois ou mais tipos de analisadores combinados. Exemplos clássicos são os analisadores de massas do tipo triplo quadrupolo (QqQ), quadrupolo-*ion trap* (LTQ) ou mesmo quadrupolo-*time-of-flight* (qTOF). Nesse tipo de experimento, elege-se um íon precursor de *m/z* conhecida e o mesmo é “bombardeado” por átomos de um gás inerte (He, Ar), que fragmenta a molécula inicial, gerando íons-produto. Esses, por sua vez, auxiliam na elucidação da estrutura do íon precursor, já que o padrão de fragmentação de uma molécula é, em grande parte, único.

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OBJETIVOS

Objetivo Geral

Propor novas metodologias de análise direta de alimentos utilizando a plataforma metabolômica em conjunto com novas técnicas de ionização e analisadores em espectrometria de massas.

Objetivos específicos

a. Utilizar as metodologias desenvolvidas tanto para monitorar compostos funcionais quanto contaminações em diferentes matrizes alimentares, resguardando aspectos de segurança alimentar e nutrição.
b. Minimizar ou eliminar o preparo de amostras na analises de alimentos, diminuindo custos, tempo e principalmente o consumo de solventes orgânicos, adequando-se aos parâmetros de química verde.

CAPÍTULO I

Análise direta e simultânea de compostos tóxicos e funcionais em amendoins

Rapid and simultaneous *in situ* assessment of Aflatoxins and Stilbenes using Silica Plate Imprinting Mass Spectrometry Imaging

Diogo Noin de Oliveira, Mônica Siqueira Ferreira, Rodrigo Ramos Catharino

Abstract: A fast and direct combination of techniques for simultaneous mycotoxin and phytoalexin identification in peanut skin and kernel is described. Silica Plate Imprinting Laser Desorption/Ionization Mass Spectrometry Imaging (SPILDI-MSI) is a powerful technique that exhibits great advantages, such as solvent-free and matrix-free characteristics, as well as no sample preparation or separation steps. It also permits accurate identification of mycotoxins and phytoalexins with unique fingerprint profiles in just a few seconds. Results are expressed as chemical images of the four identified types of aflatoxins (B1, B2, G1 and G2) and a stilbenoid (resveratrol). In addition, SPILDI-MSI allows the comparison between the spatial distribution of aflatoxins and resveratrol found in kernel and skin. This novel application has proven to be useful for instantaneous qualitative assessment of aflatoxins and stilbenoids both in the peanut skin and kernel and offers precise tracking of fungal contamination in nuts and other foodstuffs.

Keywords: aflatoxinas; SPILDI-MS; peanuts; resveratrol

Introduction

Mycotoxins have been more closely monitored in the past decades due to their harsh effects observed in humans and animals; potent toxic effects in humans and animals have been related to these molecules, such as cytotoxicity, carcinogenicity, mutagenicity, neurotoxicity, hepatotoxicity, immunosuppressive, and estrogenic effects [1-4]. As to their occurrence, aflatoxins and ochratoxins are produced mainly by *Aspergillus* sp., fumonisins, trichothecenes and zearalenone by *Fusarium* sp., patulin by *Penicillium* sp., and ergot alkaloids, produced in the sclerotia of *Claviceps* sp. [5]. Furthermore, these compounds have great financial impact. From an economic point of view, mycotoxins cause money loss to producers, processors and consumers of food and feeds. Significant reduction in foreign exchange is also an issue, as exported products are rejected in other countries due to the presence of these molecules [5-7]. In peanuts (*Arachis hypogaea* L.), *Aspergillus* sp. correspond to the main class of fungi that are associated to aflatoxin contamination, producing the types B1, B2, G1 and G2 [8].

Phytoalexins, more specifically stilbenoids, are molecules that help monitor fungal contamination [9]. They are secondary metabolites of nuts, produced in response to infections, injuries and/or other suffered attacks [10]. Many of these species are oxidation products derived from resveratrol, a phenolic compound that exhibits great antioxidant potential, especially in humans, with many potential applications for the treatment of several diseases such as cancer and cardiopathies in the past few years [11-14]. In plants, it is believed that an increased phytoalexin production is directly related to the defensive response of the vegetable, and this may also correspond to lower levels of aflatoxins [10].

Traditional analytical methods for assessing mycotoxins and phytoalexins include many steps of sample preparation, as liquid-liquid extraction (LLE), supercritical fluid extraction (SFE), solid phase extraction (SPE) and solid phase microextraction (SPME) [10, 15-21]. After these procedures, the sample is then subjected to a separation and detection system for identification and/or quantification. Generally, thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) detector are the most used approaches [11, 17, 22-26]. For these time-consuming characteristics, faster and more effective methods for high-throughput screening of mycotoxins and stilbenes in foodstuffs are necessary.

New approaches have already been developed in this field. Matrix-assisted laser desorption/ionization (MALDI) coupled with Time-of-Flight (TOF) analyzer has been successfully employed in aflatoxin screening [27]. This technique uses an energy-absorbent molecule (matrix), which is mixed with the sample or applied directly over it to assist laser ionization. Due to their characteristic structure, stilbenes have also been employed as MALDI matrices [28]. Within the most common configurations, apart from MALDI-TOF, there has recently been an increasing interest in instruments with Mass Spectrometry Imaging (MSI) [29]. This modern and interesting approach provides spatial distribution of compounds with intensities of a given ion on a coordinate system and its relative position in a physical sample, creating a sample image based on the specific molecular information measured [29].

Another recent analytical trend is the sorptive tape extraction (STE), in which a sorbent surface is used for molecular imprisonment and posterior

instrumental analysis [30]. This technique requires little sample preparation and no derivatization or liquid extractions. The STE principle was used as the basis for our procedure of sample preparation for subsequent LDI-MSI analysis, where a silica gel plate (Thin-Layer Chromatography plate) was used as a sorptive tape-like support for the imprinting of samples, in a slightly modified methodology as the ones described in previous works [31, 32].

The aim of this work is to provide, for the first time, a new method for direct and simultaneous screening of aflatoxins and a stilbenoid (resveratrol) in peanuts (*Arachis hypogaea* L.) skin and kernel using SPI as a sorptive tape-like extraction method followed by LDI-MSI. A silica gel (60 Å) plate is used as a molecular trapping surface for the samples. The greatest advantage associated to these methods is that they do not require chromatographic separation or many steps of sample preparation. This is also the first work that assesses both health hazardous and beneficial compounds to humans in a single sample, at the same time.

Materials and Methods

Reagents and Standards. Methanol and acetonitrile were HPLC grade (> 98%), purchased from J.T. Baker (Xastoloc, Mexico). Aflatoxins and resveratrol standards were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Peanut samples. Commercially available raw peanut bags were purchased from grocery stores in Campinas, Brazil. The bags were properly stored in a cabinet, free from light and at 25ºC. Samples were utilized after 1 year from the expiration date.

Sample preparation. Skin was removed from kernel and thin transversal sections of peanuts were cut with a stainless steel blade to obtain thin slices (~ 1 mm) of the sample. SPILDI experiments were carried out by pressing the samples against two silica 60 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) for five minutes. Preliminary tests with 1, 5, 10 and 15 minutes were performed; no signal improvements were observed with pressing times higher than 5 minutes (data not shown). Plates were then sent to analysis with no matrix coating. A representation of the analytical workflow is depicted in Figure 1. Standards were prepared as 1 mg/mL solutions in MeOH:H_2O (50:50). 2 μL of each standard solution were directly spotted in the TLC plate and then sent to analysis under the same MS conditions as the samples.

Mass spectrometry imaging. Samples were analyzed in a MALDI-LTQ-XL instrument (Thermo Scientific, California, USA) with imaging feature. The instrument uses an ultraviolet nitrogen laser. Typical conditions for data acquisition were as follows: 20 μJ laser power, 100 μm raster step size with laser spot size of 50 μm (factory default setting) and 30-50 normalized collision energy for collision-induced dissociation (CID) when performing MS/MS reactions. All mycotoxins data were acquired in the positive ion mode and resveratrol was analyzed in the negative ion mode (both at the *m/z* range of 100-500).

Data workup. The obtained MS/MS spectral data from standards and samples were submitted to structural analysis with Mass Frontier software (v. 6.0, Thermo Scientific, California, USA). The inputted structures are analyzed using algorithms and database information to produce fragment possibilities, which are then compared to the MS/MS spectra to assist in compound identification.

Chemical images were treated with ImageQuest software (Thermo Scientific, California, USA) and all intensities were normalized according to the total ion current.

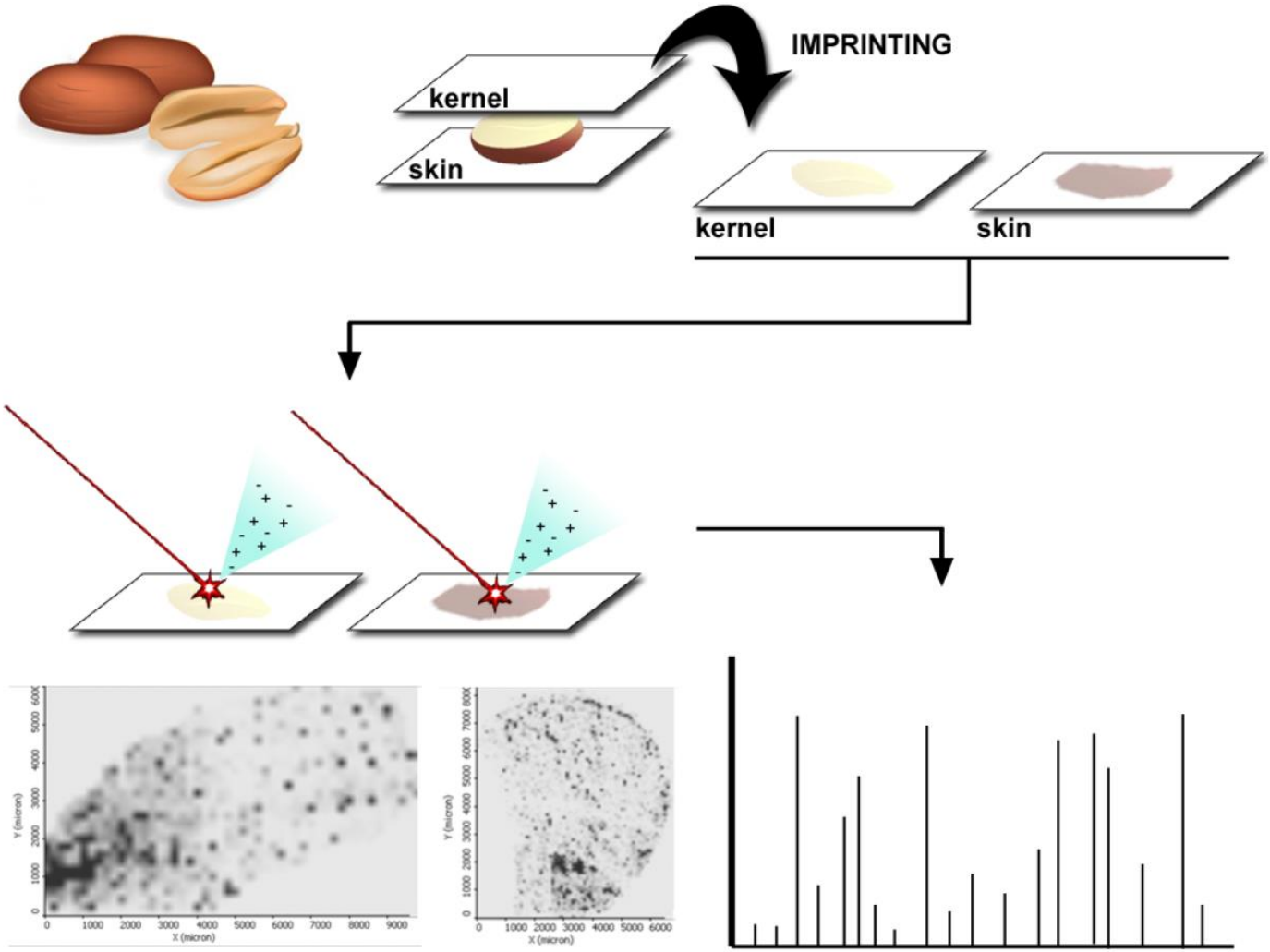

Figure 1. Detailed workflow of the SPILDI-MSI experiments for compound identification in peanut skin and kernel. Cross-sections of the kernel and the skin are imprinted in a TLC plate and then sent for MSI analysis.

Results

As the experiments were conducted with the skin and the kernel of peanuts, the spatial distribution of the different aflatoxins are compared in both regions as chemical images in Figures 2 (skin) and 3 (kernel). It was possible to observe that all types of aflatoxins were present even deeply into the internal regions of the kernel. CID was performed for the $[M + H]^+$ species for identification of the different aflatoxin types, with MS/MS data presented in figures 4 and 5 and also organized in Table 1. These data were analyzed using Mass Frontier software for fragmentation processes; they were also supported by the comparison with the MS/MS fragmentation pattern of the standards (found in Supplementary Material). Two-dimensional distributions on the surfaces of skin and kernel were collected directly via MS/MS of the characterized $[M + H]^+$ species, with results plotted as follows: AFB1 (*m/z* 313), AFB2 (*m/z* 315), AFG1 (*m/z* 329) and AFG2 (*m/z* 331).

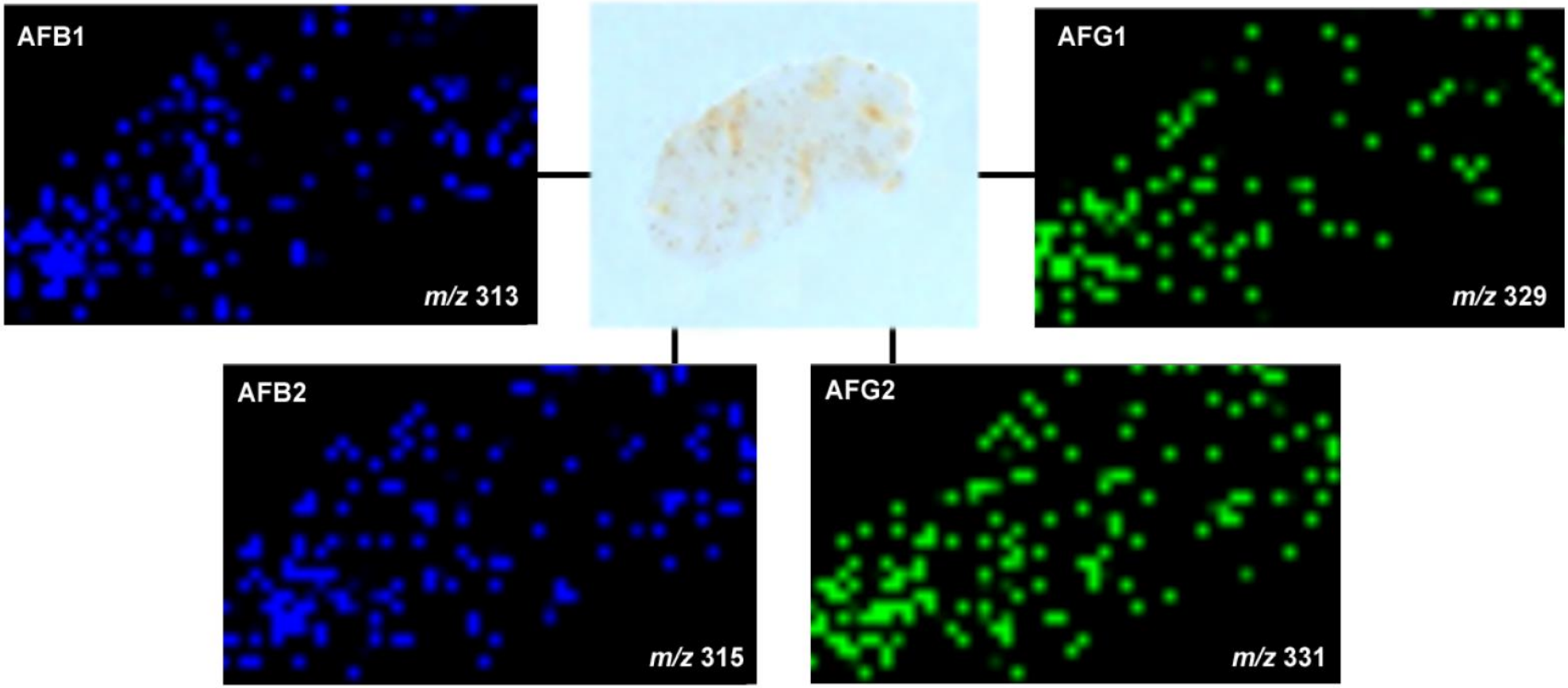

Figure 2. Example of SPILDI-Mass spectrometry images of the peanut skin: aflatoxins B1, B2, G1 and G2 are noted in their characteristic spatial distributions. Positive ion mode.

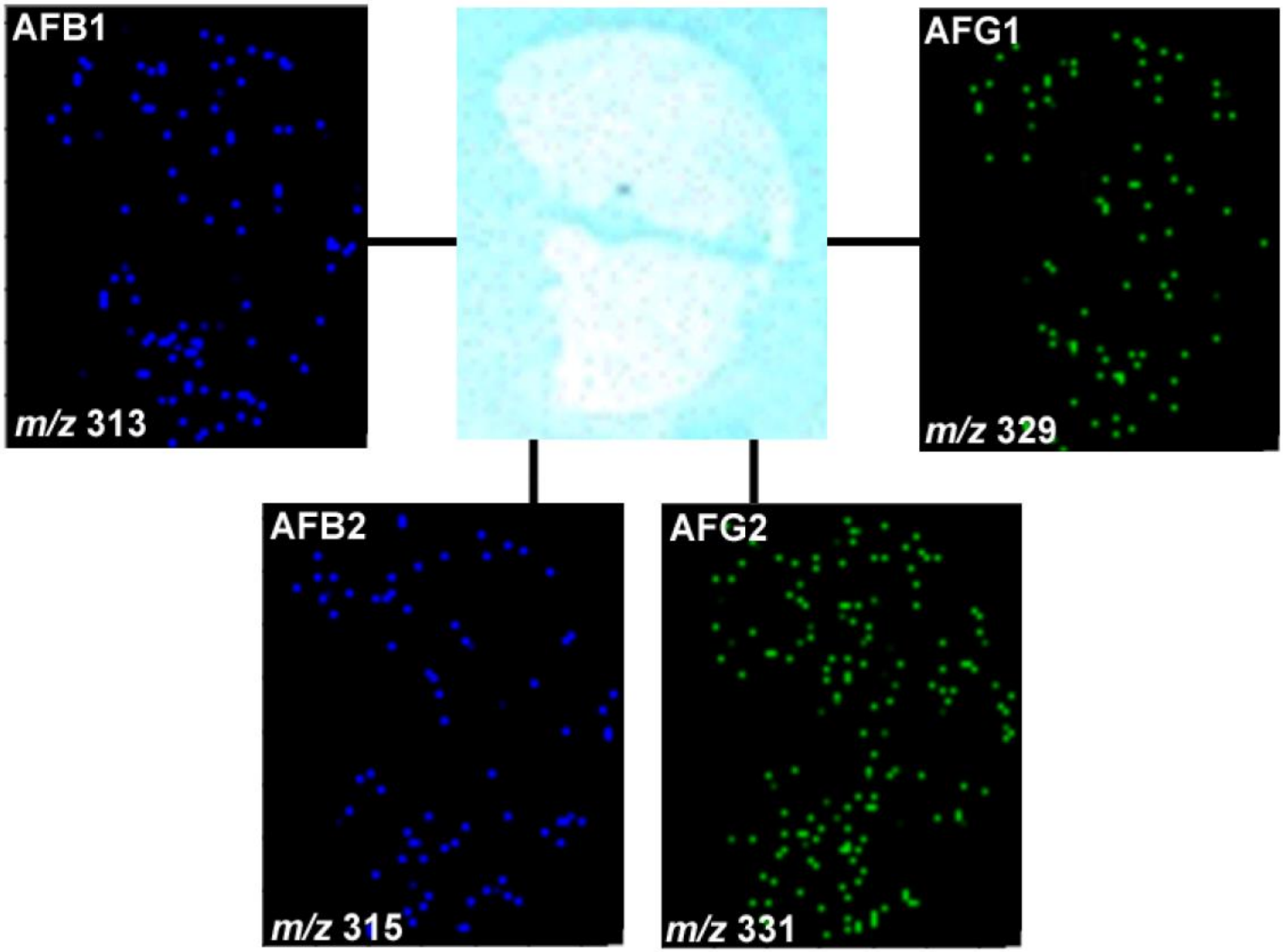

Figure 3. Sample SPILDI-Mass spectrometry images of the peanut kernel: aflatoxins B1, B2, G1 and G2 are noted in their characteristic spatial distributions. Positive ion mode.

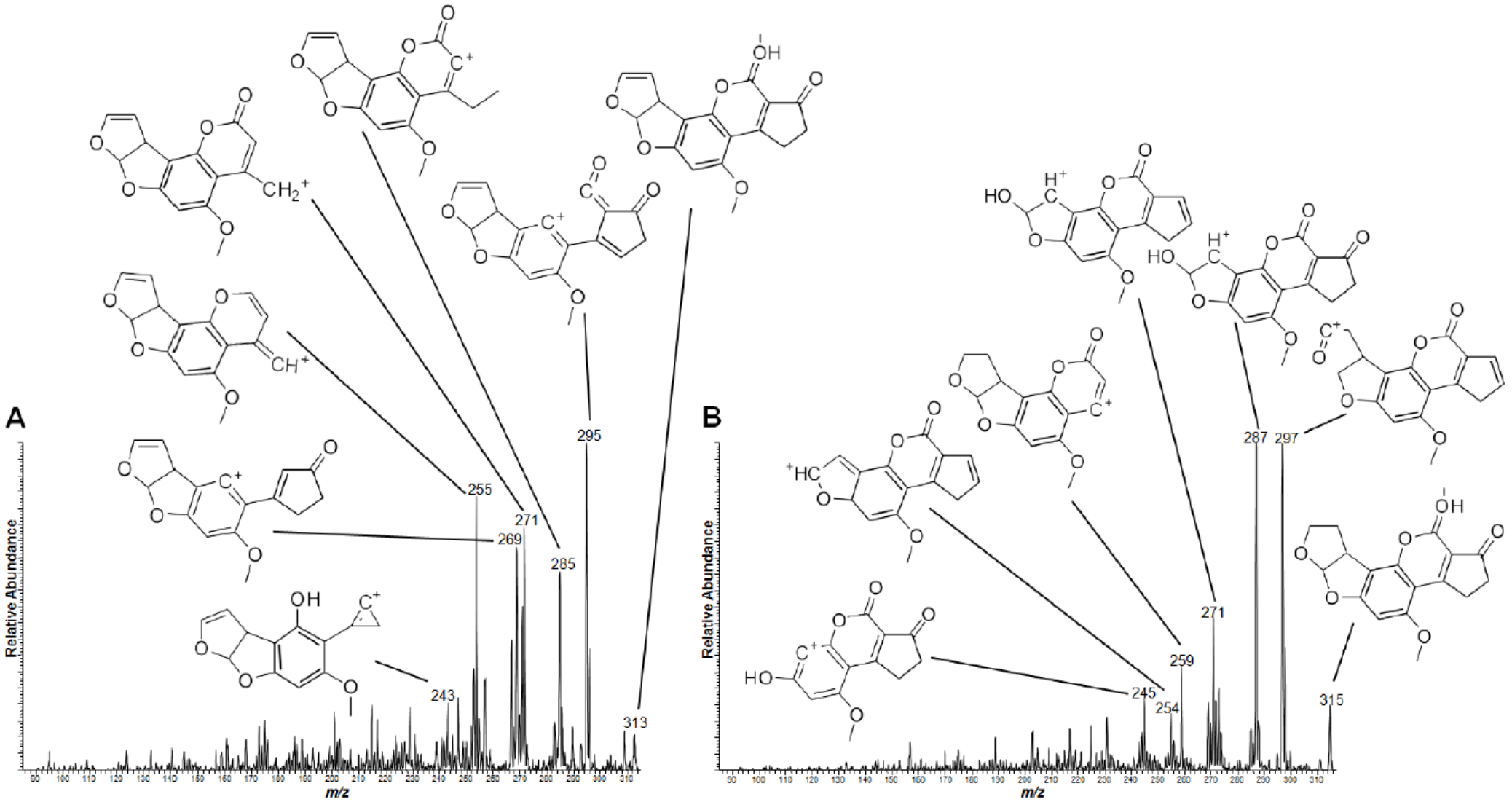

Figure 4. MS/MS spectra of aflatoxins (A) B1 and (B) B2. The characteristic fragments identified with Mass Frontier are identified along with the respective signals. Positive ion mode.

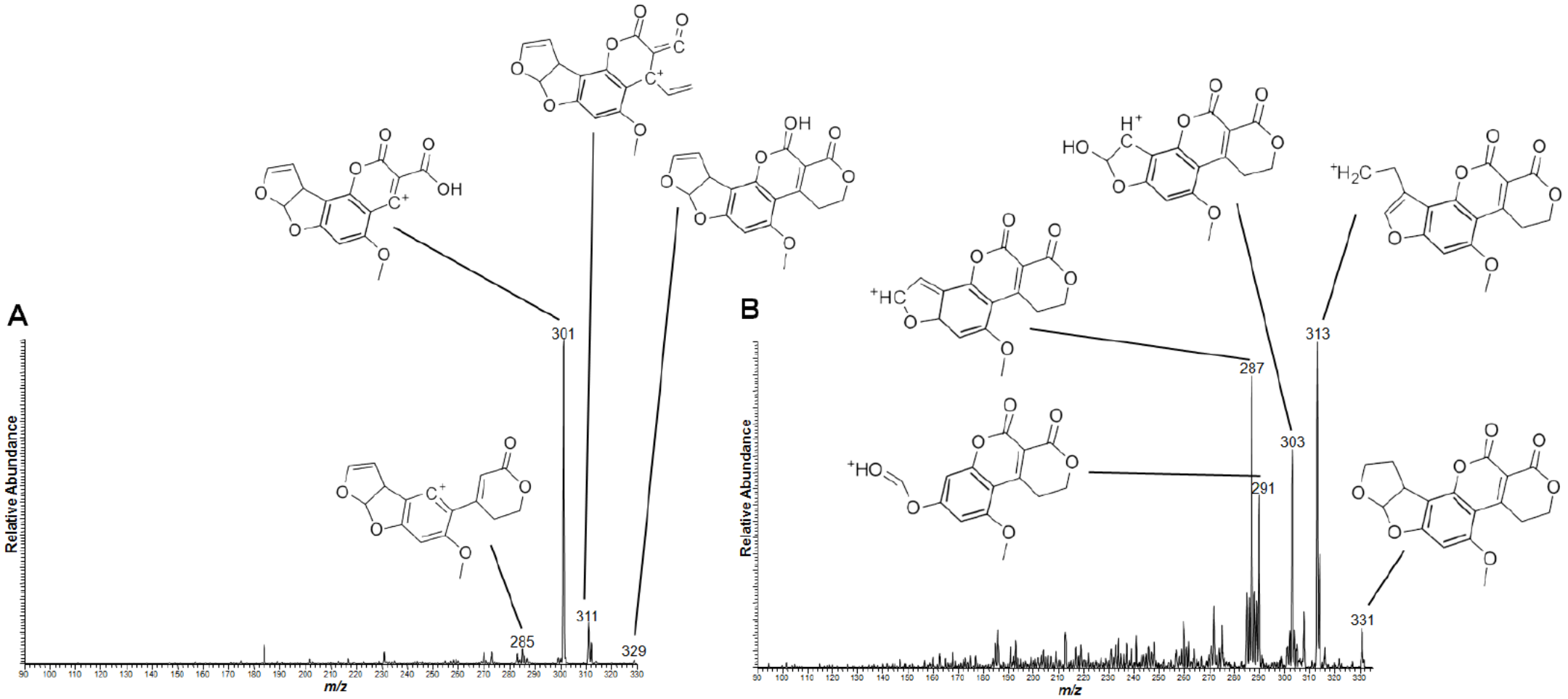

Figure 5. MS/MS spectra of aflatoxins (A) G1 and (B) G2. The characteristic fragments identified with Mass Frontier are identified along with the respective signals. Positive ion mode.

Compound	Precursor ion → Product ions	
Aflatoxins	**$[M+H]^+$** ***m/z***	**CID fragments** ***m/z***
B1	313	295, 285, 271, 269, 255, 243
B2	315	297, 287, 271, 259, 254, 245
G1	329	311, 301, 285
G2	331	313, 303, 287, 291
Stilbene	**$[M - H]^-$** ***m/z***	**CID fragments** **m/z**
Resveratrol	227	185, 159, 157, 145

Table 1. Identified species and their CID products for structural elucidation.

MSI was also utilized to assess resveratrol, evaluating its spatial distribution using the same methodology as for the aflatoxins. The stilbenoid-derivative was also identified in the negative ion mode by MS/MS at resveratrol as $[M - H]^-$ (*m/z* 227) with characteristic fragments, as elucidated in Figure 7. Figure 6 presents (A) the molecular structure of resveratrol and the spatial distribution of these compounds both in the (B) skin and (C) in the kernel of peanuts.

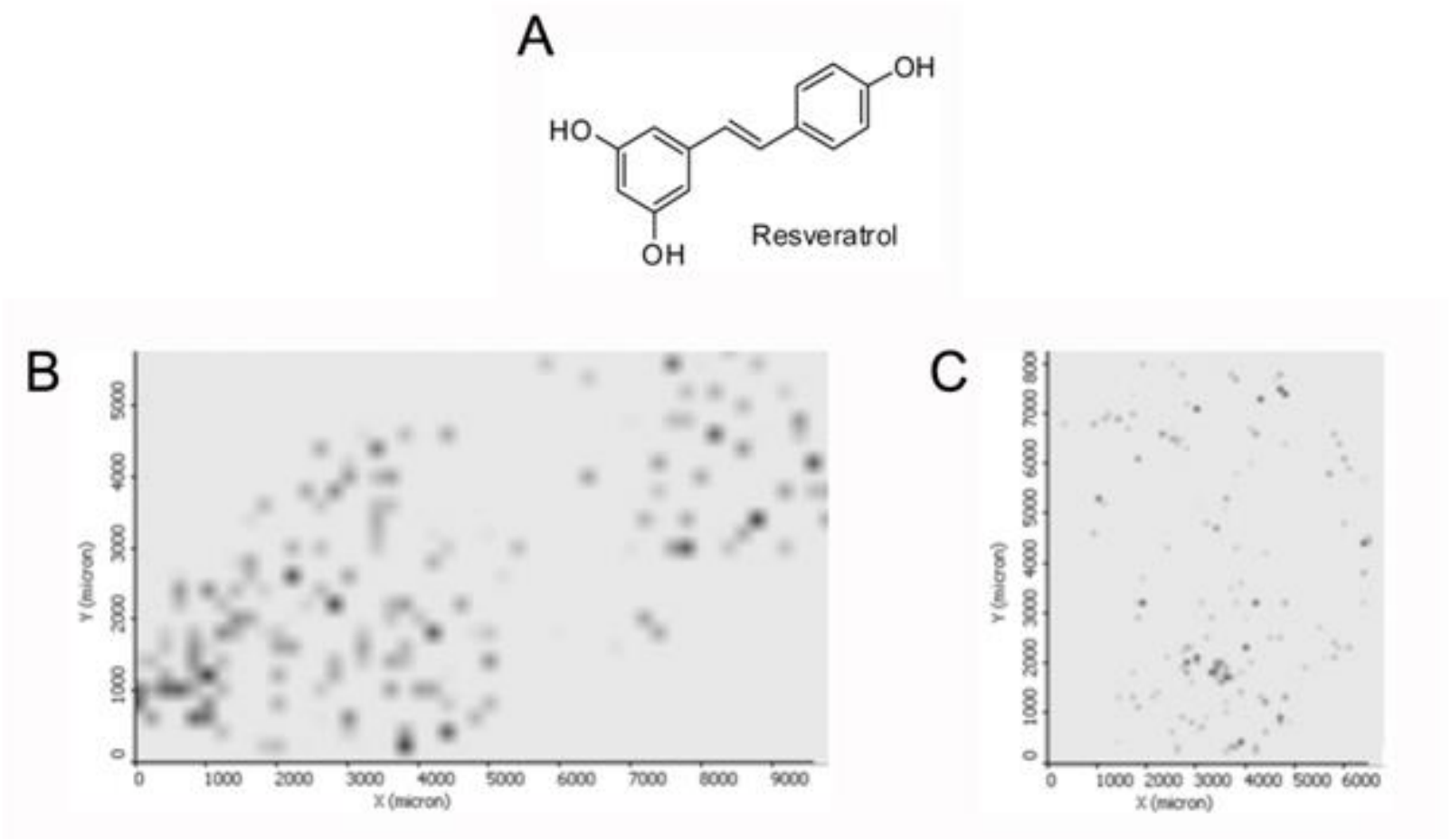

Figure 6. Schematic representation of the resveratrol molecule (A) and characteristic distribution on (B) peanut skin and (C) kernel. Data acquired in the negative ion mode.

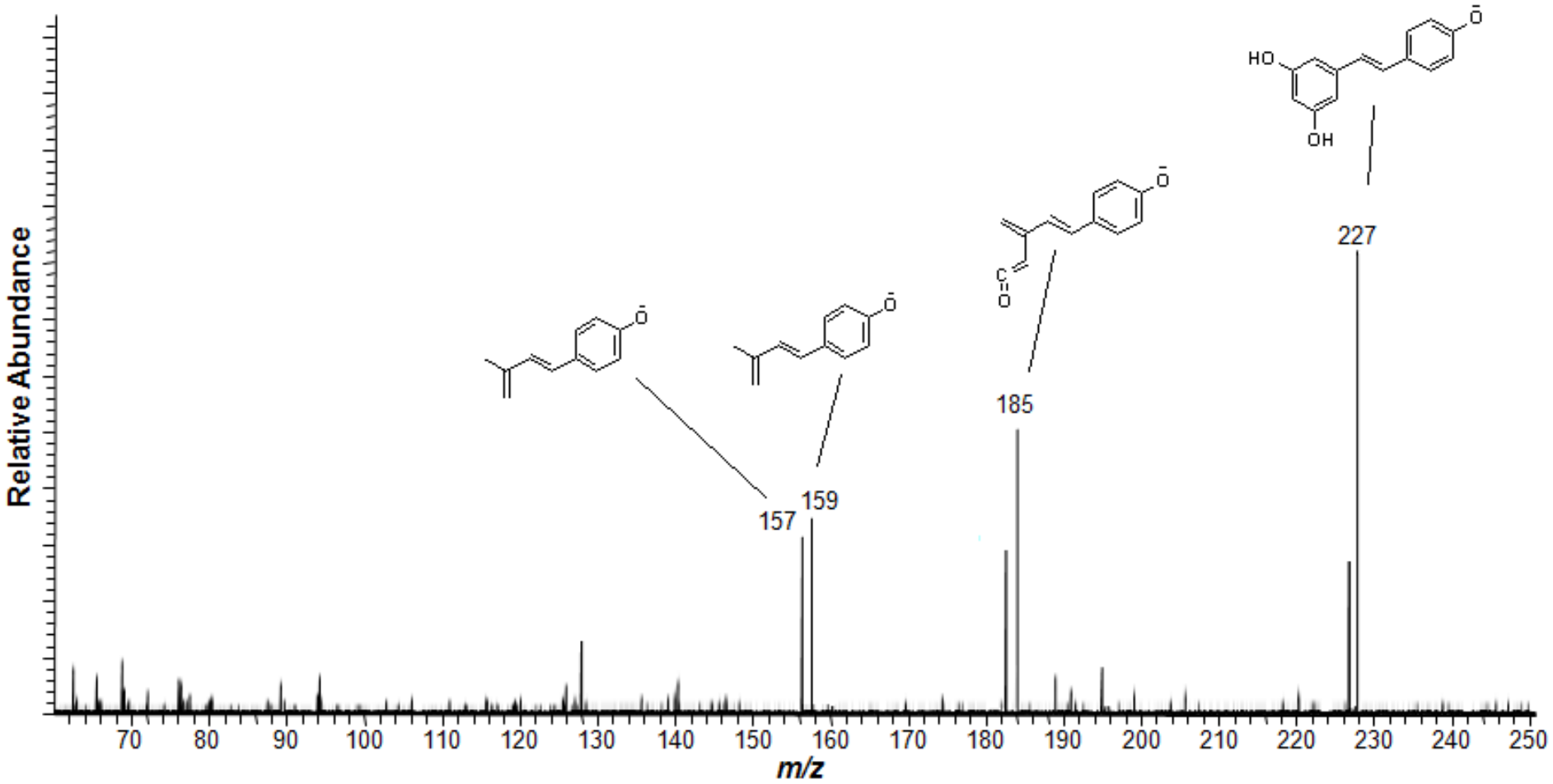

Figure 7. MS/MS spectrum of the compound identified as resveratrol at *m/z* 227 $[M - H]^-$ and the characteristic product ions. Negative ion mode.

Discussion

This novel approach on aflatoxin and phytoalexin detection directly on peanut surface has proven to be a fast and reproducible method. Without extensive sample preparation steps and no organic solvent employment, this technique shows great compromise with green chemistry trends [33]. Furthermore, this also avoids analyte losses in extraction and clean-up phases [34].

The use of MSI to identify both toxic and benefitial molecules in the same sample run is an effective and simpler approach. The possibility of identifying the colocalization of the targeted molecules directly from the sample surface is very interesting and may be appealing in terms of quality control and assurance.

Tandem mass spectrometry also provides accurate structural information for the analyzed molecules, especially when compared to chemical standards. The use of MS/MS as the main identification tool for small molecules is largely described as a very useful and reliable approach [35, 36] and the use of a linear-trap quadrupole for these purposes is also feasible and compatible with this application, and is especially a well-established routine with MSI [37-40]. To the extent of this work, structural elucidation of the targeted molecules was supported by software-predicted molecular fragmentation. Mass Frontier is an expert system where CID products and fragmentation mechanisms can be modeled. To do so, it uses MS databases as well as algorithm calculations to propose fragmentation pathways and final product ions [41]. For this work, aflatoxin structures were proposed based on the matches between all obtained MS/MS experimental data and the calculated Mass Frontier fragments. To support even further the given information, structures of the product ions are presented in the sample spectra from Figures 4 and 5. For resveratrol, the same principles have been applied, and the results are plotted in Figure 7. This

reinforces the high specificity of our methodology, where MS information is given with a high level of certainty.

Aflatoxin analysis is extremely relevant in terms of public health, as they are known for their carcinogenic effects and hepatotoxicity [42]. For the first time, the spatial distribution of these molecules is reported with information obtained directly from the skin and the kernel of peanuts, as illustrated by Figure 1. Interestingly, these mycotoxins present higher density towards the extremities of the skin and a more thorough distribution in the kernel. The analyzed phytoalexin, resveratrol, is a phenolic-derived compound. As well as an important role in plant defenses [10], this molecule is also important for human health and nutrition [43].

The overall amount of time dedicated to all analytical steps altogether (sample preparation, plate imprinting, instrumental analysis and data interpretation) can take as long as 15 minutes. This makes the presented approach a very fast and viable alternative for compound assessment directly from sample surface, with minimum sample preparation steps.

In summary, this work has demonstrated an effective analytical approach using SPILDI-MSI for direct assessment of aflatoxins and phytoalexins in peanut samples that has proven to be a simple and accurate strategy. This can be especially interesting for product treatment and toxin-removal processes, since it is possible to see that aflatoxins are not only present on the skin surface, but also in the inner parts of the kernel.

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CAPÍTULO II

Identificação direta de componentes lipídicos em azeites de oliva e determinação de adulterações através de *metabolic fingerprinting*

Direct Metabolic Fingerprinting of Olive Oils using STELDI-MS

Diogo Noin de Oliveira, Rodrigo Ramos Catharino

Abstract: A new and rapid approach for analysis of olive oil has been developed using sorptive tape-like extraction in combination with laser desorption ionization mass spectrometry (STELDI-MS). This powerful combination has some great advantages, as no-separation steps, solvent-free, matrix-free, and no sample preparation. The olive oil compounds are analyzed by LDI-MS, directly from the sample spot in a thin-layer chromatography (TLC) plate. Chosen samples represent products commonly used as adulterants in extra virgin olive oil (EVOO) and the main monitored ions were lipid adulteration markers. Analytical procedures consisted of profiling the main fatty acids (*m/z* 255 – palmitic acid, 279 – linoleic acid, 281 – oleic acid and 283 – stearic acid), triacylglycerols (*m/z* 901 – LLL and 907 – OOO) and some phenolic compounds (*m/z* 169 – gallic acid, 193 – ferulic acid and 195 – 2(4-hydroxyphenyl) ethyl acetate) in extra virgin olive oil (EVOO), olive oil (OO), hazelnut oil (HO) and soybean oil (SO). Compound identification was confirmed by analysis of collision-induced dissociation (CID) products in positive (ion $[M+Na]^+$) and negative mode (ion $[M-H]^-$). This method is simple, fast and efficient in identifying compounds that can be used to recognize different levels of adulteration, oxidation and hydrolysis of vegetables oils.

Keywords: olive oil; adulterations; food analysis; mass spectrometry; thin-layer chromatography; laser desorption ionization; sorptive tape-like extraction laser desorption ionization mass spectrometry; STELDI-MS

Introduction

Olive oil is widely distributed and highly appreciated worldwide due to its perceived beneficial effects on human health and its gastronomic uses. It is the major fat component of the Mediterranean diet, and Greece, Portugal, Spain and Italy are major suppliers of olive oils on the world market. It shows great economic importance, representing one of the main export products from these countries. The quality of olive oils covers many factors, such as genetic variety, geographical origin, climatic conditions, agronomic techniques and production technologies. These factors affect total fatty acid composition – particularly the concentration of oleic acid – and the concentration profiles of many other oil components [1, 2].

European Mediterranean countries have adopted common regulations to protect olive oil growers and consumers from fraud. According to the European Union Legislation [3], virgin olive oils are classified as extra virgin olive oil (EVOO), virgin olive oil (VOO) and lampante virgin olive oil (LVOO). EVOO is the highest-priced of all olive oil grades. Thus, adulteration of higher quality olive oils with either seed oils or lower-quality olive oils is a relatively common fraudulent practice, aiming to increase the product value. One of the most common adulterations in Europe is based on the addition of hazelnut oil (HO) or lower-quality grade olive oils because of the similarities in their chemical composition (triacylglycerol, sterol and fatty acid compositions). In Brazil, the most common form of adulteration of EVOO is addition of soybean oil (SO), a product widely available in the internal market. It can be added right before the process of filling, and this is usually done in the Brazilian territory by companies that buy large volumes of EVOO. Apart from the abusive character of such practices, using unrefined HO and SO has potential hazard for consumers who are allergic to hazelnut and soybean proteins [4-7].

Traditionally, gas chromatography (GC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE) are used in the authentication and analyses of olive oil. Recently, other techniques have been employed, especially using mass spectrometry. A few examples are Direct Analysis in Real Time coupled with high-resolution Time-of-Flight (DART-TOF-MS) [8], Easy Ambient Sonic-spray Ionization in negative ion mode (EASI-(-)-MS) [9], and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) [10]. These techniques present an attractive analytical alternative for their minimal requirements of sample preparation and no chemical derivatization or chromatographic separation.

Sisalli *et al.* (2006) [11] and Bicchi *et al.* (2007) [12] introduced sorptive tape extraction (STE) to study compounds in biological matrices. In these applications, the analytes were trapped in polydimethylsiloxane (PDMS) tapes and then recovered by either thermal or solvent desorption and then analyzed by GC or GC–MS. This technique was the basis for the procedure of compound-trapping for subsequent LDI-MS analysis, where a silica gel sheet (Thin-Layer Chromatography plate) was used as a sorptive tape-like support to the samples, according to a modified approach described by de Oliveira *et al.* (2013) [13] and Ferreira *et al.* (2014) [14].

The aim of this work was, for the first time, to describe a fast technique that can characterize compounds of interest – quality markers – in vegetable oils, as well as readily identify frauds and adulterations in EVOO using OO, HO and soybean oil SO directly from a spot in a thin-layer chromatography (TLC) plate, using LDI-MS with no matrix and no sample preparation steps.

Material and Methods

Samples. This study employed samples of extra virgin olive oil (EVOO), olive oil (OO), soybean oil (SO) and hazelnut oil (HO), available at regular market stores in Campinas, São Paulo, Brazil. Extra virgin olive oil (EVOO) with Protected Designations of Origin (PDO) and olive oil (OO) were produced in Spain, soybean oil (SO) in Brazil and hazelnut oil (HO) in Denmark. Mixtures of EVOO + HO and EVOO + SO are prepared at 80:20 proportions and EVOO + OO at 50:50. Trademarks and producers are suppressed due to ethical and legal purposes.

Sample Preparation. One microliter (µL) of each sample was directly spotted onto a thin-layer chromatography (TLC) silica gel 60 plate (Merck, Darmstadt, Germany), which was embedded on a special plate suitable for MALDI Mass Spectrometry Imaging (Thermo Fisher, San Jose, California, USA) and immediately sent for analysis. No matrix was applied and no further steps were taken.

LDI Mass Spectrometry. A MALDI-LTQ-XL instrument (Thermo Fisher) was used to acquire mass spectrometric data. Typical operation conditions were 20 µJ Nitrogen laser power, three laser shots per step and 30–50 eV of collision-induced dissociation (CID) for MS/MS experiments. All spectra were acquired in the positive and negative ion modes. Full scan analyses were performed in the *m/z* range of 100 to 600 in negative mode and 600 to 1200 in positive mode. The proposed identification of the compounds was based on both MS/MS spectra and software calculations with Mass Frontier (v. 6.0, Thermo Fisher).

Results and Discussion

Figure 8 shows STELDI-MS spectra in negative mode. The proposal for compound identification was based on the search of the main $[M-H]^-$ ion, as well as the interpretation of characteristic collision-induced dissociation (CID) products. Table 2 summarizes the data obtained for each of the analyzed sample in negative mode. Positive mode spectra are shown in figure 9 and the identification of compounds is based on search of the main $[M+Na]^+$ ion with CID products (Table 3). In some cases, the ionic species $[M-H]^-$ or $[M+Na]^+$ were neither detected nor observed (assigned as 'nd' in Table 2 and 3), which may be due to either unfavorable MS ionization, signal suppression or high intrinsic resistance towards dissociation.

Table 2. Components identified by STELDI-MS using the direct analysis approach. Ions and products were analyzed in the negative mode to assess fatty acid and phenolic composition.

Components	$[M-H]^-$ (*m/z*)	CID Products (*m/z*)	Samples						
			EVOO	OO	HO	SO	EVOO + HO	EVOO + OO	EVOO + SO
Fatty Acids									
Myristic	227	199	nd[a]	nd	nd	nd	nd	X	nd
Palmitoleic	253	235	X	nd	nd	X	nd	nd	nd
Palmitic	255	237	X	X	X	X	X	X	X
Linolenic	277	259	X	nd	nd	X	X	nd	nd
Linoleic	279	261	X	X	X	X	X	nd	X
Oleic	281	253	X	X	X	X	X	X	X
Stearic	283	269	X	nd	X	nd	X	X	X
Phenolic Compounds									
Gallic acid	169	151	nd	X	nd	nd	nd	nd	nd
Ferulic acid	193	165	X	X	X	nd	X	X	X
2-(4-hydroxyphenyl) ethyl acetate	195	177	X	X	X	X	X	X	X

[a] nd: not detected

Table 3. Components identified by STELDI-MS using the direct analysis approach. Ions and products were analyzed in the positive mode to assess triacylglycerol (TAG) composition.

Components[a]	$[M+Na]^+$	CID	Samples						
	(*m/z*)	Products (*m/z*)	EVOO	OO	HO	SO	EVOO + HO	EVOO + OO	EVOO + SO
Triacylglycerols									
PLL	877	597	nd	nd	nd	X	nd	nd	X
PLO	879	623, 601, 555	nd	X	X	nd	X	X	X
POO	881	625, 599, 577	X	X	X	nd	X	X	X
POS	883	627, 601, 579	X	X	X	nd	X	X	X
LLL	901	621	nd	nd	X	X	nd	nd	X
LLO, LLn	903	625, 623, 621	nd	nd	X	X	nd	nd	X
OOL, LLS	905	623, 601 621	X	X	X	X	X	X	X
OOO, SOL	907	625, 603	X	X	X	nd	X	X	X
OOS, SSL	909	627, 605	X	X	X	nd	X	X	X

[a] Fatty acids abreviations: P, palmitic acid; L, linoleic acid; O, oleic acid; S, stearic acid; Ln, linolenic acid.

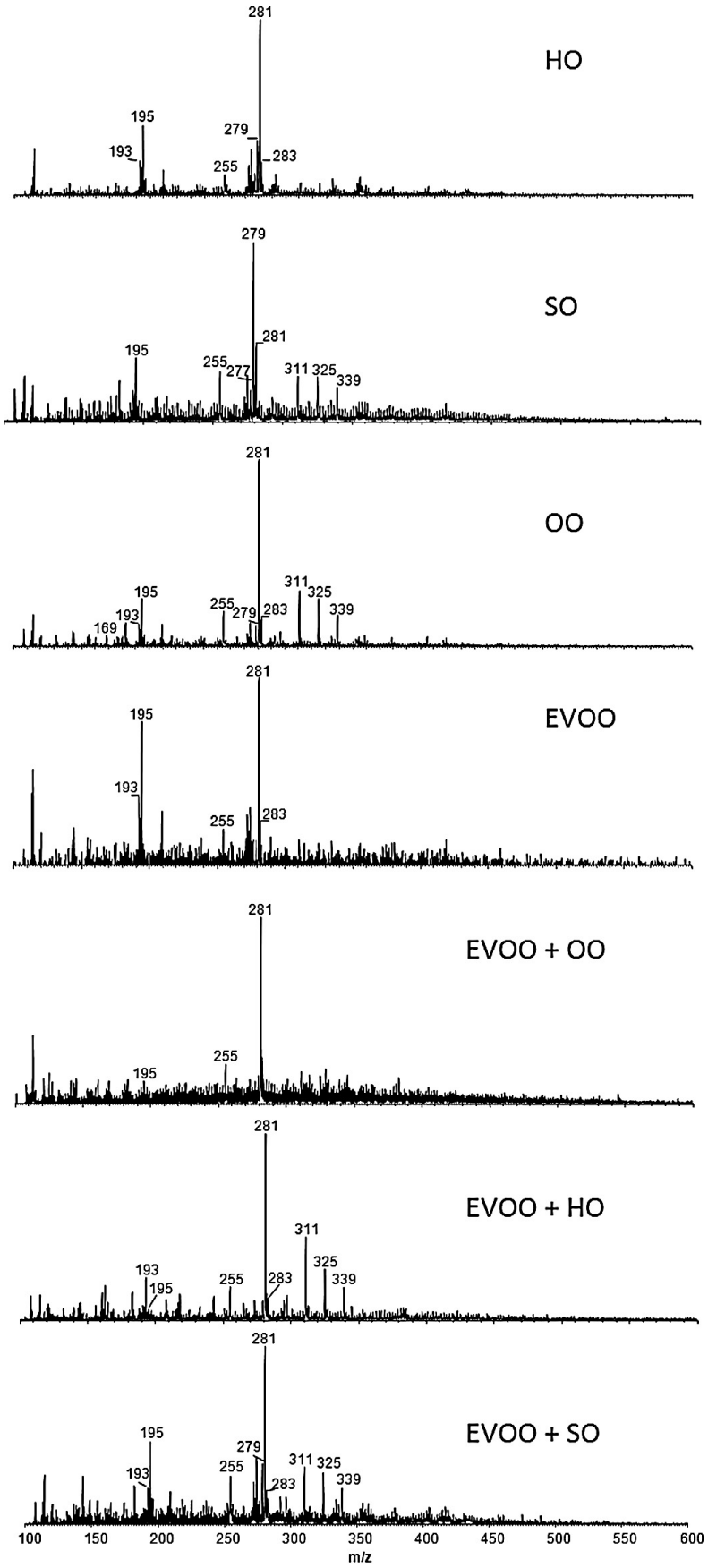

Figure 8. STELDI-MS spectra in negative ion mode.

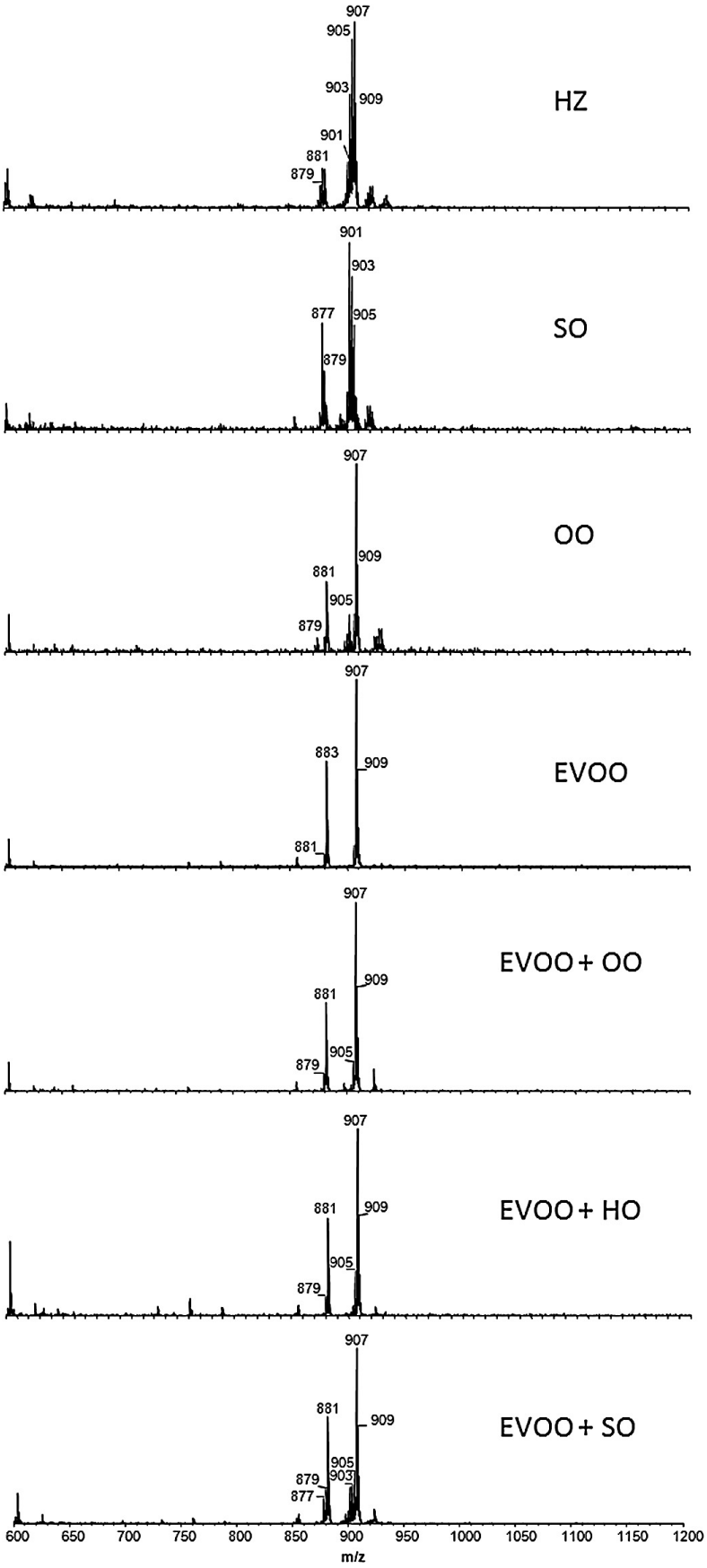

Figure 9. STELDI-MS spectra in positive ion mode.

The identified ions within the *m/z* range of 100 to 600 in the negative mode represent both free fatty acids and phenolic compounds. The presence of the latter in vegetable oils is intimately related to potential health benefits due to their potent antioxidant activity [15]. For this experiment, three of them were chosen for their considerable distribution in vegetable oils: 2-(4-hydroxyphenyl) ethyl acetate (*m/z* 195), ferulic acid (*m/z* 193) and gallic acid (*m/z* 169). Generally, determination of phenolic species is performed using chromatographic techniques, such as liquid chromatography coupled with mass spectrometry (LC-MS) [16]. In the present work, the capability of analyzing this class of compounds simultaneously with lipids (free fatty acids) is placed as one of its most interesting features. It was observed that OO is the only sample in which gallic acid was found, which can probably represent increased antioxidant potential for this product. Moreover, as far as antioxidant content, SO was the only sample that did not present ferulic acid.

For the free fatty acids within this range, oleic acid (*m/z* 281), linoleic acid (*m/z* 279), stearic acid (*m/z* 283) and palmitic acid (*m/z* 255) were the main species observed, which is attributable for their wide distribution among vegetable oils. Oleic acid was the major component of HO, OO and EVOO, whereas linoleic acid was the major fatty acid in SO. At first glance, it is possible to differentiate SO from all the others simply by looking at its spectral profile, and even further, adulteration of EVOO with SO can be inferred (but not confirmed) by doing so (Figure 8).

The positive mode shows the triacylglycerol (TAG) profiles of each sample, as seen on Figure 9. All structure assignments are based on MS/MS reactions. While it is not possible to establish oil adulterations simply by analyzing the negative ion mode, its combination with the positive mode results allows evidential results. CID has shown

characteristic fragmentations with losses equivalent to acyl moieties, confirming the negative mode results and even introducing new information on fatty acid composition. This enables, in this case, the identification of HO and OO adulteration in EVOO and thus reaffirms the importance of a thorough analysis.

The method presents the possibility of performing MS/MS reactions to confirm proposed compound structures. Hence, the analysis is specific enough to the target compounds, which in this case are mainly free fatty acids (negative mode) and triacylglycerols (positive mode).

Conclusions

The proposed methodology is rapid, easy to implement and requires virtually no sample preparation steps. Interestingly, without using any matrix, it is possible to apply the same experimental conditions to a MALDI instrument. The TLC plate has shown characteristics similar to a sorptive tape, with excellent compound trapping. The combination between these two techniques has proven to be very useful in the metabolic fingerprinting of different vegetable oils. Therefore, this metabolomic strategy can be a useful tool applicable to the analysis of adulterations and, since structural analysis is feasible to be performed, oxidative and hydrolytic processes can potentially be explored in future contributions.

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CAPÍTULO III

Determinação do perfil de degradação térmica do adoçante de mesa sucralose

Thermal degradation of sucralose: a combination of analytical methods to determine stability and chlorinated byproducts

Diogo Noin de Oliveira, Maico de Menezes, Rodrigo Ramos Catharino*

Abstract: In the late years, much attention has been brought to the scientific community regarding the safety of sucralose and its industrial applications. Although it is the most used artificial sweetener in foods and pharmaceuticals, many questions still arise on its potential to form chlorinated byproducts in high temperatures, as demonstrated by several recent studies. In the present contribution, we use a combination of differential scanning calorimetry and thermogravimetric analysis coupled with infrared spectroscopy (DSC/TGA/IR), Hot-stage microscopy (HSM) and high-resolution mass spectrometry (HRMS) on samples submitted to water bath at mild temperatures to evaluate a broad spectrum of hazardous compounds formed in the degradation of this product. TGA/IR has revealed that there is effective decomposition in form of CO_2 along with the formation of hydrogen chloride and other minor compounds. HSM results have provided accurate information, where the melting of the crystals was observed, followed by decomposition. Chlorinated derivatives, including polychlorinated aromatic hydrocarbons (PCAHs) were also confirmed by HRMS. These findings not only corroborate the suspected instability of sucralose to high temperatures, but also indicate that even exposed to mild conditions the formation of hazardous polychlorinated compounds is observed.

Keywords: sucralose; chlorinated compounds; high-resolution mass spectrometry; differential scanning calorimetry; thermogravimetric analysis; infrared spectroscopy

Introduction

Sucralose (1,6-Dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside) is currently the most utilized artificial sweetener for both industrial purposes and personal use [1]. Although initially considered safe for use [2], recent literature raised awareness regarding the intrinsic biological effects exhibited by sucralose [3], as well as the potential that its structure has to hydrolyze into toxic compounds when exposed to severe temperature conditions, forming chloropropanols and other related chlorinated compounds [4]. Important contributions have significantly broadened the knowledge on the accurate conditions under which these undesirable molecules emerge. In 2009, Bannach *et al.* performed thermogravimetry experiments that presented a characteristic decomposition profile for sucralose, indicating that the molecule is unstable (decomposes) in considerably mild temperatures [5]. Further studies utilizing gas chromatography approaches showed that it is possible to assess and monitor the formation of chloropropanols (CPs) in the presence of glycerol [6], and chlorinated polycyclic compounds in the presence of oils [7] and metal oxides [8, 9]. This has intensified the amount of evidence that support the hypothesis that sucralose cannot be suitable for processes that involve temperatures above 120ºC up to conditions near pyrolisis.

There is therefore urge to raise questions regarding the safety of non-nutritive sweeteners and bring them to public attention; in past contributions, our group was able to present the formation of potentially health-hazardous byproducts of stevia (*Stevia rebaudiana*) when mixed in low-pH solutions [10]. In this report, we employed a powerful combination of differential scanning calorimetry/thermogravimetric analysis coupled with Fourier transform infrared spectroscopy (DSC/TGA-FTIR), hot-stage microscopy (HSM) and high-resolution mass spectrometry (HRMS) to evaluate the

decomposition and elucidate the chemical profile of the compounds that are formed when sucralose is submitted to a relatively mild temperature. Our findings expand the knowledge on chlorinated byproducts, providing strong evidence that the formation of polychlorinated aromatic hydrocarbons (PCAHs) is feasible when isolated sucralose is exposed to temperatures that are even lower than those previously reported in literature.

Methods

Reagents and solvents. Technical-grade sucralose from two distinct manufacturing processes was purchased from local suppliers. Names of the manufacturers remain undisclosed due to legal and ethical issues. Both brands were sampled as follows: 1 g destined to MS analyses and 10 mg for DSC/TGA-FTIR analyses. All utilized solvents were HPLC grade, purchased from J.T. Baker (Xalostoc, Mexico), unless otherwise noticed.

Thermal analyses. Sucralose samples were introduced into a customized DSC/TGA-FTIR system (DSC-822e/STARe System, Mettler Toledo GmbH, Greifensee, Switzerland; Nicolet^{TM} FTIR module, Thermo Electron Corporation, MA, USA), in a DSC cell previously calibrated using the following high purity metallic standards: indium (T_{melt} = 156.56ºC; H_{melt} = 29.13 J.g^{-1}) and zinc (T_{melt} = 419.40ºC H_{melt} = 109.53 J.g^{-1}).Both DSC and TGA curves were performed in a dynamic process, with temperatures ranging from 25°C to 250°C, in a heating program of 10°C/min for scan study. Data acquisition was performed both under inert atmosphere (N_2) and synthetic air atmosphere, with a flow rate of 50 mL/min in both cases. Samples were deposited in standard aluminum pans with perforated lid, containing 10 mg of sample for DSC/TGA analyses. FTIR results were monitored on-line, and spectra were recorded all along the process, focused on endothermic peaks (DSC) and on the maximum mass variation (TGA). Spectral data were compared to software libraries (HR Aldrich Vapor and HR TGA Vapor Phase) to propose molecular identities. HSM was performed with a hybrid device equipped with a Zeiss Scope.A1 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) with polarized light as the image analysis system and a Mettler Toledo FP82HT electrical furnace, controlled by a

central processor (FP90, Mettler Toledo). Images were recorded using an AxioVision camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). The heating ramp for HSM was the same as the one utilized for DSC/TGA.

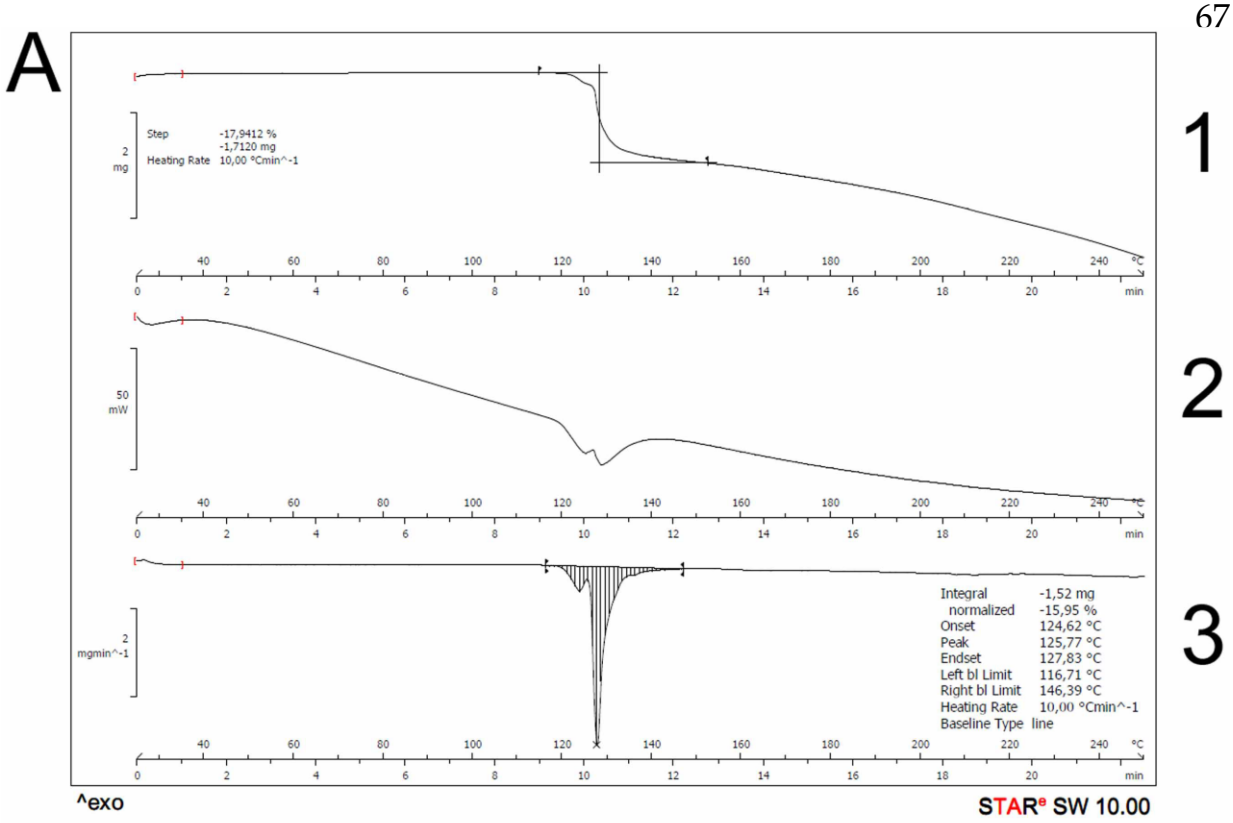
Mass spectrometry. Samples were placed in a glass vial with a large headspace, capped with a gas-tight lid with silicon septum. The vial was then submerged into a temperature-controlled water bath, where it was allowed to raise temperature at a rate of 1ºC/min (from 25ºC to 98ºC – boiling temperature). After reaching the boiling temperature, the vial remained submerged in the bath for 15 minutes and, after this time, a sample of 10 mL of the air was collected from the headspace using a gas tight syringe (Hamilton Company, Reno, Nevada, USA). The collected air was then dissolved in a 50:50 MeOH/H_2O solution (1 mL), through the septum of a glass vial. A schematic figure of this experimental setup is available as supplementary material (Figure S1). The final solution was then directly infused into an ESI-LTQ-XL Orbitrap Discovery (Thermo Scientific, Bremen, Germany) high-resolution mass spectrometer (30,000 FWHM). Data acquisition was performed under the following conditions: 4.5 kV spray voltage, 285ºC capillary temperature, sheath gas at 10 arbitrary units and a flow rate of 15 mL/min. Negative ion mode was explored, with *m/z* ratios ranging from 50 to 1000. The experiment was conducted in triplicates for both sucralose samples.

Structure elucidation. High resolution was the parameter of choice for the identification of the chlorinated species in MS analyses. The comparison between theoretical and experimental masses for mass accuracy are given in terms of parts per million (ppm) for error distribution. HRMS (*m/z*): $[M]^-$ calcd. for $C_6H_5Cl_2O$, 162.9723 found, 162.9729 (3.68 ppm). HRMS (*m/z*): $[M]^-$ calcd. for $C_6H_{10}ClO_5$, 197.0222 found,

197.0227 (2.53 ppm). HRMS (*m/z*): $[M]^-$ calcd. for $C_{13}Cl_7O$, 416.7774 found, 416.7780 (1.43 ppm).

Results

Thermal Analyses. DSC/TGA results are portrayed in figure 10, where A and B are the two distinct sucralose manufacturers; A1 and B1 present the mass loss observed in the TGA curves, which can be attributable to the loss of several moieties of the parent molecule. It is possible to see that the decomposition of sucralose happens around 125ºC (T_{onset} ~124.5ºC; T_{peak} ~125.5ºC) for both brands, in a 17-20% rate. Both experiments using N_2 and synthetic air have presented the same outcome regarding the thermal behavior exhibited, as well as the spectroscopic profile. Figure 11 presents the image profile of sucralose crystals obtained throughout the process of HSM analysis, showing that its crystalline structure is affected at the same temperature range as the one observed in DSC/TGA experiments (around 125ºC), in a fusion followed by decomposition process. A time-lapse video depicting the HSM process is available as supplementary material.



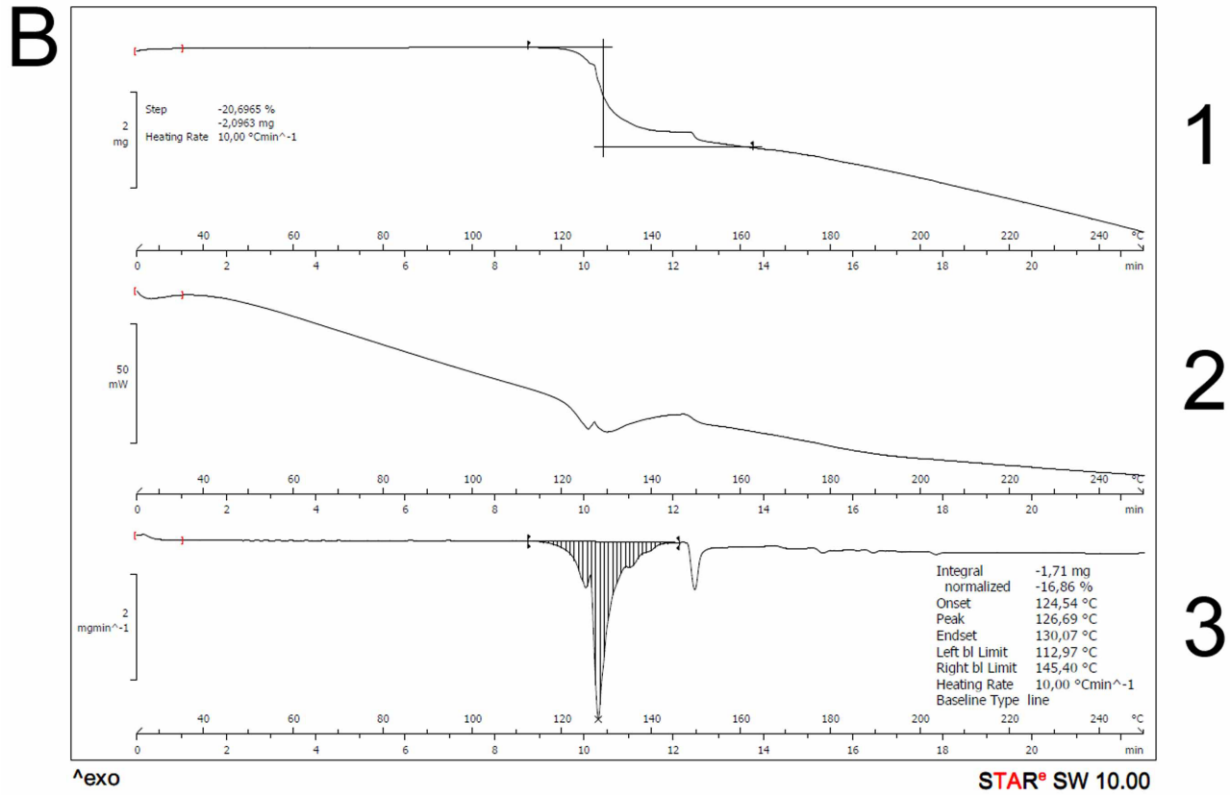


Figure 10. TGA (1 and 3) and DSC (2) curves from both sucralose brands (A and B). It is possible to notice that the mass loss (TGA) and endothermic peak (DSC) happen at the same time during the heat ramp.

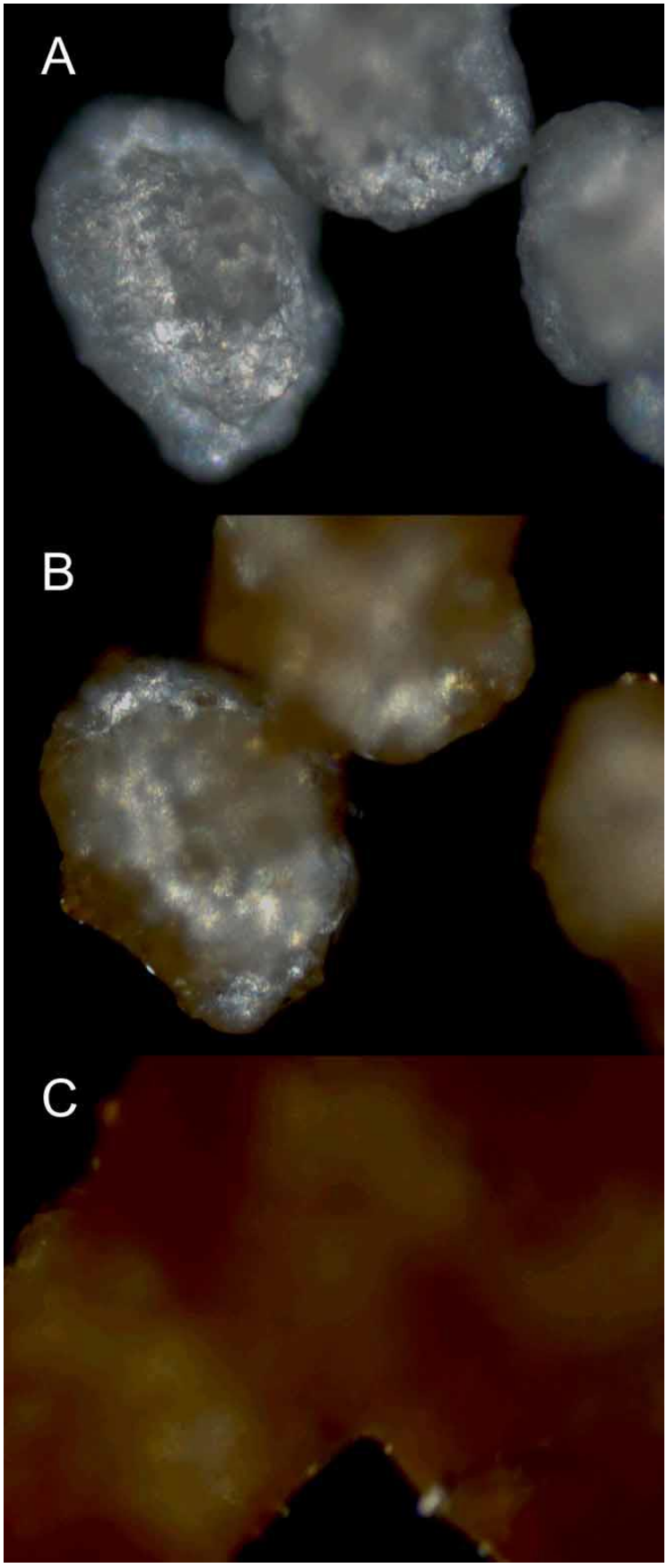


Figure 11. Images of sucralose crystals submitted to HSM analysis. (A) presents the crystals after heating; (B) shows the pre-melting stage and (C) shows the complete melting/caramelization of the crystals.

Fourier-transform Infrared Spectroscopy. Sample spectra from coupled FTIR analysis are presented on figure 12. It is possible to notice that both samples from different manufacturers presented the same peak profile (fingerprinting). The depicted spectra are linked with the same runtime where DSC/TGA presented the mass loss curves.

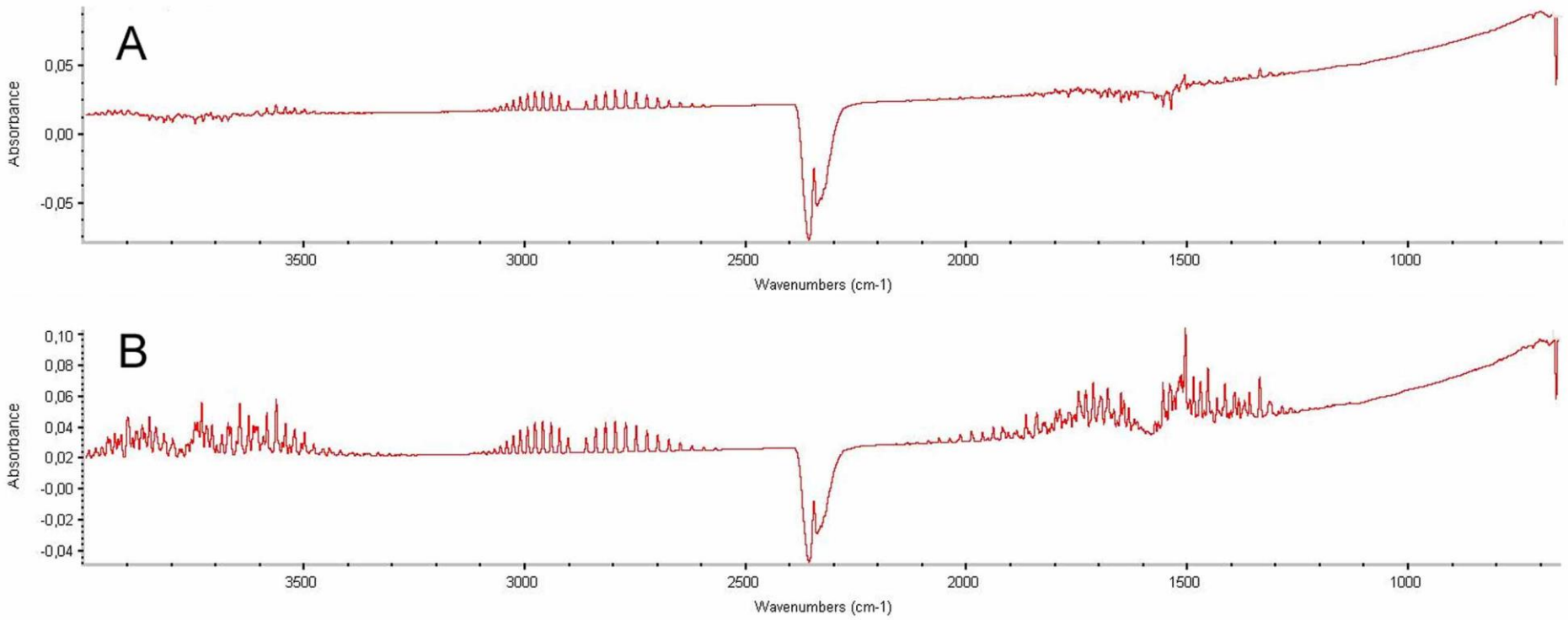


Figure 12. FTIR spectra of both sucralose brands (A and B). Spectral profiles were recorded at the same time (~11 min) for both cases, and present characteristic stretchings for the elucidated compounds.

Mass Spectrometry. Aromatic chlorinated byproducts were observed in the gaseous phase of the thermal decomposition experiment: a chlorinated furan derivative, similar to hydroxymethylfurfural (HMF) at *m/z* 162, and a compound from the PCAH class at *m/*z 418. Furthermore, a chlorinated tetrahydropyran (equivalent to the glucopyranosyl moiety on the sucrose equivalent) was also observed at *m/z* 197. A spectral sample is available is available on figure 13, where identified structures are assigned to the observed signals.

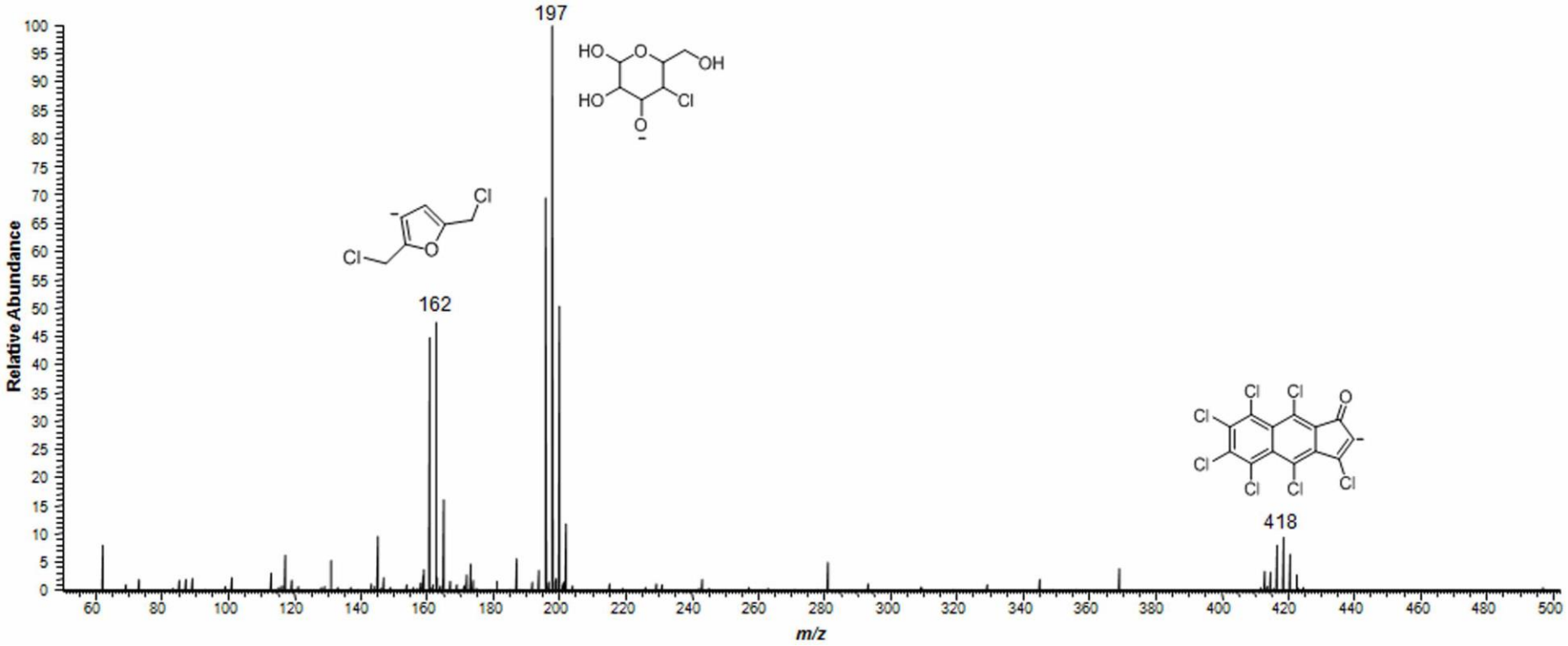


Figure 13. Sample mass spectrum of the air collected from the headspace after heating of sucralose. Identified structures are shown as $[M-H]^-$ species. Negative ion mode.

Discussion

DSC curves show an endothermic peak at the same temperature, which may be attributable to the fusion followed by decomposition of the molecule. This is confirmed by HSM data: figure 11 captures the exact transition stage between solid and liquid state. Supplementary data provided present a short movie showing the moment of transition between phases (movie 1). It is possible, therefore, to see that sucralose decomposition occurs right after the fusion of crystals begins, in an almost-simultaneous process. Bannach *et al.* (2009) [5] have postulated that sucralose decomposes once it reaches the melting point; indeed, in a simple physical state transition there would not be any mass change. Our combination of techniques of thermal analyses with crystal imaging, however, indicates that there is effective melting, even if for a very quick period, prior to decomposition.

FTIR results were processed with software matching using correlation algorithms from spectra libraries. Spectral analysis provide absorbance bands at peaks with characteristic wavenumbers, inferring that, at the decomposition point (linked spectrum at ~11 min), it is possible to observe characteristic profiles of water (ranges from 4000-3200 cm^{-1} and 2000-1200 cm^{-1}), carbon dioxide (main peak at 2400-2300 cm^{-1}), hydrogen chloride (range from 3100-2600 cm^{-1}) and chloroacetaldehyde (main peak at 1850-1700 cm^{-1}). This complements the data from DSC/TGA, providing the information that these low molecular weight species must be directly linked to the mass loss events observed in thermal analyses. Furthermore, the observed species are consistent with the expected behavior of a carbohydrate derivative under oxidative conditions (H_2O and CO_2 loss), with the addition of chlorinated species due to the presence of such atoms in the molecule.

Spectral analysis of acquired data from HRMS was performed taking into account previous works describing degradation pathways of both sucralose[6] and sucrose [11] to guide the search for compounds. Spectral information on figure 13 presents a clear cleavage of the main molecule (chlorinated disaccharide) into two semi-complementary $[M-H]^-$ moieties at *m/z* 197 and 162 – the latter being an advanced aromatic degradation product, which follows a hydroxymethylfurfural (HMF)-like pathway from the modified fructose moiety, and the first being a derivative from the modified glucose moiety. Furthermore, at *m/z* 418 it was possible to find a characteristic profile for PCAHs, assigned to a derivative from the complete thermal degradation of sucralose. Interestingly, previous reports have provided information on polychlorinated aromatic species from sucralose[9], but those experiments were carried out under more sophisticated and particular conditions. Our findings are more closely related to a pyrolytic environment, with molecules developing a mechanism similar to the one that occurs with regular dissacharides, such as sucrose, under these conditions [12]. Since sucralose is a molecule that has three chlorine atoms replacing the usual hydroxyl groups in sucrose, the addition of the halogen may be the key to PCAH formation under considerably mild conditions; there is previous evidence [12] that carbohydrates that are submitted to harsh temperatures exhibit the tendency to rearrange into thermodynamically stable configurations, resulting in aromatic compounds (either simple or polycyclic). Furthermore, the presence of chlorine increases the potential for reactivity due to an increase in the bond length, especially for the atoms bonded to non-cyclic carbons. These findings indicate, therefore, that potentially hazardous byproducts can effectively emerge even in conditions that can be considered mild, showing that degradation can occur well below the melting point. ADespite being a qualitative view, we found strong evidence that PCAHs are formed

from sucralose at boiling-water temperatures (up to 98ºC), which is the usual temperature reached when preparing hot beverages such as tea or coffee.

This is the first work that reports the thermal behavior of isolated sucralose, with no additional compounds, encompassing a wide range of analytical approaches. Our findings indicate that it is mandatory that the chronic exposure of humans to these chlorinated derivatives be further investigated regarding health-hazardous effects. The use of this artificial sweetener deserves, therefore, close attention, and further research on other food products must be conducted.

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CAPÍTULO IV

Determinação de percentuais de cacau em chocolates comerciais brasileiros utilizando a estratégia de metabolic fingerprinting

A semi-quantitative approach of catechin/epicatechin content in chocolates using MALDI-MSI

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Abstract: Chocolate is a popular food bearing a number of different classifications, which differentiate the proportions of cocoa solids, milk and cocoa butter. Literature brings evidence that some types of chocolate contribute to good health maintenance due to the presence of phenolics in cocoa. Phenolics contents depend on processing methods, which influence the level of these substances in the final product; therefore, accurate strategies to measure the levels of this molecular class are key to assure quality aspects of this food product. Mass spectrometry is an analytical tool of high sensitivity and specificity that is leading the research in food analysis towards new directions. By using mass spectrometry imaging in direct food analysis, this contribution developed an effective methodology for comparatively establishing the levels of phenolics catechin/epicatechin as selective quality markers of cocoa content in commercial chocolates, rendering a useful tool for quality control and counterfeit/ adulteration identification in these products.

Keywords: chocolate; quality; phenolic compounds; adulterations; mass spectrometry

Introduction

Chocolate is a food product that is commercially classified by the cocoa content declared by manufacturers. This results in four main different types of product, namely bitter, semisweet, milk, and white, which present varying amounts of cocoa solids, milk, sugar and cocoa butter [1], in addition to different resulting proportions of carbohydrates, fat and protein contents. The consumption of cocoa and chocolate contributes to nutrition by providing constituents for several metabolic functions; chocolate fat, for instance, is a rich source of saturated triacylglycerlols, whereas cocoa solids provide protein, minerals and phenolic compounds [1].

Cocoa and derived food products present, as a general rule, considerable flavonoid content, with highlights to flavan-3-ols, especially catechin/epicatechin [2]. The health benefits of these bioactive molecules in humans has been widely studied, especially a number of protective implications over the cardiovascular system [3] and the prevention of cognitive function decay with aging [4].

Assessing the different ingredients in chocolate for quality purposes is critical from a commercial point of view, as ensuring that consumers are purchasing the right product based on their dietary needs has transcended the economic aspect only. With abundant information on food products available over the internet and other sources, consumers are more prone to choose their products by relying on labels that contain ingredients or contents that meet their own criteria [5], thus making both the quality control and counterfeiting issues even more relevant.

There is a variety of studies in the literature with analytical approaches that assess different aspects of chocolate; FTIR, for instance, has shown great potential in the evaluation of adulterations in fat content [6], as did GC-MS for volatile compounds [7].

Quality assessment of chocolates regarding cocoa content, however, is still impaired by the lack of diversity and versatile methods that encompass both qualitative and quantitative approaches in literature. Chromatographic approaches are being widely used for phenolics in chocolates [8, 9] with consistent quantitative results. Nonetheless, alternatives aimed at saving both analysis time and solvent consumption are being sought, which ultimately presented MALDI mass spectrometry as a potential suitable method for assessing cocoa content and quality through phenolics [10]. Developing analytical strategies that are able to provide quali-quantitative approaches are therefore a growing trend in determining the quality of commercial chocolates through cocoa content.

In this sense, mass spectrometry imaging (MSI) has been emerging as a preponderant analytical tool that is robust enough to provide not only qualitative, but also quantitative aspects in solid-state samples, with minimal preparation requirements [11, 12]. Additionally, the careful choice for a suitable and selective quality marker, such as phenolic compounds that are specific for chocolate, plays a major role at assisting in a thorough and accurate analysis for quality control purposes. The aim of this contribution, therefore, was to explore this feature in actual chocolate samples, assessing cocoa content by the relative quantification of catechin/epicatechin, which were also understood as quality parameters.

Materials and methods

Chocolate samples

Commercially available samples of chocolate at five different declared percentages of cocoa content in the label were purchased from a Brazilian manufacturer. The percentages used for this study were 28%, 41%, 55%, 70% and 85%. Cocoa liquor,

obtained from the same manufacturer, was used as the 100% standard for comparison purposes.

Sample preparation

Samples were imprinted onto a silica plate (Merck, Darmstadt, Germany), covered with a 10-mg/mL solution of MALDI matrix α-cyanohydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO) in MeOH:AcN (1:1), and sent for direct analysis, in a similar approach as developed by de Oliveira, Ferreira [11]. A general scheme of the workflow is depicted in Figure 14. Samples were prepared in quintuplicates for each cocoa percentage.

MALDI-MSI analyses

The phenolic species catechin/epicatechin were elected as the selective quality markers for cocoa content. An LTQ-XL MALDI instrument (Thermo Scientific, San Jose, CA) was used to monitor the $[M+H]^+$ ion at *m/z* 291 after characterization through MS/MS reactions, compared to the fragments from a catechin standard (Sigma-Aldrich, St. Louis, MO) and calculated collision-induced dissociation products using the Mass Frontier software (Thermo Scientific, San Jose, CA). Chemical images with 600 square pixels were generated through selection of desired areas in the silica plate in the software prior to analyses. Images were then processed in grayscale using the ImageQuest software (Thermo Scientific, San Jose, CA), and ultimately cropped and submitted for relative quantification using the open-source software ImageJ (US-NIH, Bethesda, MD), in an adaptation of de Oliveira, de Bona Sartor [13].

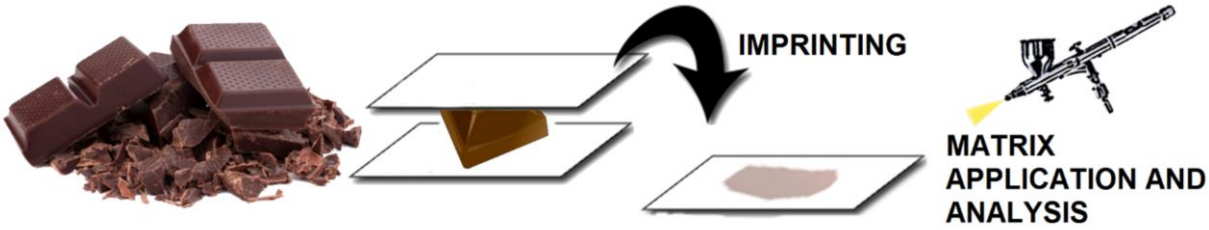

Figure 14. A simplified workflow for the preparation of chocolate samples followed by MALDI-MSI analyses.

Results and discussion

MALDI-MSI was utilized in the MS/MS mode, i.e. only the selective ion at *m/z* 291 $[M+H]^+$ was monitored during the analyses, after characterization. The main transitions observed in MS/MS were *m/z* 275, 247 and 197, which matched those calculated using Mass Frontier (data not shown). This strategy was performed to increase the levels of specificity for the target ions; as the instrument is set to acquire only the desired species, there is little or no interference of the other existing species in the sample, which is consistent with a method that is intended and designed to provide accurate results in cocoa content. As chocolate is a complex sample that is comprised of several molecular classes, the monitoring of a single ion may pose a challenge due to ionization issues, ion suppression, etc., which are eliminated by this feature.

To explore the semi-quantitative aspect of MSI, ImageJ software assigns non-dimensional values to images, similarly to the area under the curve (AUC) values obtained with chromatographic approaches. Comparatively, the higher the amount in a sample that undergoes chromatography, the higher will be the AUC value; in contrast, ImageJ provides higher values for darker pixels, indicating that this intensity variation is directly proportional to the amount of the selected ion in that particular sample. Values were then used to build an analytical curve, as per Table 4. The dataset from

each percentile was analyzed, which revealed a normal distribution according to the Kolmogorov-Smirnov test; a linear profile with a Pearson r-value of 0.962 and a two-tailed p-value of 0.002 was obtained from the relative quantification of samples, as provided in Figure 15.

Table 4. Mean values obtained from the quintuplicates of the non-dimensional values derived from the measurements with ImageJ software.

Declared cocoa content (%)	Mean values of pixel intensity*	Relative standard deviation (RSD, %)
100	9.3E+07	11.5
85	8.9E+07	15.1
70	8.2E+07	7.2
55	6.8E+07	23.4
41	5.3E+07	23.3
28	3.0E+07	6.5

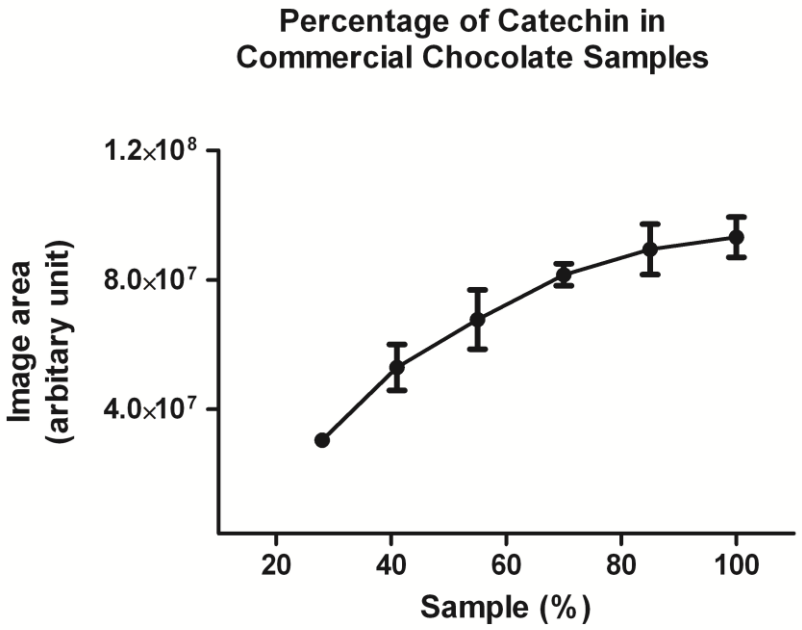

Figure 15. Analytical curve discriminating the percentage of catechin/epicatechin in commercial chocolate samples, as determined by the relative quantification with MALDI-MSI. The bars represent the standard errors of the means (SEM).

Significant intra-sample variation was observed, i.e. the results obtained from the areas were not uniform in all analyses, evidenced by high values of relative standard deviation, as per Table 4. This finding is quite interesting from an analytical point of view and can be explained by the intrinsic variation in the MALDI ionization. Although the quantification strategy is largely based on the same premise as that used in high performance liquid chromatography (HPLC), where the generated peaks are integrated and the area under the curve is used as a parameter directly proportional to the analyte concentration in the sample, in the case of MSI, two predominant factors directly influence the result of the analyses: (i) the fact that the sample is in the solid phase, which makes it difficult to obtain a degree of uniformity as high as in the liquid phase, and (ii) the fact that the "detector", a mass spectrometer as opposed to a diode array detector, is coupled to a laser pulsed ionization system (MALDI). These factors, together, determine that, despite the good results found, there are still technical impairments that may lead to fluctuations in results. Despite this, the MALDI-MSI technique proved extremely promising in a practical application precisely because of its simplicity of execution and relative ease in the preparation of samples.

Observed results ultimately showed that there is a linear trend behavior regarding the amount of cocoa present in commercial chocolates, and the amount of catechin/epicatechin is directly proportional to the amount of cocoa in chocolate. This is an important aspect for the proposed strategy, as MALDI-MSI is being consolidated as a versatile and accurate strategy, simple enough to be implemented with ease in process and quality controls for fast assessment of samples from the whole productive chain. Finally, as previous contributions have stated [14], there is an actual correspondence between the declared cocoa content in the label and the actual values

for selected manufacturers; this tool, therefore, emerges as a viable and reliable alternative for both quality and counterfeit detection purposes in chocolates.

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ANEXO I

Considerações finais

As metodologias analíticas propostas por este trabalho de doutorado, baseadas em plataformas “ômicas”, apresentam uma alternativa real e viável para serem implementadas em análises de alimentos. Seja em rotinas de alta performance e em monitoramento industrial, ou mesmo em pesquisa e desenvolvimento, a rapidez e simplicidade das estratégias desenvolvidas são características-chave que fundamentam sua aplicabilidade.

O escopo da utilização da espectrometria de massas em análise de alimentos deve, portanto, englobar desde análises de processo produtivo, estudos de estabilidade e *shelf-life* de produtos, processos de engenharia reversa (deformulação) e identificação de fraudes, além de estudos de compatibilidade entre ingredientes. Isso coloca a espectrometria de massas como uma ferramenta extremamente versátil e com alto poder de resolução de problemas relacionados à qualidade dos alimentos. Isso não somente auxilia resultando em menores perdas financeiras relacionadas a problemas industriais e incompatibilidades, como também garante a segurança alimentar do consumidor, contribuindo ainda para melhor entendimento de mecanismos envolvidos em toxicidade alimentar.