UNIVERSIDADE FEDERAL DE LAVRAS

ELISÂNGELA DO CARMO DOMINGO

DETERMINAÇÃO DE MELAMINA EM LEITE EM PÓ POR INFRAVERMELHO PRÓXIMO E ESPECTROMETRIA DE MASSAS

LAVRAS-MG

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Tese a ser apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência dos Alimentos, para a obtenção do título de Doutor.

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DEDICO

Aos meus pais, Nair e Adílio.
Aos meus sobrinhos.
Aos meus irmãos, em especial e *in memorium* ao Edimar.
Ao grande amor da minha vida, Claudimar.

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“Não fui eu que ordenei a você? Seja forte e corajoso! Não se apavore nem desanime, pois o Senhor, o seu Deus, estará com você por onde você andar".

Josué 1:9

RESUMO

A adição de melamina no leite em 2008 causou prejuízos ao setor lácteo e à saúde dos consumidores. A ingestão destes produtos causou a formação de cristais nos rins em crianças e bebês. Neste sentido diversos métodos foram desenvolvidos para detectar contaminações com esta substância. Contudo, a maioria exige longa preparação da amostra, utiliza reagentes nocivos à saúde humana, gera resíduos e possui alto custo, o que muitas vezes torna inviável o uso dessas técnicas. Dentro deste contexto, este trabalho teve por objetivo aplicar a espectrometria de massas com ionização por eletrospray (ESI-MS/MS) e a espectroscopia na região do infravermelho (IRS) na determinação de melamina em leite em pó. No primeiro artigo foram utilizados três tratamentos na preparação das amostras para análise por ESI-MS/MS, contudo apenas um pré-tratamento foi eficiente em remover a interferência dos constituintes do leite na análise. Os produtos desta extração apresentaram correlação univariada com a concentração de melamina no leite em pó, mostrando que a melamina pode ser quantificada. A extração da melamina baseou-se na formação de precipitado pela adição de ácido tricloroacético e solubilização em acetato de etila, sendo que esta técnica não havia sido relatada na literatura. Desta forma, a melamina pode ser extraída de forma simples e rápida para análise em ESI-MS/MS. No segundo artigo as amostras de leite em pó sem pré-tratamento foram avaliadas por IRS e calibração multivariada. A região 4500-5100 cm^{-1} apresentou melhor modelo de calibração, apesar de os modelos para as outras regiões do espectro terem sido precisos. Amostras comerciais de leite em pó avaliadas não estavam contaminadas com melamina. Portanto, os resultados mostraram que a análise do espectro de infravermelho por calibração multivariada permite obter resultados confiáveis de forma simples e rápida, sem que haja a necessidade de preparação das amostras.

Palavras-chave: Espectroscopia de Infravermelho. Espectrometria de massas. Fraude.

ABSTRACT

The melamine addition in milk on 2008 caused losses to the dairy industry and consumer health. The ingestion of these contaminated products caused the formation of crystals in the kidney in children and babies. In this regard various methods have been developed to prevent further contamination by this substance. However, most require lengthy sample preparation, using harmful reagents, generate waste, and are expensive, which often makes it impossible to use these techniques. Within this context, this work aimed to apply electrospray ionization mass spectrometry (ESI-MS/MS) and infrared spectroscopy (IRS) in the determination of melamine in powdered milk. In the first article three treatments were used in the preparation of samples for analysis by ESI-MS/MS, however only one pretreatment was efficient in removing the milk interference in analysis. The products of this extraction showed a univariate correlation with the concentration of melamine in milk powder, showing that melamine can be quantified. The extraction of melamine was based on the formation of precipitated by addition of trichloroacetic acid and solubilization in ethyl acetate, and this technique has not been reported in the literature. Thus, melamine can be extracted in a simple and quick way to ESI-MS/MS analysis. In the second article milk powder samples without pretreatment were evaluated by IRS and multivariate calibration. The region 4500-5100 cm $^{-1}$ showed the better calibration model, although the model for the other regions of the spectrum have been accurate. The commercial powdered milk powder evaluated was not contaminated with melamine. Thus, the results showed that the use of infrared spectroscopy and multivariate calibration allows to obtain reliable results simply and quickly, without the need for sample preparation.

Keywords: Infrared spectroscopy. Mass spectrometry. Fraud.

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1 INTRODUÇÃO

Nos últimos anos as exigências de leis e do mercado mundial pela melhoria da qualidade contínua no setor lácteo têm sido constantes, incentivando assim a maior produtividade, a criação de programas de capacitação e assistência técnica aos produtores, baseando-se principalmente na qualidade da matéria-prima (CASSOLI, 2011).

Nesse sentido algumas indústrias adotaram a remuneração do leite em função da qualidade, ou seja, o preço deixou de se basear apenas no volume fornecido e passou a ter outros parâmetros, incluindo o teor de proteína e gordura, a contagem de células somáticas e a contagem bacteriana total. Contudo, também houve o estimulo a prática de fraudes, dentre as quais a adição de melamina ao leite em 2008.

A melamina é uma substância rica em nitrogênio (Figura 1) e foi adicionada ao leite na China com o objetivo de aumentar o teor de proteína aparente, já que o método oficial se baseia na quantificação do teor de nitrogênio total e por isso considera que todo nitrogênio quantificado provém da hidrólise das proteínas, ou seja, o método de Kjeldahl é indireto.

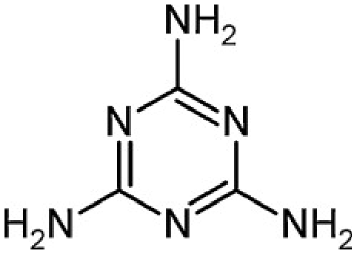

Figura 1 Estrutura molecular da melamina.

A contaminação se espalhou para outros alimentos, isso porque o leite é ingrediente em diferentes indústrias alimentícias. Por consequência a ingestão destes produtos pela população Chinesa ocorreu durante meses e resultou na formação de cristais nos rins e insuficiência renal em bebês e crianças, sendo que 294.000 ficaram doentes, 50.000 foram hospitalizadas e seis morreram (DING et al., 2010; INGELFINGER, 2008; MAUER et al. 2009).

Diversos países proibiram a importação de produtos lácteos chineses. E, além da proibição, o Brasil definiu que produtos lácteos chineses importados anteriormente deveriam comprovar a ausência desta substância. Desta forma a fraude acarretou prejuízos para o setor lácteo, comércio internacional e para a saúde dos consumidores, ressaltando a importância da atuação efetiva de órgãos fiscalizadores para garantir a autenticidade dos alimentos.

Alguns órgãos fiscalizadores definiram métodos para a detecção de melamina em alimentos, dentre os quais se destaca a cromatografia líquida de alta eficiência (HPLC), cromatografia líquida com espectrometria de massas (LC-MS/MS) e cromatografia gasosa com espectrometria de massa (GC-MS/MS) (Organização Internacional para Padronização - ISO 2010). Contudo, tais métodos exigem longa preparação da amostra e execução da análise, utilizam reagentes nocivos à saúde, geram resíduos e possuem alto custo, o que muitas vezes inviabiliza a utilização dessas técnicas para grandes lotes de amostras, principalmente por aumentar o tempo de liberação de produtos em portos e aeroportos.

A espectroscopia na região do infravermelho apresenta algumas vantagens, tais como: simples preparação e não destruição da amostra, ausência de reagentes nocivos à saúde, avaliação de diferentes características e obtenção de resultados confiáveis de forma simples e rápida. Diversos autores relataram a eficiência deste método na determinação da autenticidade e qualidade dos alimentos (BABALIN & SMIRNOV, 2011; BORIN et al, 2006; DOWNEY et al., 1997; FERRÃO et al., 2007; HE, RODRIGUEZ-SAONA, GIUSTI, 2007).

A espectrometria de massas é uma técnica precisa, utiliza pequenas quantidades de amostras e os resultados obtidos são precisos (DANE e CODY, 2010; DESMARCHELIER et al., 2009; HUANG et al., 2010). Apesar da disponibilidade de diferentes métodos para detecção de melamina, no Brasil ainda não foi definido método oficial para detecção e monitoramento de melamina em alimentos.

Dentro deste contexto este trabalho teve por objetivo aplicar a espectrometria de massas com ionização por eletrospray (ESI-MS/MS) e a espectroscopia na região do infravermelho (IRS) na determinação de melamina em leite em pó.

Dentre os objetivos específicos destacam-se:

a) Parte I:

- Desenvolver metodologia analítica para extração de melamina do leite em pó
- Avaliar a eficiência da extração por ESI-MS/MS

b) Parte II:

- Avaliar a quantificação de melamina em leite em pó sem preparação prévia por espectroscopia na região do infravermelho próximo
- Construir modelo de calibração multivariada em três regiões do espectro
- Quantificar a melamina em amostras comerciais de leite em pó, utilizando o melhor modelo de calibração.

2 REFERENCIAL TEÓRICO

2.1 Leite em pó

O leite apresenta importância social, econômica e sob aspecto nutricional é considerado um dos alimentos mais equilibrados e completos, sendo consumido em todas as partes do mundo e responsável por proporcionar o atendimento de parte das necessidades calóricas diárias (VEIGAS et al., 2006).

O leite em pó destaca-se por ser saudável, nutritivo, prático, possui longo período de validade, facilidade no transporte e grande impacto na exportação e importação. Além disso, é o terceiro derivado lácteo mais produzido no Brasil, o que corresponde à industrialização de 18 % da produção de leite e com consumo médio anual no país de 2,6 kg por pessoa (Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA, 2007).

Os parâmetros de qualidade do leite em pó são regulamentados pela Portaria nº 369 do Ministério da Agricultura Pecuária e Abastecimento (MAPA), a qual define que o leite em pó é o produto apto para a alimentação humana, obtido por desidratação do leite e classificado de acordo com o teor de gordura, sendo desta forma proibida a adição de soro ou proteína e gordura de origem não láctea (BRASIL, 1997). Entretanto, diversos adulterantes têm sido adicionados com o intuito de mascarar a má qualidade ou aumentar o rendimento, o que exige o monitoramento constante da qualidade e autenticidade dos alimentos pelos órgãos de fiscalização, já que a intensificação do comércio mundial permite que o consumidor tenha acesso a alimentos de diferentes partes do mundo, o que aumenta a exposição a riscos.

No Brasil a legislação estabelece penalidades para adulteração de alimentos, como forma de manter a qualidade nutritiva original, preservar o conceito de pureza e tornar justa e leal a concorrência entre as indústrias, sendo a penalidade aplicada a quem fabrica, vende, expõe à venda, tem em depósito pra vender ou de qualquer forma distribui e entrega o produto falsificado para o consumo (BRASIL, 1998).

No ano de 2007 a operação conhecida como Ouro Branco investigou a adulteração de leite cru no estado de Minas Gerais, a qual identificou que indústrias adicionavam soro de queijo e água para aumentar o volume, peróxido de hidrogênio para reduzir a carga microbiana, citrato de sódio como estabilizante e bicarbonato de sódio e hidróxido de sódio para corrigir o pH do leite, o qual era destinado para a produção de leite UHT ou vendido a outras indústrias (CASSOLI, 2010).

Em 2013 a operação Leite Compensado investigou a prática de fraude em leite cru no estado do Rio Grande do Sul, sendo que a adulteração se baseou na adição de água e ureia para aumentar o volume do leite e manter falsamente os parâmetros de proteína, respectivamente (Agência Nacional de Vigilância Sanitária - ANVISA, 2013). Além disso, acredita-se que o formaldeído foi adicionado acidentalmente, isso porque estava presente na ureia.

De acordo com Ferrão et al. (2007) a maltodextrina, a sacarose e o amido são adicionados na adulteração de leite em pó, sendo as concentrações de 20 % a 25 % as mais comuns por não promoverem alterações sensoriais, ou seja, os consumidores não percebem mudanças no sabor, porém as adulterações podem chegar a 60 %.

A melamina é utilizada na fertilização de solos e fabricação de plásticos, adesivos, resinas, colas, laminados e revestimento. Desta forma, esta substância pode ser encontrada naturalmente em baixas concentrações ($mg\ kg^{-1}$) em alimentos e bebidas, principalmente pela migração de materiais plásticos para os alimentos ou ainda como metabólito do inseticida ciromazina, o qual é utilizado em animais e plantações de legumes e frutas, porém estes níveis não constituem riscos à saúde humana (Organização Mundial de Saúde - WHO, 2008). Entretanto, a fraude na China promoveu a ingestão de melamina em altas concentrações durante três a seis meses até que o aparecimento de crianças doentes foi associado ao consumo destes alimentos. As concentrações variaram conforme a marca do produto consumido (0,09 mg/kg a 2.563 mg/kg) (WHO, 2008).

As principais vítimas foram crianças, isso porque possuem baixo peso corporal e o leite é a principal fonte de caloria nos primeiros anos de vida, além disso, a função renal não é bem desenvolvida (MAUER et al., 2009).

Neste sentido limites para a concentração de melamina em alimentos foram definidos. De acordo com a agência responsável por fiscalizar alimentos e medicamentos (*Food and Drug Adminstration* - FDA) nos Estados Unidos da América (EUA) 1 ppm é o limite máximo para fórmulas infantis e 2,5 ppm para outros alimentos. No Brasil ainda não foram definidos parâmetros que delimitem a concentração máxima desta substância e não há relatos de órgãos fiscalizadores sobre contaminação (MAUER et al., 2009). Além disso, pesquisas que avaliem a presença da melamina em produtos lácteos brasileiros não foram reportadas.

A expansão da exportação de produtos lácteos brasileiros está diretamente relacionada à adequação aos parâmetros internacionais, à qualidade dos produtos, garantia à saúde dos consumidores e à utilização de métodos precisos e rápidos, o que ressalta assim a importância de pesquisas que utilizem métodos práticos e eficientes para a detecção da melamina.

2.2 Método de Kjeldahl

O método de Kjeldahl é a principal técnica utilizada na quantificação de proteína em alimentos e baseia-se na quantificação do nitrogênio total e conversão para o nível de proteína, ou seja, é indireto. Esta técnica tem sido descrita como precisa, simples e rápida (Moore et al. 2010). Entretanto, possui a limitação de não diferenciar nitrogênio proteico do não proteico, e por isso a adição de substâncias nitrogenadas aos alimentos aumenta falsamente o teor de proteína.

A adição de melamina aumenta falsamente o nível de proteína sem que haja a identificação por este método, o que explica o fato da fraude com melamina ter sido descoberta apenas após o aparecimento dos sintomas nas crianças chinesas.

O método baseia-se em quatro etapas com objetivo de quantificar o nitrogênio total, conforme Figura 2.

$$\text{Amostra} \xrightarrow[\text{Digestão}]{} (NH_4)_2SO_4 \xrightarrow[\text{Neutralização}]{} NH_3 \xrightarrow[\text{Destilação}]{} NH_4H_2BO_3 \xrightarrow[\text{Titulação}]{} \text{Nitrogênio Total x Fator}$$

Figura 2 Etapas da execução do método de Kjeldahl.

Na primeira etapa a amostra é digerida com ácido sulfúrico e catalisadores, sendo que a matéria orgânica é oxidada a dióxido de enxofre, dióxido de carbono e sulfato de amônio. Na segunda etapa a solução é neutralizada e destilada, sendo que o sulfato de amônio reage com hidróxido de sódio e há a liberação de amônia, a qual é coletada no erlenmeyer contendo ácido bórico, formando borato de amônio. A terceira etapa é a titulação com ácido clorídrico, sendo que a partir do volume gasto na titulação, concentração e fator de correção dos reagentes utilizados obtém-se o teor de nitrogênio total na amostra.

A quarta etapa é a determinação da proteína bruta, a qual é obtida pela multiplicação do fator de correção com o nitrogênio quantificado. O fator de correção varia conforme o alimento, isso porque considera o nível de nitrogênio das proteínas. No caso de produtos lácteos utiliza-se 6,38, já que as proteínas do leite contêm aproximadamente 15,67 % de nitrogênio.

De acordo com Moore et al. (2010) o número de etapas na execução do método de Kjeldahl pode introduzir erros na quantificação, principalmente a precisão da pesagem e o uso de reagentes contaminados. Finete et al. (2013) utilizaram diferentes substâncias nitrogenadas para verificar a eficiência do método de Kjeldahl e métodos espectrofotométricos na quantificação de proteína do leite. Estes autores confirmaram a influência do nitrogênio não proteico nos níveis de proteína pelo método de Kjeldahl, mas sugeriram que a combinação destes métodos é capaz de monitorar a qualidade do leite e evitar adulterações. Entretanto, o uso de dois métodos analíticos na quantificação de proteína exige maior número de equipamentos, aumenta o custo e tempo de execução das análises.

Desta forma, o método de Kjeldahl é específico para quantificação de nitrogênio e por isso a adição de substâncias nitrogenadas aumenta falsamente o teor de proteína dos alimentos.

2.3 Espectrometria de massas

A espectrometria de massas (EM) baseia-se na geração de íons gasosos e posterior separação de acordo com a razão massa/carga (m/z), sendo possível obter informações específicas a respeito do analito (VESSECCHI et al., 2008).

A técnica de espectrometria com ionização por eletrospray (ESI-EM) é útil no estudo de biomoléculas com alto peso molecular e outros compostos lábeis e não voláteis. Na ESI-EM, a amostra é borrifada na ponta do tubo capilar fino para dentro da câmara aquecida e em pressão quase atmosférica, sendo que há potencial de alta voltagem na superfície do tubo capilar e com isso pequenas gotículas carregadas são expulsas para a câmara de ionização (HOLLER, SKOOG, CROUCH, 2009; PAVIA et al., 2010).

As gotículas carregadas enfrentam contra fluxo de gás secante que evapora as moléculas e assim a densidade da carga de cada gotícula aumenta até que as forças repulsivas eletrostáticas excedam a tensão superficial da gotícula, sendo que o processo continua até que os íons da amostra, livres de solvente, sejam deixados na fase gasosa (CROTTI et al., 2006; PAVIA et al, 2010). Após a ionização, o feixe de íons é acelerado por um campo elétrico e entra no analisador de massa, a qual é a região do espectrômetro de massa em que os íons são separados de acordo com as razões massa/carga (m/z) (HOLLER, SKOOG, CROUCH, 2009; PAVIA et al., 2010).

A versatilidade e sensibilidade da espectrometria de massas a torna importante em diversas áreas, dentre as quais na medicina, biologia, química e alimentos. Neste âmbito diversos autores demonstraram a eficiência da espectrometria de massas, como técnica única ou acoplada com outros métodos, na identificação e/ou quantificação de adulterantes em alimentos infantis, produtos cárneos e derivados lácteos (DANE, CODY, 2010; DESMARCHELIER et al., 2009; FILIGENZI et al., 2008; HUANG et al., 2010; SAVINDO et al., 2010; YANG et al., 2009; ZHU et al., 2009).

Nunes e Guerreiro (2012) ao avaliarem própolis por espectrometria de massas por ionização eletrospray demonstraram que este método é eficiente na caracterização de compostos fenólicos. Souza et al. (2014) utilizaram espectrometria de massas por ionização eletrospray para avaliar a autenticidade do tempo de envelhecimento de cachaças e reportaram que este método é rápido e direto, sendo por isso indicado por estes autores para monitorar a prática de fraudes. Huang et al. (2010) ao avaliarem a identificação de melamina em matrizes lácteas por espectrometria de massas verificaram que a rápida detecção pode ser realizada sem a preparação da amostra.

Hsieh et al. (2012) utilizando espectrometria de massa para detectar melamina em fórmula infantil e grãos em pó demonstraram que este método é simples, rápido e possui potencial para rastrear melamina em alimentos. Dane e Cody (2010) avaliaram a quantificação da melamina em leite em pó por espectrometria de massas e obtiveram limites de detecção de 1 ppm. Desmarchelier et al. (2009) utilizaram a cromatografia líquida de alta eficiência com espectrometria de massas na quantificação de melamina em leite e alimentos infantis de base láctea e observaram eficiência nos resultados obtidos.

Diferentes solventes foram utilizados na preparação das amostras para detecção de melamina por espectrometria de massas, sendo a maioria dos reagentes nocivos à saúde humana. Desta forma, o desenvolvimento de métodos simples contribui com a diminuição do tempo na execução das análises e obtenção dos resultados, retirando a influência da matriz na identificação do adulterante.

2.4 Espectroscopia na região do infravermelho

A espectroscopia na região do infravermelho estuda a interação entre a radiação e a matéria com o objetivo de avaliar as ligações químicas. A técnica consiste na incidência de energia eletromagnética sobre a molécula, a qual, de acordo com a variação do momento dipolo elétrico, adquire movimento vibracional e origina os espectros (SANTOS, 2005). Contudo, a complexidade dos espectros exige a utilização de análises estatísticas na identificação, classificação ou quantificação.

O espectro infravermelho é considerado uma propriedade físico-química, isso porque duas moléculas de estruturas diferentes podem absorver frequências iguais, mas os espectros serão diferentes, já que cada tipo de ligação possui sua própria frequência de vibração (PAVIA et al., 2010). O princípio fundamental de todos os analisadores de infravermelho é baseado na capacidade de absorção de diferentes comprimentos de ondas dos grupos químicos específicos, ou seja, as bandas correspondem às frequências de vibração entre os átomos e a altura da banda corresponde à intensidade da frequência de vibração (OLIVEIRA, 2010).

A espectroscopia na região do infravermelho compreende de 10 cm^{-1} a 10.000 cm^{-1} e dividi-se em três regiões: o infravermelho próximo (NIR) abrange 4000 cm^{-1} a 10.000 cm^{-1}, o infravermelho médio (MIR) abrange 400 cm^{-1} a 4000 cm^{-1} e o infravermelho distante (FIR) que abrange de 10 cm^{-1} a 200 cm^{-1} (PINTO, 2011).

Existem três tipos de espectrômetros: o instrumento dispersivo, o baseado em filtros e a transformada de Fourier. O instrumento dispersivo utiliza monocromador para dispersar a radiação infravermelha em frequências individuais de radiação que, sequencialmente, irão atravessar a amostra e com isso a absorção de cada frequência poderá ser mensurada, sendo que o detector mensura a quantidade de energia absorvida durante a passagem de cada frequência pela amostra e dá origem ao espectro (LANHER, 1996). O espectrofotômetro baseado em filtros utiliza filtros ópticos para aumentar a velocidade de análise e tais filtros restringem algumas frequências garantindo que apenas os comprimentos de onda desejados passem pela amostra (LANHER, 1996).

Os instrumentos baseados na transformação de Fourier (FTIR) utilizam interferômetro e todas as frequências são examinadas simultaneamente, sendo que o interferograma digital é convertido a espectro utilizando o logaritmo da transformada de Fourier (LANHER, 1996).

A espectroscopia na região do infravermelho é bem difundida em análise de alimentos e diversas pesquisas foram desenvolvidas com o intuito de classificar, identificar, quantificar ou avaliar a qualidade de alimentos

(BALALIN, SMIRNOV, 2011, BORIN et al., 2006; BRANDÃO et al., 2010; DOWNEY et al., 1997; HE, RODRIGUEZ-SAONA, GIUSTI, 2007; HUCK, GUGGENBICHLER, BONN, 2005; MAUER et al., 2009; NAVRÁTIL, CIMANDER, MANDENIUS, 2004; OLIVEIRA, 2010; RODRIGUES-SAONA et al. 2006; SANTOS, 2005; SILVEIRA, FONSECA, LAGO, 2005; SHAO, HE, 2009; WU et al., 2008; WU, FENG, HE, 2008; WOODCOCK et al. 2008).

Zhang et al. (2014) avaliaram a utilização da espectroscopia na região do infravermelho para a discriminação de leite contaminado e observaram que este método associado com a quimiometria é específico e rápido na determinação da autenticidade de leite cru.

Rodrigues-Saona et al. (2006) avaliaram o grau de maturação de queijo suíço e mostraram a importância da espectroscopia na região do infravermelho no desenvolvimento de protocolos simples e rápidos para o monitoramento das alterações bioquímicas e previsão da qualidade final. Woodcock et al. (2008) evidenciaram a importância da técnica no monitoramento da composição, processamento e maturação de queijos.

A espectroscopia na região do infravermelho foi utilizada na avaliação de iogurte, incluindo o monitoramento da fermentação, o agrupamento de iogurtes de acordo com as propriedades físico-química e a determinação do pH e sólidos solúveis (NAVRÁTIL, CIMANDER, MANDENIUS, 2004; SHAO, HE, 2009).

Balabin e Smirnov (2011) e Mauer et al. (2009) relataram a eficiência da espectroscopia na região do infravermelho na determinação de melamina em produtos lácteos e alimentos infantis. Contudo, não existem trabalhos que relatem a aplicação deste método na detecção de melamina em produtos lácteos brasileiros ou mesmo método oficial para detecção desta substância no Brasil, o que ressalta a importância de pesquisas que utilizem métodos confiáveis na detecção de melamina. Além disso, tais estudos podem ser utilizados pelas agências e órgãos reguladores na definição de método oficial, já que o monitoramento previne a comercialização de produtos adulterados.

2.4.1 Métodos Quimiométricos

A quimiometria é a aplicação de métodos matemáticos e estatísticos a dados complexos para obtenção de informações úteis, incluindo o planejamento de experimentos, reconhecimento de padrões e a calibração multivariada (SOUSA, POPPI, 2012).

O planejamento de experimento objetiva avaliar a influência de variáveis em determinado processo e pode ser realizada utilizando planejamento fatorial, superfície de resposta, simplex e modelagem de mistura. O reconhecimento de padrões agrupa as amostras de acordo com a similaridade das características avaliadas, incluindo análise de componentes principais (PCA e análise de agrupamento hierárquico (HCA).

A calibração multivariada relaciona a propriedade de interesse com duas ou mais respostas instrumentais. Além disso, possibilita a análise mesmo na presença de interferentes da amostra e permite determinações simultâneas em análise sem resolução (BRAGA, POPPI, 2004). Os modelos de calibração multivariada são: regressão por quadrados mínimos parciais (PLS), máquina de vetor suporte (SVM), maquina de vetor suporte por mínimo quadrados (LS-SVM) e redes neurais (ANN), entre outros.

A espectroscopia de infravermelho produz espectros com sinais provenientes de mais de mil comprimentos de onda, o que inviabiliza a aplicação de métodos univariados e por isso é necessária a aplicação de métodos de regressão multivariados (SANTOS, 2005; ROBINSON, 2001). Diversos autores reportaram a associação entre a quimiometria e espectroscopia na região do infravermelho (ADAMOPOULOS, GOULA, PETROPAKIS 2001;

MORGANO, FARIA, FERRÃO, 2005; MORGANO et al. 2007; SAUER-LEAL, OKADA, 2008).

Snyder et al. (2014) utilizaram espectroscopia na região do infravermelho e métodos quimiométricos na avaliação de suco de uva. Estes autores observaram que mesmo que haja falta de uniformidade entre os lotes avaliados o modelo PLS pode predizer a concentração da uva no suco e, além disso, que a modelagem de classe por componentes principais (SIMCA) define alto nível de discriminação dos sucos de uva.

Borin et al. (2006) avaliaram a detecção de soro de leite, açúcar e amido em leite em pó por espectroscopia na região do infravermelho utilizando máquinas de vetores suporte por mínimos quadrados (LS-SVM) e verificaram eficiência nos resultados, ressaltando a importância da técnica na determinação da autenticidade de alimentos.

Bassbasi et al. (2014) utilizaram métodos quimiométricos e espectroscopia na região do infravermelho na determinação dos sólidos não gordurosos do leite cru, sendo que os resultados mostraram que PLS e SVM apresentaram alta performance na predição das concentrações.

A quimiometria tem se mostrado promissora para análise de espectro de infravermelho, promovendo a redução da complexidade dos dados e facilitando o entendimento dos resultados. Neste sentido a espectroscopia na região do infravermelho e a quimiometria são importantes ferramentas na identificação da autenticidade dos alimentos.

3 CONSIDERAÇÕES GERAIS

O monitoramento de melamina em produtos lácteos é imprescindível na garantia da saúde dos consumidores e, por isso, métodos precisos e rápidos são importantes no monitoramento e identificação de adulterantes.

Neste trabalho para análise de espectrometria de massas (ESI-MS/MS) foram utilizados três pré-tratamento das amostras, contudo apenas um foi eficiente em remover a interferência dos constituintes do leite na análise. Os resultados desta extração apresentaram correlação linear com a concentração de melamina no leite em pó, mostrando que a melamina pode ser quantificada. Esta extração de melamina baseou-se na formação de precipitado pela adição de ácido tricloroacético e solubilização em acetato de etila, sendo que até agora esta técnica não havia sido relatada na literatura. Desta forma, a melamina pode ser extraída de forma simples e rápida para análise em ESI-MS/MS.

Na análise por espectroscopia na região do infravermelho utilizaram-se três regiões do espectro na quantificação de melamina e observou-se que a região 4500-5100 cm^{-1} apresentou melhor modelo de calibração multivariada. As amostras comerciais de leite em pó avaliadas não estavam contaminadas com melamina. Assim, os resultados mostraram que a análise do espectro de infravermelho por PLS permite obter resultados confiáveis de forma simples e rápida, sem a necessidade de preparação das amostras.

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SEGUNDA PARTE

ARTIGOS

ARTIGO 1

RAPID EXTRACTION OF MELAMINE IN POWDERED MILK FOR DIRECT ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY ANALYSES

Artigo submetido à revista Talanta, sendo apresentado segundo as normas de publicação desta revista

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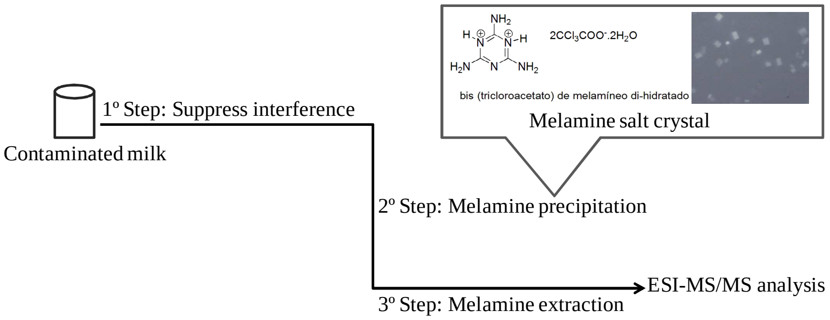
GRAPHICAL ABSTRACT



ABSTRACT

A combination of a simple pretreatment for melamine extraction and direct analysis in electrospray ionization tandem mass spectrometry (ESI-MS/MS) is proposed. Three pretreatments were evaluated. The first was based on suppressing interference using acetonitrile. The second used sulphuric acid and trichloroacetic acid to suppress interference and for melamine extraction, respectively. The third used sulphuric acid to suppress milk interference, trichloroacetic acid for melamine precipitation, and ethyl acetate for melamine extraction. However, only the last pretreatment suppressed milk interference in melamine detection. Thus, a linear correlation between melamine concentration and the absolute mass spectra intensity was verified, showing that adulteration can be detected. Further, the research was extended to elucidate the nature of the melamine in the extract through infrared spectroscopy and microscopy analyses. The precipitate was characterized as melaminium bis(trichloroacetate) dehydrate, which is generated through hydrogen bound formation in an interaction between melamine and trichloroacetic acid. Therefore, a simple, fast, and easy method for melamine extraction and direct ESI-MS/MS analysis was developed.

Keywords: Adulteration; Melaminium bis(trichloroacetate) dihydrate;
Trichloroacetic acid precipitation; Ethyl acetate extraction;

List of abbreviations
EA- Ethyl acetate
ESI-MS/MS- electrospray ionization tandem mass spectrometry

FDA-Food and Drug Administration
HB- Hydrogen bond
HMF- 5-Hydroxymethylfurfural
HTST- High temperature short time
LC-MS/MS- Liquid chromatography-mass spectrometry
TCA-Trichloroacetic acid
USA- United States of America

1. Introduction

Milk is a complete food and source of essential nutrients, including carbohydrate, protein, fatty acids, calcium, phosphorus and magnesium. Moreover, the milk constituents can affect dairy products performance and quality; therefore, the industries usually define milk quality based on the nutrient levels, mainly protein and fat. These parameters have been used to calculate the payout to the supplier [1]. Unfortunately, adding nonauthentic nutrients has been used to adulterate milk and, consequently, increase economic gain [2].

This adulteration is defined as the removal or replacement of milk components and addition of substances without a purchaser's knowledge, including water, whey, sucrose, starch, salt, sodium hydroxide, and formaldehyde [2, 3]. Melamine is a nitrogen-rich compound commonly used to manufacture plastics, laminates, glues and adhesives [4]. Although it is not an ingredient in food, it was reported as present in Chinese powdered milk and infant formula in 2008. It was used to falsely increase the apparent milk protein and was not discovered because the routinely used methods cannot distinguish between nitrogen from protein and non-protein sources, which results in incorrectly high protein measurements [5]. Consuming tainted products by the Chinese population affected 294,000 infants and children with more than 50,000 hospitalizations and at least 6 deaths [6]. This scandal scared the world and created the need for sensitive, specific, rapid, and reliable screening methods.

The Food and Drug Administration (FDA) from the United States of America (USA) established a liquid chromatography-mass spectrometry (LC-MS/MS) method to detect melamine in infant formula. This technique is accurate, but it is time consuming and requires hazardous solvent [7, 8]. Many researchers have reported analytical methods for melamine detection and quantification, such as gas chromatography-single quadrupole mass spectrometry [9], a spectrophometric method [10], liquid chromatography-electrospray ionization tandem mass spectrometry [11], an enzyme-linked immunosorbent assay [7], liquid chromatography tandem mass spectrometry [4, 1, 12], and high-performance liquid chromatography-triple quadrupole mass spectrometry [13, 14]. However, most available methods are time-consuming, labor-intensive and expensive [15, 16, 17, 18]. Therefore, to simplify the sample preparation and improve the methods for detecting melamine, it is important to monitor contamination and prevent damage to consumer health.

Recent advances in ambient ionization mass spectrometry facilitate direct, rapid, real-time, and high-throughput analyses with little or no sample pretreatment [19, 20]; it has been used to characterize food and identify adulterants, and authors report it as a reliable method [21, 22, 23, 24]. In this paper, we proposed a simple and rapid sample preparation to detect melamine in powdered milk using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Further, the research was extended to infrared spectroscopy and microscopy analyses to identify the nature of the precipitate formed during extraction.

2. Material and Methods

2.1. Sample and reagents

Powdered milk was prepared from pasteurized whole milk (Gran Lait ®, Perdões, Brazil; high temperature short time [HTST]). Melamine was obtained from Sigma ® (St. Louis, USA). The chemicals' purity was greater than 98%, and they were used with Milli-Q purified water.

2.2. Powdered milk preparation

Three replicates of powdered milk were produced from pasteurized milk concentrated in a rotary evaporator (70ºC, TE-0581, TE-210, Tecnal ®, Piracicaba, Brasil) until 52% of total solids; thereafter, the concentrate was dried in mini spray dryer (MSD 1.0, Labmaq ®, Ribeirão Preto, Brasil). The temperature for the inward and out ward drying air was 160ºC and 100ºC, respectively, and the feed flow rate was 0.79L h^{-1} in systems with dual fluid nozzle atomization.

2.3. Melamine-added milk standards

Melamine stocks solutions (500mg L^{-1}) were prepared by dissolving melamine in water. The powdered milk was diluted in water and contaminated with melamine at different concentrations (0mg L^{-1}; 2.5mg L^{-1} 5mg L^{-1}; 10mg L^{-1}; 15mg L^{-1}; and 20mg L^{-1}).

2.4. Melamine extraction

Three extraction procedures were evaluated. The first pretreatment was performed in accordance with the methodology described by Desmarchelier et al. [12]. The powdered milk (1g) and melamine aliquots were placed into a falcon polypropylene tube that was mixed and allowed to stand for 10 minutes. The water (5mL) and acetonitrile (5mL) were added successively, and the resulting slurry was thoroughly mixed after each solvent was added. The slurry was further diluted with acetonitrile (30mL) and water (10mL) and placed onto an automated shaker for 5 min. The tube was then centrifuged at 4000g and room temperature for 10 min (Centrifuge, Beckman ®, GS-15R), and the liquid supernatant was used for direct injection into ESI-MS/MS.

The second pretreatment was performed by adding 2 mol L^{-1} sulphuric acid until the pH reached 4.6 (pHmeter, Quimis ®, Q400AS); thereafter, the samples were filtered (quantitative filter paper, Pró-análise ®, porous 7µm). The filtered aliquot (5mL) was added to 24% trichloroacetic acid (TCA) (5mL) and left to stand for 15 minutes. The mixture was filtered and the permeate was divided in two parts. The first aliquot of extract was used for injection into ESI-MS/MS. The remaining portion (4mL) was added to ethyl acetate (EA) (4mL) followed by stirring (Vortex, Phoenix-AP56); thereafter, the supernatant was used for direct injection into ESI-MS/MS (the third pretreatment sample). Fig. 1 describes the extraction steps.

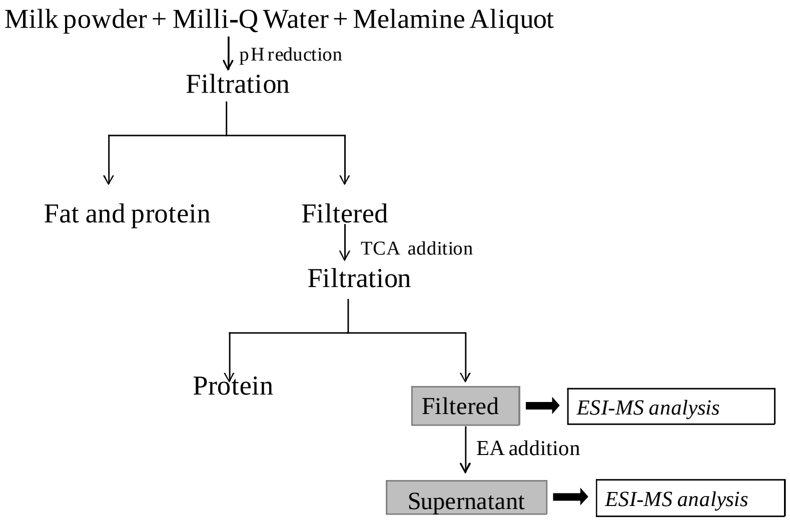


Fig. 1. The second and third pretreatment steps for powdered milk. TCA-Trichloroacetic acid; EA-Ethyl acetate; ESI-MS/MS- Electrospray ionization tandem mass spectrometry.

2.5. ESI-MS/MS analysis

The ESI-MS/MS analyses were performed using Agilent 1100 Series LC/MSD trap equipment in positive ion mode to identify the melamine. The spectra were obtained through 50 scans at 0.2s each. The analysis conditions were as follows: heat capillary temperature 350°C, dry gas flow 6L min^{-1}, pressure 15psi, and capillary voltage -3.5kV.

2.6. Infrared spectroscopy analysis

The infrared spectra were recorded on a Shimadzu FT-IR Prestige 21 spectrometer. The melamine (500 mg L^{-1}) and trichloroacetic acid (24%) solutions were in a 1:1 ratio. The precipitate (2 mg) were dried and mixed with KBr powder (200 mg) and pressed to form a thin, transparent disk. Infrared absorption spectra within the range 4000-400cm^{-1} at a 2cm^{-1} resolution using 32 scans were recorded at room temperature.

2.7. Optical and electronic microscopy analysis

The melamine (500mg L^{-1}) and trichloroacetic acid (24%) solutions were at a 1:1 ratio, and the crystals images were obtained using optical microscopy (Meiji ML 5000, Meiji Techno América, Santa Clara, USA) coupled with a camera (Cle-Palmer 49901-35, Cole-Palmer, Vernon Hills, USA).

The melamine (15mg L^{-1}) and trichloroacetic acid (24%) solutions were at a 1:1 ratio, and 60 μL of this mixture was added to a blade, to which Poli-L-Lisin was previously added. After 30 minutes, the Karnovsky solution was added until the blade was covered and stored at 8ºC over 3 hours. The sample was washed with cocodilato buffer (0.05M) three times over 10 minutes and washed with water three times. Next, the dehydration process began by adding 25%, 50%, 75%, 90% acetone over 10 minutes then three times in 100% acetone for 10 minutes each, followed by drying in a critical point dryer. The sample was placed in the gold bath then observed in microscope (LEO Evo 040).

3. Results and Discussion

3.1. Melamine extraction and detection

Melamine, 1,3,5-triazine-2,4,6-triamine, has 126.1 Da molecular mass. Detecting of this compound in milk can be affected by matrix interference because powdered milk has a compound with similar molecular mass, namely 5-hydroxymethylfurfural (HMF), which has a 126.1 Da molecular mass. HMF is a natural compound formed when milk is heated; its ESI-MS spectrum is characterized by the presence of the m/z 127.1 [23]. However, HMF fragmentation produces the MS/MS fragments 109, 84, and 71, whereas protonated melamine generated ions with m/z 110, 85, and 60, showing that fragmentation can be used to differentiate these compounds [25].

To analyse the melamine in powdered milk samples and suppress matrix interference, extraction procedures were performed. First, the extraction procedure by Desmarchelier et al. [12] was performed for samples with melamine added. The obtained mass spectrum for this extract is presented in Fig. 2.

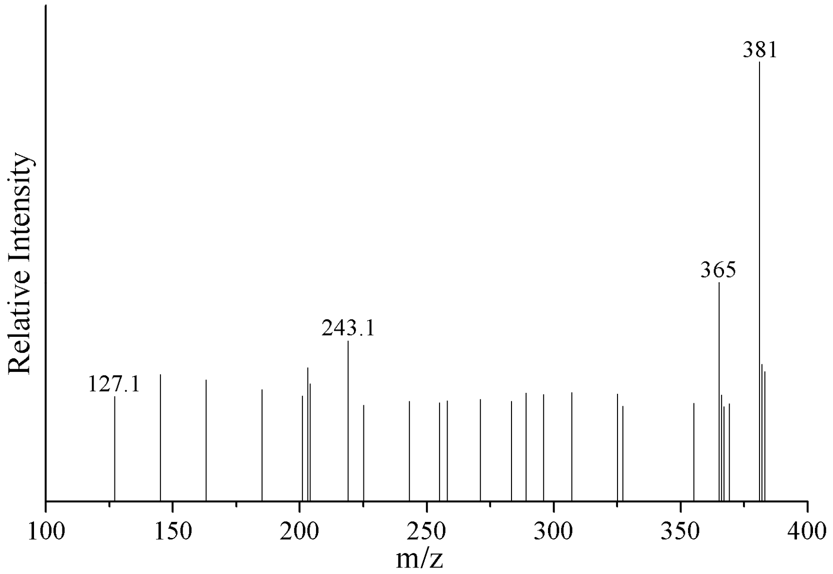


Fig. 2. The mass spectrum for the melamine extract obtained according Desmarchelier et al. [12]. The sample includes 15mg L^{-1} of melamine, and the peaks show the m/z 381 $[Lactose+ Na]^+$ and m/z 365 $[Lactose + K]^+$.

The mass spectrum presented a number of m/z signals related to the presence of disaccharide from milk. Lactose is the main carbohydrate in milk and could be identified from two peaks in the spectrum. The signal detected at m/z 381 and 365 were generated from lactose association with sodium and potassium, respectively. However, the low 127.1 signal hampers the fragmentation, which does not show whether this signal is from melamine or HMF, indicating that the high lactose signal disrupts the detection; this occurred at all concentrations evaluated. The additional ions in the spectrum arose from the other powdered milk components.

Desmarchelier et al. [12] successfully used this method to detect melamine through liquid chromatography, which separates matrix components from melamine and, consequently, suppresses matrix interferences. On the other hand, ESI-MS/MS does not provide proper separation, which favours matrix effects during melamine detection.

To assess melamine in powdered milk through ESI-MS/MS by suppressing milk interference, another pretreatment procedure was evaluated, which was based on eliminating milk interference; this procedure was performed in two steps. The first step consisted of eliminating the insoluble protein and fat. The decrease in pH for reconstituted milk from 6.6 to 4.6 promoted protein precipitation; and filtering this mixture formed an extract without casein micelles and fat. The fat was eliminated based on the solubility difference from the extract, and the insoluble protein was eliminated based on the characteristic isoelectric points at pH 4.6 [26]. In the second step, the trichloroacetic acid reacted with melamine to form crystals; thereafter, it was filtered to eliminate the soluble protein and directly injection into the ESI-MS/MS. The mass spectrum for this extract is shown in Fig. 3.

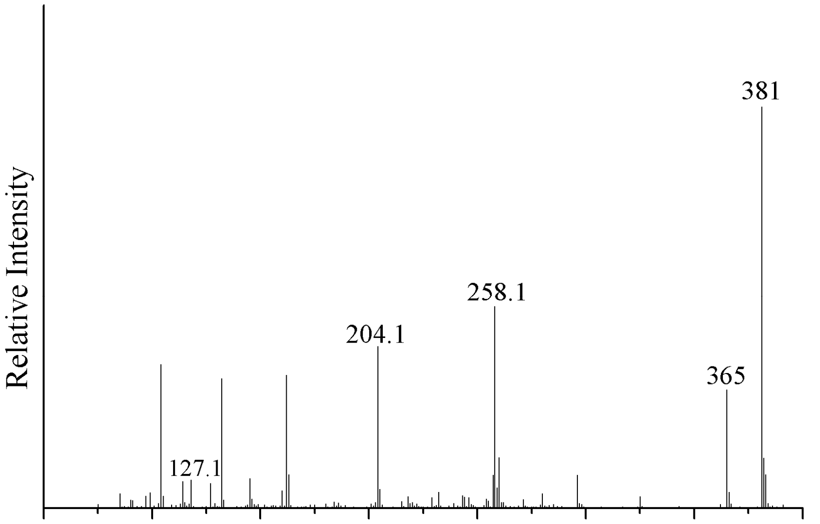


Fig. 3. The mass spectrum for the extract obtained by precipitating melamine using trichloroacetic acid (samples with 15mg L^{-1} of melamine). m/z 381 $[Lactose+ Na]^+$; m/z 365 $[Lactose + K]^+$.

Melamine detection is affected by interference from milk compounds, mainly the ions from lactose and mineral interactions. Moreover, the m/z 127.1 has a small intensity, which limited the fragmentation and distinction between melamine and HMF, demonstrating that this pretreatment does not efficiently suppress the interference. Many authors have reported interference by the milk matrix during melamine detection. Ibáñez, Sancho and Hernández [27] reported that the matrix interference affected melamine detection in mass spectrometry. Xia et al. [28] reported that the matrix affected melamine determination even using hydrophilic interaction liquid chromatography coupled to ESI-MS. He et al. [4] observed that the melamine signal was suppressed by milk compounds. González-Antuña et al. [29] described how the matrix interferes with the mass spectrometry, showing that the competition between matrix analyte compounds and modifying the physical properties of the droplets can affect analysis efficiency.

Nevertheless, different solvents have been used to clean the sample for melamine detection, including acetonitrile, methanol, diethyl ether, and dichloromethane [1, 9, 13, 14, 30, 31, 32, 33, 34]. Thus, an additional step in the extraction procedure was performed to suppress the matrix interference. The sample was prepared over three steps; the first consisted of suppressing the insoluble protein and fat followed by melamine precipitation and ethyl acetate addition to the melamine extract. Thereafter, the supernatant was injected into an ESI-MS/MS, and the result is shown in Fig. 4 and 5.

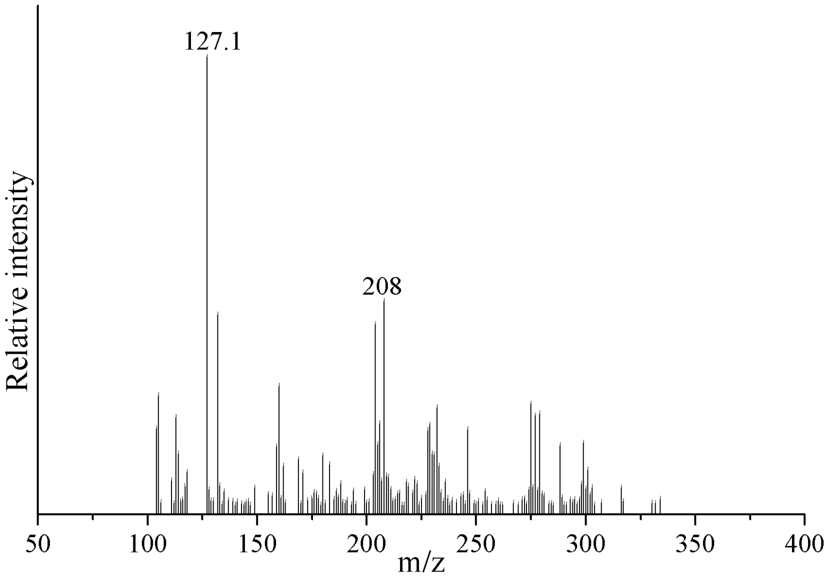


Fig. 4. The mass spectrum for the extract obtained by melamine precipitation with trichloroacetic acid and solubilized in ethyl acetate (sample with 15mg L^{-1} of melamine) as well as m/z 127.1 fragmentation.

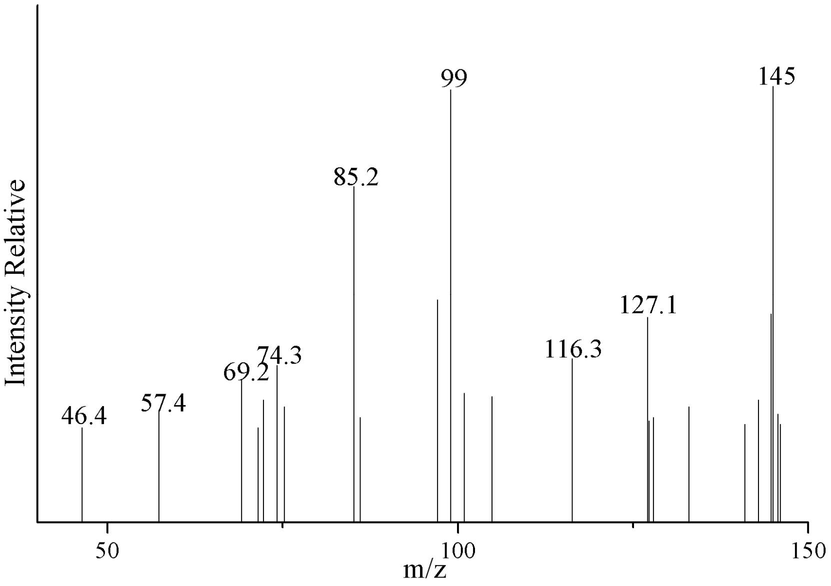


Fig. 5. Mass spectrum of m/z 127.1 fragmentation for the extract obtained by the third extraction technique (powdered milk contaminated with 15mg L^{-1} of melamine).

This extract produced a mass spectrum with high intensity for the m/z 127.1 (Fig. 4), which favoured fragmentation (MS/MS) (Fig.5). As expected for the melamine added to the milk sample, the m/z 127.1 fragmentation confirms the presence of melamine through identification of the MS/MS 85.2 peak, which is produced through loss of the NH_2CH group [12].

Many authors have reported melamine detection in milk and its discrimination from HMF in milk. Yang et al. [25] showed interference by HMF in melamine detection and proposed that the fragment MS/MS 85 can be used to exclude false positive signals. Dane and Cody [23] reported that the low levels of melamine may be difficult to identify in the presence of HMF. Chen and Yan [35] used capillary electrophoresis coupled with diode array detection to differentiate melamine from HMF and demonstrated melamine detection.

In addition, a calibration curve was obtained using 5 concentrations of milk contaminated with melamine. The response range was discerned, and the calibration curve is represented by the equation shown in Fig. 6.

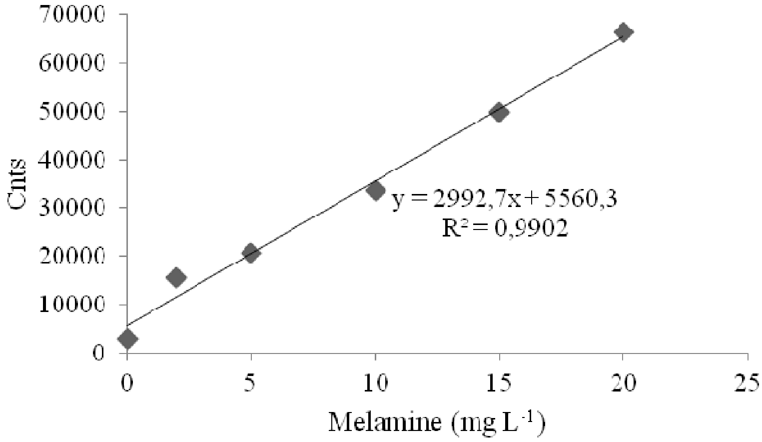


Fig. 6. The relationship between mass intensity and melamine concentration.

A linear relationship was observed between the melamine concentration and absolute mass spectra intensity, which indicated that the ESI-MS/MS can be efficiently used for melamine detection.

3.2. The nature of the melamine in the extract

Melamine precipitation was based on a reaction with trichloroacetic acid, which forms of melamine salt crystals. The interaction between melamine and trichloroacetic acid facilitates crystal formation, and this complexation adds an interesting aspect to the intramolecular hydrogen bond (HB) system formed [36, 37, 38, 39]. Thus, to investigate the nature of the precipitate, infrared spectroscopy was employed. Fig. 7 shows the infrared spectrum for melamine and its precipitate.

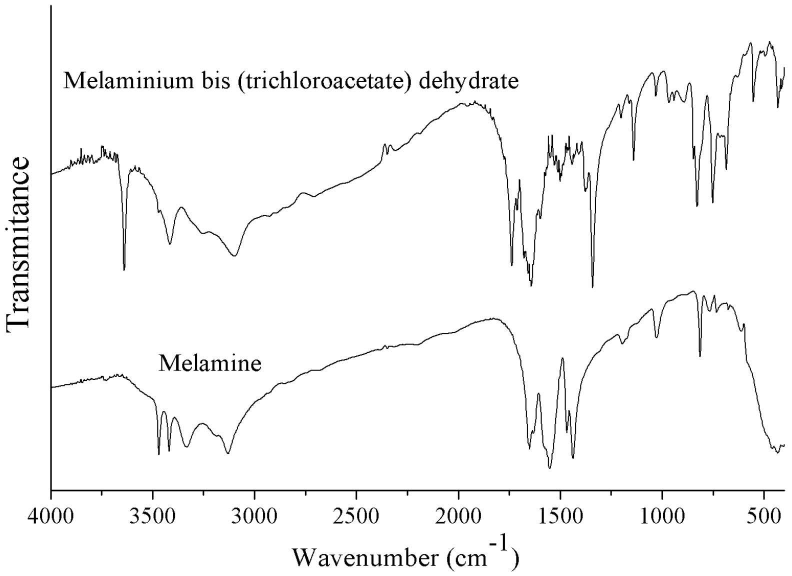
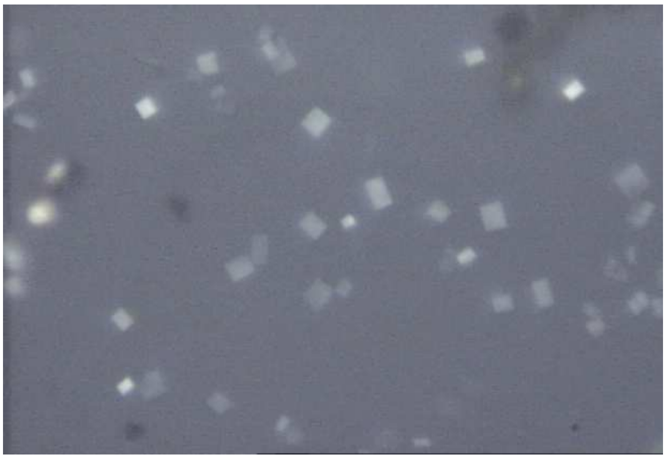


Fig. 7. The near infrared spectrum for melamine and its precipitate using trichloroacetic acid.

The melamine N-H stretching vibrations absorb in an interference-free region (3500-3300 cm^{-1}) of the infrared spectrum; thus, they are suitable bands for HB assignment. The presence of HB moves the N-H stretching band to a lower wavenumber due to a weaker of N-H bond. The strong peak at 3419 cm^{-1} (melamine spectrum) is attributed to NH_2 symmetric stretching (ν_{sym}), which shifted to 3414 cm^{-1} in the melaminium salt, as observed by Kanagathara et al. [42], while asymmetric stretching (ν_{asym}= 3470 cm^{-1}) did not change the wavenumber. In addition, the side chain C-N stretching [40], which occurs at 1439 cm^{-1} is shifted to 1335 cm^{-1}, which provides insight into the presence of HB during formation of the precipitate, melaminium bis (trichloroacetate) dehydrate, as reported by Perpétuo and Janczak [41] and Kanagathara et al. [42]. The melamine salt crystals are shown in Fig. 8.

A



B

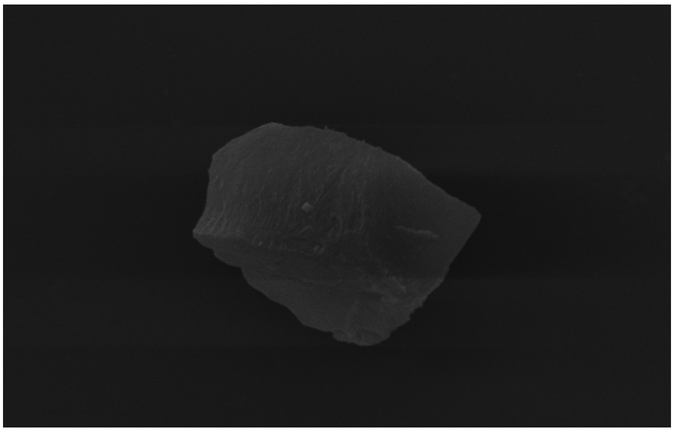


Fig. 8. A melaminium bis (trichloroacetate) dehydrate crystal observed using optical microscopy (A) (500 mg L^{-1} of melamine) and electronic (B) microscopy (15 mg L^{-1} of melamine).

The melaminium bis (trichloroacetate) dehydrate crystal was detected using different microscopy techniques, which demonstrates the characteristic structure of the salt formed during the second sample pretreatment step, which consists of adding trichloroacetic acid and a reaction with melamine. However, Kanagathara et al. [42] reported a different morphology, which can be explained by the using of trichloroacetic acid at a different concentration.

4. Conclusion

The first and second pretreatments were based on melamine extraction using acetonitrile and trichloroacetic acid, respectively. However, these techniques did not efficiently suppress milk interference for melamine detection.

For the third pretreatment sulphuric acid was used to suppress protein and fat, and trichloroacetic acid and ethyl acetate were used for melamine precipitation and extraction, respectively. The ESI-MS/MS analysis showed that the matrix interference was removed, and a linear correlation was observed between the melamine concentration and the mass spectrometry results, showing that the adulteration can be detected.

The protocol for melamine extraction was based on melaminium bis (trichloroacetate) dehydrate formation by trichloroacetic acid addition and solubilisation with ethyl acetate, which was not previously reported; and we shows that it as a simple, fast, and easy method for melamine extraction from powdered milk and direct ESI-MS/MS analysis. Future studies should use this extract for additional analytical methods to extend the sample pretreatment application.

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ARTIGO 2

MELAMINE DETECTION IN POWDERED MILK BY INFRARED SPECTROSCOPY AND CHEMOMETRICS: STUDY ON COMMERCIAL BRAZILIAN SAMPLES

Artigo submetido à revista Journal of Food Composition and Analysis, sendo apresentado segundo as normas de publicação desta revista

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ABSTRACT

Milk is an important source of nutrients for all ages. However, milk is a major target of fraud, including the addition of melamine which has been detected by different methods. However, most of these methods are time-consuming and generate chemical waste. Therefore, the objectives of this research were to quantify melamine in powdered milk using near infrared spectroscopy (NIR) and chemometrics and to use a calibration model to evaluate commercial Brazilian powdered milk from different states. In addition, this study has been extended to evaluate the use of different regions of the spectra for melamine quantification. All partial least square (PLS) models (at different spectral regions) exhibited high correlations coefficients for cross validation, calibration and prediction (R^2>0.90) and minimal root mean square errors of cross validation, calibration and prediction (RMSEC<0.8926). The 5100-4500 cm^{-1} region represented the best calibration model. For all PLS models, the parameters ${}^{c}r^2_p$ and r^2_m were greater than 0.5, thereby demonstrating the robustness and good correlation between experimental and predicted results for quantifying melamine in powdered milk. The Brazilian powdered milk samples that were evaluated were not contaminated. The results indicated that infrared spectroscopy (IR) and a chemometric method allow for attaining reliable results quickly and easily.

Keywords: Adulteration; Brazilian powdered milk; Multivariate calibration; Rapid method

List of abbreviations
GC- Gas chromatography

GC-MS- Gas chromatography mass spectrometry

HPLC- High performance liquid chromatography

IR- Infrared spectroscopy

ISO- International Organization for Standardization

LC-MS/MS- Liquid chromatography mass spectrometry

MID- Mid infrared spectroscopy

NIR- Near infrared spectroscopy

PLS-DA- Multi way partial least square

PLS- Partial least square

R^2- Correlation coefficient

RMSEC- Root mean square error of calibration

RMSECV- Root mean square error of cross-validation

RMSEP- Root mean square error of prediction

USA- United States of America

1. Introduction

Food authenticity can be described as the use of ingredients allowed by regulatory agencies; the absence of processing not described in the law; and coherence among labels, products and legislation (Moore, Spink, & Lipp, 2012). Regulatory agencies and industries have analytical methods to determine authenticity and to remove contaminated products. However, routine methods have failed to detect adulterants, mainly because the sophistication of fraud has increased, thus undermining the reliability of analytical results. Moreover, adulteration can introduce toxic substances and decrease the nutritional value of food.

Melamine adulteration has highlighted the weaknesses of the official method in detecting the illegal changes in food composition (Botros et al. 2013). It was added to milk in China with the objective of falsely increasing the milk protein content. The contamination spread to other products because milk is a common ingredient in food industries. These products were consumed by the Chinese population and caused the formation of kidney stones in hundreds of thousands of infants and children, 6 of whom died (Ma et al. 2013; Pei et al. 2011; WHO, 2008). This incident scared the world and created the need for monitoring the absence of melamine in milk.

Until now in Brazil, melamine had not been detected in milk, which could be explained by the absence of monitoring because the Kjeldahl methods are not efficient. However, a large amount of contaminant has been found. In 2007, whey, water, hydrogen peroxide, sodium citrate, sodium bicarbonate and sodium hydroxide were found in raw milk. In 2013, formaldehyde and urea were found in three brands of commercial milk (Brasil, 2013). Melamine detection in commercial Brazilian milk can prevent health risks for consumers around the world because this country is a major milk producer (FAO, 2013). In addition, in a globalised and dynamic market of food, it is extremely important to use rapid and reliable methods.

Several researchers have reported efficient methods for melamine detection (Abernethy & Higgs, 2013; Deng et al. 2010; Desmarchelier, Cuadra, Delatour, & Mottier, 2009; He et al. 2014; Khedr, 2013; Lutter et al. 2011; Tran, Okoniewski, Storm, Jansing, & Aldous, 2010; Venkatasami & Sowa Junior, 2010; Wang et al. 2010; Zhang et al. 2014a). However, most of them require intensive sample pretreatment and are time-consuming, which limits screening a large number of samples (Mauer, Chernyshova, Hiatt, Deering, & Davis, 2009). As an alternative to melamine detection in milk, infrared spectroscopy (IR) possesses some advantages, such as reduced sample treatment and analysis time, feasibility for nondestructive and noninvasive analysis, and high sensitivity and specificity, serving as a “fingerprint” technique (Santos, Pereira-Filho, & Rodriguez-Saona, 2013; Xu, Yan, Cai, & Yu, 2013). Its association with chemometric methods has made IR a powerful tool for determination of the quality and authenticity of food (Ferreira, Pallone, Poppi, 2013; Liu, Wen, Dong, Lai, & Zhao, 2013; Rodriguez-Saona & Allendorf, 2011). It has been used successfully for melamine detection (Balabin & Smirnov, 2011; Fu et al. 2014; Huang, Min, Duan, Wu, & Li, 2014; Jawaid, Talpur, Sherazi, Nizamani, & Khaskheli, 2013; Mauer, Chernyshova, Hiatt, Deering, & Davis, 2009; Yang, Liu, & Kexin, 2013). However, until now, melamine detection in Brazilian milk has not been reported in the literature. Thus, in this paper, the objectives were to quantify melamine in powdered milk using IR and chemometrics and to use the calibration model to evaluate commercial Brazilian powdered milk from different states. In addition, this study was extended to use different regions of the spectra for melamine quantification.

2. Material and Methods

2.1 Raw materials and reagents

Three replicates of pasteurised milk were purchased from Gran Lait ® (Perdões, Brazil; high temperature short time [HTST]). Melamine was obtained from Sigma ® (St. Louis, USA). All chemicals had purity greater than 98%.

2.2 Standard samples

Three replicates of powdered milk were produced by concentration in a rotary evaporator (70ºC, TE-0581, TE-210, Tecnal ®, Piracicaba, Brasil) until 52% of total solids was obtained, after which the concentrate was dried in a mini spray dryer (MSD 1.0, Labmaq ®, Ribeirão Preto, Brasil). The temperature of the drying air in and out was 160ºC and 100ºC, respectively, and the feed flow rate was 0.79L h^{-1} in systems with dual fluid nozzle atomisation.

The standard powdered milk were prepared with 23 concentrations of melamine, including the control, 0.01%, 0.02%, 0.1%; 0.2%, 0.5%, 0.75%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 8%, and 8.5% (w/w). All samples were prepared in triplicate and stored in polypropylene vials to avoid moisture.

2.3. Commercial samples

Powdered milk was purchased from local supermarkets in Campinas in São Paulo state, Lavras in Minas Gerais state, Porto Velho in Rondônia state and Gramado in Rio Grande do Sul state. Eight samples were selected from different states of Brazil, including 1 sample from São Paulo, 3 samples from Minas Gerais, 2 samples from Rio Grande do Sul, and 2 samples from Rondônia. For each sample, 3 independent batches were collected, and the means were used.

2.4. Infrared spectroscopy analysis

The 47 samples of standard and commercial powdered milk were analysed by near infrared spectroscopy (NIR) (10000-4000 cm^{-1}). The spectra were obtained in Fourier transform IR spectrometers with OPUS 6.5 software (Tensor series, Bruker ®, Brasil). The samples were placed in a flask accessory, and the measurement was performed by diffuse reflectance. For each spectrum, 260 scans were applied. The samples were scanned in triplicate, and the means were used.

2.5. Statistical analysis

Multivariate calibration models were developed by the partial least squares using Chemoface software (Nunes, Freitas, Pinheiro, & Bastos, 2012). The spectra were preprocessed by multiplicative scatter correction, followed by the selection of the following regions: 10000-4000 cm^{-1} (full spectra), 6818-6563 cm^{-1} (NH_2 stretching), and 5100-4500 cm^{-1} (C-N stretching).

The calibration model was developed using 45 standard samples (15 concentrations in triplicate) and 24 standard samples (8 concentrations in triplicate) that were used for external validation. The number of latent variables was chosen by considering the lower root mean square error of cross-validation (RMSECV).

The quality of the models was checked by several parameters, including the root mean square error of calibration (RMSEC), RMSECV, root mean square error of prediction (RMSEP), and determination coefficients (r^2).

In addition, a y-randomisation test (the mean of 10 repetitions to guarantee abroad representativeness) was carried out to attest the model robustness (statistically evaluated using $RMSEC_{rand}$ and r^2_{rand}). The y-randomisation test consists of several runs for which the original descriptor matrix is kept fixed, and only the vector y is scrambled (randomised). The model obtained under such conditions should be of poor quality and without real meaning (Roy, Paul, & Roy, 2009).

Additional statistical parameters, such r^2_m and r^2_p (Eqs. 1 and 2), were also used for validation purposes (Mitra et al., 2010):

$$r^2_m=r^2[1-(r^2-r^2_0)^{1/2}] \qquad (1)$$

where r^2 and r^2_0 correspond to the squared correlation coefficient values between the observed and predicted values for the test set with and without an intercept, respectively.

$$r^2_p = r^2 \, (r^2 - r^2_{rand})^{1/2} \qquad (2)$$

The parameter r^2_m is used to guarantee that not only would a good correlation coefficient in the external validation be achieved but also that the absolute actual and predicted values would be congruent, i.e., r^2_m gives insight about the slope of the curve for the experimental versus predicted values; $r^2_m \geq 0.5$ is considered acceptable. $r^2_p \geq 0.5$ guarantees a valid difference between the r^2 from calibration and the r^2 from the y-randomisation test (r^2_{rand}) (Mitra & Roy, 2010).

3. Results and Discussion

3.1. NIR Analysis

IR is a simple technique that provides detailed information about the structure of compounds (Jawaid, Talpur, Sherazi, Nizamani, & Khaskheli, 2013). The vibrational absortion peaks in the near infrared spectra correspond to the vibrational transactions between the fundamental energy level and higher energy levels, which contain overtone information and combinations of these vibrations (Ferreira, Galão, Pallone, Poppi, 2014). Figure 1 shows the IR spectra of melamine and powdered milk.

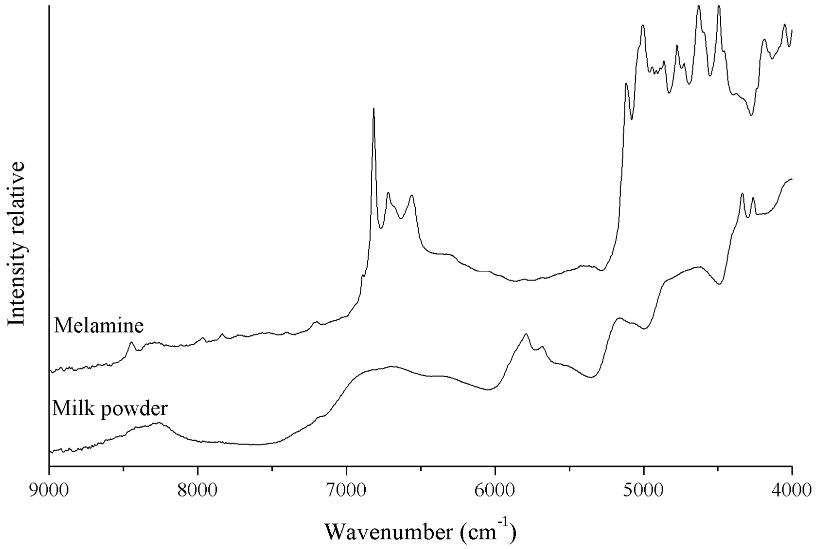

Figure 1 IR spectra of melamine and powdered milk.

The regions of interest to identify melamine in powdered milk are 6818 to 6563 cm^{-1}, and 5114.8 to 4497.5 cm^{-1}. The peak at 6818 cm^{-1} in the melamine spectrum is attributed to NH_2 stretching. The absorption band present at 6563 cm^{-1} and 6727 cm^{-1} is from symmetric and asymmetric stretching of NH_2, respectively. The peak at 5114.8 cm^{-1} is from the stretching vibrations of C=N bonds (Mauer, Chernyshova, Hiatt, Deering, & Davis, 2009). The stretching vibration of the side chain C-N bond is located at 4851 cm^{-1}, and the peak at 5004 cm^{-1} arises from asymmetric stretching vibrations of C-N (Marchewka, 2002).

The characteristics of the near infrared spectra for powdered milk represent functional groups from protein, lactose and fat. The region at 5200 and 5100 cm^{-1} is characteristic of O-H vibration (lactose). The peaks at 5600 and 5000 cm^{-1} are related to the C-H functional group (fat), and those at 5000 and

4500 cm^{-1} correspond to the vibrations of protein functional groups (N-H) (Ferreira, Galão, Pallone, Poppi, 2014).

To quantify melamine in powdered milk by NIR, different spectral regions (in addition to entire spectra) were regressed against melamine content in standard samples using models.

3.2 Multivariate calibration

A calibration model was developed to attain the lowest RMSEC and highest r^2 in melamine quantification. PLS regression was applied in three regions of the NIR spectrum to construct the models that are shown in Table 1.

Table 1 Statistical parameters of PLS models in different regions of the NIR spectra.

Parameter	10000-4000 cm^{-1}	6563-6818 cm^{-1}	4500-5100 cm^{-1}
Latent variables	9	4	9
r^2	0.9886	0.9287	0.9972
RMSEC	0.3027	0.7576	0.1476
r^2cv	0.9793	0.9023	0.9855
RMSECV	0.4079	0.8926	0.3410
r^2_{rand}	0.5206	0.2614	0.7334
$RMSEC_{rand}$	19.527	24.361	14.576
$^{c}r^2_p$	0.6763	0.7586	0.5122
RMSEP	0.3654	0.7777	0.3291
r^2_{pred}	0.9849	0.9278	0.9840
r^2_m	0.9833	0.8834	0.9688

The number of latent variables was defined based on the lowest RMSECV. All models possessed a high determination coefficient ($r^2>0.92$) and low regression error (RMSEC<0.76) in calibration. Additionally, acceptable parameters were exhibited for validation ($r^2_{cv}>0.90$; RMSECV<0.8926). The values of r^2_p for all models were >0.5, indicating that when y blocks are scrambled, a poor correlation is observed, which confirms a valid difference between the r^2 and r^2_{rand} values. In addition, $RMSEC_{rand}$ resulted in higher values than RMSEC, and r^2_{rand} had a lower value than r^2, thus showing the robustness of the model and that the real calibration was not due to a chance correlation (Nunes, & Freitas, 2013a).

The external validation was acceptable for the results of all regions of the infrared spectra, with r^2_{pred}>0.92 and RMSEP≤0.78. The r^2_m values were >0.88 for all models, which guarantees that the slopes of the regression lines for the test set, when forced to pass through the intercept or not, do not differ significantly from each other, thus indicating a good correlation between experimental and predicted results for the test set of all models (Nunes, Freitas, 2013b).

Figures 2, 3 and 4 show the predicted and measured results of the PLS models for cross validation, calibration and prediction.

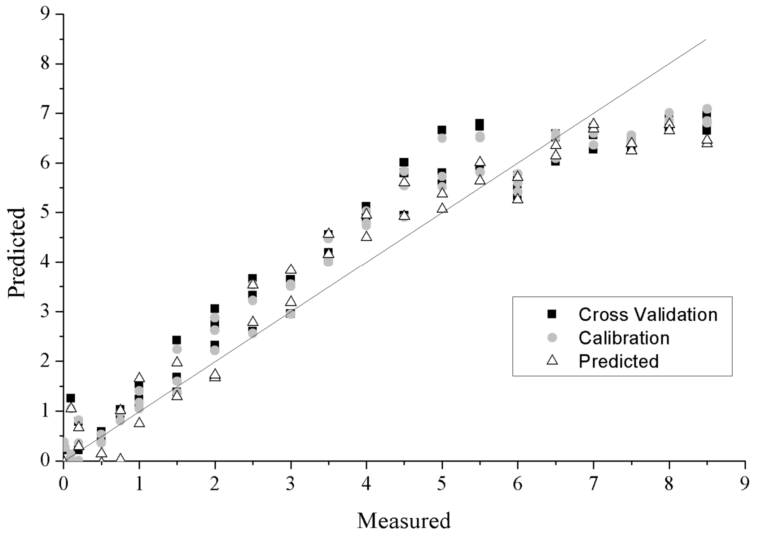

Figure 2 Measured and predicted results of the melamine concentration in powdered milk using the 6563-6818 cm^{-1} region of the infrared spectrum.

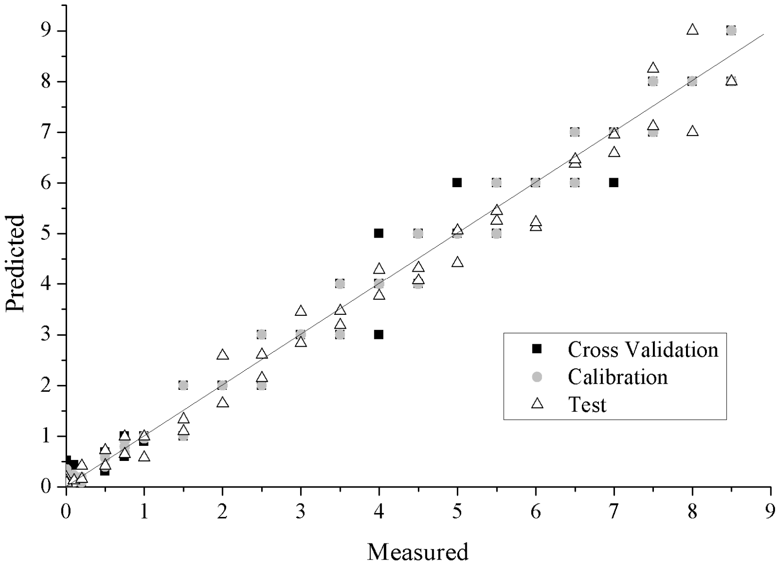

Figure 3 Measured and predicted results of melamine concentration in powdered milk using a PLS model constructed in the 4500-5100 cm^{-1} region.

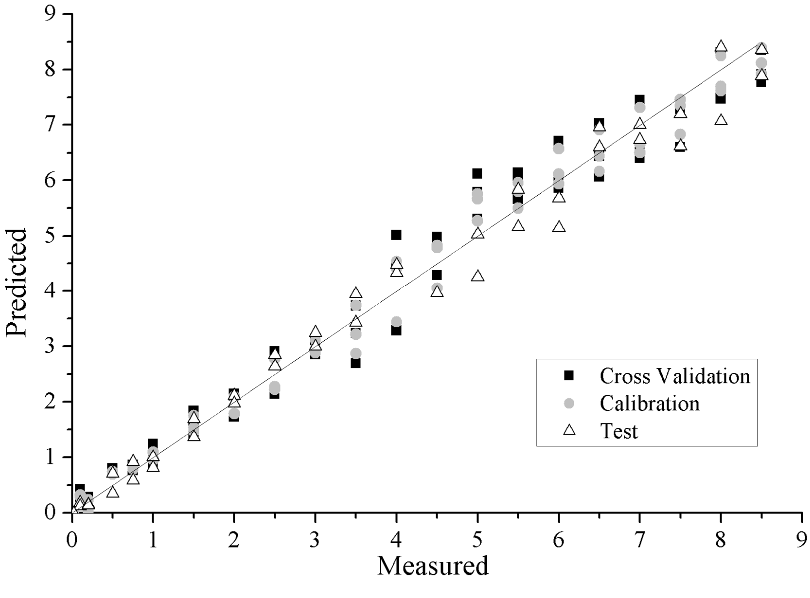

Figure 4 Measured and predicted results of the melamine concentration in powdered milk using a PLS model constructed in the 10000-4000cm^{-1} region.

For all models constructed, a high correlation has been observed between the measured and predicted results of melamine concentration in powdered milk. Among the models, the 5100-4500 cm^{-1} region showed the best determination coefficient (r^2) of cross validation and calibration, and the lowest values of the root mean square error of cross validation, calibration, and prediction, thus indicating that it is the best region of the near infrared spectrum to quantify melamine in powdered milk. Liu, Lv, He, and Xu (2011) used two regions of the near infrared spectrum to show that the region of 5896-4000 cm^{-1} was more efficient to construct a calibration model for melamine detection. Jawaid, Talpur, Sherazi, Nizamani, and Khaskheli (2013) used four regions in mid infrared spectroscopy (MID) to report that a band between peaks 840.7 and 726.0 cm^{-1} improved the calibration results. The PLS model was established based on a correlation of R^2>0.99, RMSEC 0.37%, RMSECV 5.75%, and RMSEP 1.55%.

Haughey, Graham, Cancouët, and Elliott (2013) detected melamine in soybean meal using NIR and PLS, and they showed a high correlation coefficient (0.89-0.99%) and acceptable square error of calibration (0.081-0.276%). Yang, Liu, and Kexin (2013) used two-dimensional correlation spectroscopy with multi-way partial least squares discriminant analysis (NPLS-DA) to detect melamine in milk and demonstrated a higher accuracy for predicting adulteration. These authors emphasised IR as short analysis time and low cost.

Zhang et al. (2014b) used IR to classify milk adulterated with different pseudo proteins, and the models were built by an improved support vector machine (I-SVM) and improved and simplified K nearest neighbours (IS-KNN). The discrimination of milk with melamine was greater than 93% and 91% for I-SVM and IS-KNN, respectively. Thus, these authors reported that the IR combined with non-linear supervised pattern recognition methods are objective and rapid methods for authentication of milk. Moreover, many others studies reported the high efficiency of the association IR and chemometric methods to detect the authenticity of food (Cozzolino, 2014; Miao, & Liu, 2013; Nunes, 2014; Quiñones-Islas, Meza-Márquez, Osorio-Revilla, & Gallardo-Velazquez, 2013; Teye, Huang, Lei, & Dai, 2014; Xu, Shi, Ye, Yan, & Yu, 2013; Yang, Liu, & Kexin, 2013). However, studies that used IR and chemometrics to detect melamine in Brazilian milk were not found.

3.3. Melamine quantification in commercial samples

The calibration model of the region from 5100 to 4500 cm^{-1} was used to quantify melamine in commercial Brazilian powdered milk. Figure 5 shows the infrared spectra of the samples evaluated.

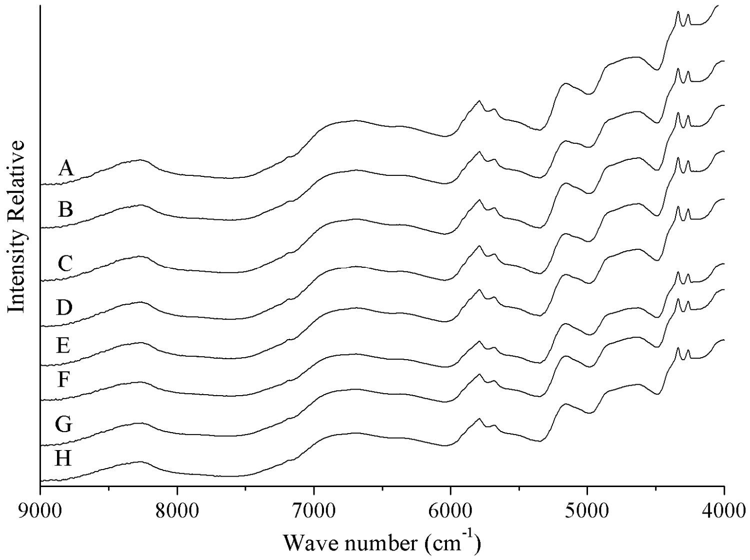

Figure 5 NIR absorbance spectra of commercial powdered milk samples.

The spectra do not show characteristic vibration peaks from melamine functional groups (6818 to 6563 cm^{-1} and 5114.8 to 4497.5 cm^{-1}). According to the predictions of the PLS calibration model, the samples evaluated were not contaminated with melamine.

Schoder (2010) evaluated powdered milk and infant formula commercialised in the East Africa market and reported different results than those shown here. These authors observed that 11% of the samples were contaminated with melamine. The same results were found by Filazi et al. (2012), who reported that dairy products from Turkey were contaminated with melamine. Hassani et al. (2013) evaluated different brands of milk products from Iranian markets and showed that only 6.6% of the samples were not contaminated with melamine. Studies describing the detection of melamine in Brazilian food were not found, although Brazil is the fourth largest milk producer in the world (FAO, 2014). However, in the last year, urea was used to falsely increase the protein levels in Brazilian milk, thus showing melamine could be an imminent danger because this type of fraud has the same principle as melamine addition.

4. Conclusion

The association between IR and PLS was efficient for melamine quantification in powdered milk and supported the attainment of reliable results quickly and easily, without sample pretreatment. The region from 5100 to 4500 cm^{-1} resulted in the best calibration model, but all models presented a high correlation coefficient for cross-validation, calibration and prediction and a minimal root mean square error of cross validation, calibration and prediction. Furthermore, all PLS models were robust and accurate.

The commercial Brazilian samples evaluated were not contaminated with melamine. However, considering the dangers caused by melamine ingestion, governmental agencies must take action to prevent contamination, mainly by using reliable methods to constantly monitor the levels of adulterants in dairy products.

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