Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 239342, 7 pages
http://dx.doi.org/10.1155/2013/239342

Research Article

A Simple HPLC-DAD Method for the Analysis of Melamine in Protein Supplements: Validation Using the Accuracy Profiles

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Received 7 May 2013; Accepted 15 July 2013

Academic Editor: Pranav S. Shrivastav

The study presents a fully validated simple high-performance liquid chromatography method with diode array detection (HPLC-DAD), able to accurately determine the melamine, fraudulently added, in protein supplements, commonly used from healthy adults to enhance exercise or sport performance. The validation strategy was intentionally oriented towards routine use and the reliability of the method rather than extreme performance. For this reason, validation by accuracy profile, including estimation of uncertainty, was chosen. This procedure, based on the concept of total error (bias + standard deviation), clearly showed that this method was able to determine melamine over the range of 0.05–3.0 mg Kg^{-1}, selected by taking into account the maximum residue levels (MRLs) proposed by European legislation to distinguish between the unavoidable background presence of melamine and unacceptable adulteration. The accuracy profile procedure established that at least 95% of the future results obtained with the proposed method would be within the ±15% acceptance limits of the validated HPLC-DAD method over the whole defined concentration range.

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine) is commonly used as a raw material or additive in the manufacture of melamine-formaldehyde plastics or can coatings for food contact materials. It can be found at low levels as a contaminant in a variety of foods due to the leaching from food contact materials [1–4].

For its nephrotoxicity [5–10] this chemical should not be intentionally added into foods or edible materials, in any circumstances. On the contrary, it is fraudulently added to food and/or feed ingredients to boost their total protein content and increase their market value. Such adulteration exploits a limitation of the most common nonspecific protein tests such as Kjeldahl method, which use total nitrogen as an indicator of protein content (melamine contains 66.6% of nitrogen by weight). In recent years the illegal addition of melamine to animal feedstuffs and infant milk formula caused illness and deaths of human infants and pet animals, who had taken the contaminated foods, primarily as a result of the accumulation of melamine-uric acid crystals in the urinary tract [8, 11–15]. That is why various international health organizations have established regulations on melamine content over a wide variety of foods in order to safeguard public health [9, 16, 17]. In order to harmonize maximum residue levels (MRLs), set in many countries all over the world, European Food Safety Authority has proposed a legal maximum residue level (MRL) of 1.0 mg/Kg for infant foods and 2.5 mg/Kg for other foods to distinguish between the unavoidable background presence of melamine (from food contact materials, pesticide use, etc.) and unacceptable adulteration [18].

Protein supplements are largely used by athletes and physically active individuals to enhance nitrogen retention, to increase muscle mass, to prevent protein catabolism during prolonged exercise, and to promote muscle glycogen resynthesis following exercise. Therefore it is likely that the adulteration of this kind of supplements with melamine increases their apparent protein content.

In the scientific literature many methods have been proposed for the analysis of melamine in food and pet foodstuffs

[19]. They are mainly based on common methodologies as gas chromatography (GC) and high-performance liquid chromatography (HPLC) in combination with different selective detection techniques [19–27]. More recent methods using electrochemical [28], surface-enhanced Raman spectroscopy (SERS) [29], infrared spectroscopy [30], colorimetric sensor based on polydiacetylene (PDA) liposomes [31], and quantum dots-based fluorescence method [32] have been published to detect melamine. All above mentioned methods are generally sensitive and accurate but they are not suitable for routine analysis because they are expensive and time consuming.

Based on these considerations, the first objective of the present study was to develop a rapid, simple, and accurate method for the quantitative determination of fraudulently added melamine to protein supplements, commonly used from healthy adults to enhance exercise or sport performance, to increase their total nitrogen concentration and consequently the calculated protein content. To authors' knowledge, only a method based on the use of micellar mobile phases for the chromatographic determination of melamine in dietetic supplements and validated following a traditional approach has been proposed [33].

Consequently, we selected an inexpensive HPLC-DAD technique due to its common availability also in laboratories, with limited financial resources, that have to perform regular monitoring of contaminants in food. The use of hydrophilic interaction liquid chromatography (HILIC) has been applied to melamine analysis to enhance its retention.

The second objective was to validate the method according to the strategy proposed by a Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) for the validation of quantitative analytical procedures in order to assess a routine food control technique [34–42]. The main advantage of this validation strategy, in comparison with other traditional validation approaches, is not only to take decision in accordance with predefined acceptance limits but also to control the risk associated with the future use of the method. This risk, which expresses the probability of measurements that are expected to fall outside the acceptance limits during routine analysis, gives analysts the guarantee that the method will be able to quantify with accuracy [37, 38, 43, 44], without applying interlaboratory studies [45].

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents were of analytical or HPLC grade and were purchased from Sigma-Aldrich (Milan, Italy). Melamine standard was obtained from Sigma-Aldrich (Milan, Italy).

The matrix was selected among dietetic supplements used in gyms; it contained milk and albumen proteins.

2.2. Instrumentation and Chromatographic Conditions. LC experiments were performed on a liquid chromatograph (Thermo Finnigan P4000) equipped with a 7125 Rheodyne injection valve (fitted with a 20 μL loop). Detection was carried out by a Finnigan Spectra System UV 6000 LP photodiode-array detector, setting the wavelength at 220 nm. Chromatograms were acquired using the ChromQuest version 4.2 software program (ThermoQuest, Milano, Italy). The analytical column was a Spherex 5 OH (diol, 250 mm × 4.6 mm I.D., 5 μm particle size) from Phenomenex (Torrance, CA, USA), with a Phenomenex C_{18} guard cartridge (4 × 8 mm). The mobile phase was acetonitrile/acetate buffer (pH 5.0) 95 : 5 (v/v). The flow rate was 1.0 mL/min.

2.3. Method Validation with Accuracy Profile. The objective of a quantitative analytical method is to be able to quantify as accurately as possible the solute of interest in its matrix. The difference between the result and the unknown true value of the sample (total error) must be lower than a predefined acceptance limit, which was fixed to ±15% [46, 47]. The validation process is then to give guarantees that each of the future results that will be generated by the method will remain close enough to the true value. The proportion of measurements inside the acceptance limit must be larger than a given β-expectation tolerance interval fixed at 95%. This means that the method can be considered as validated if the analyst can guarantee that, at least, 95% of measurements will fall within ±15% of the actual value of the sample. The method validation was performed in accordance with the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP) [35–41], using the total error approach [36, 48]. Finally, the uncertainty of measurements derived from β-expectation tolerance intervals was estimated at each concentration level of the validation standards.

Because of the lack of specific regulatory references for melamine in dietary supplements, validation domains were established according to EFSA [18] and ranged from 0.05 up to 3.0 mg Kg^{-1}. This allows us to cover the entire range to distinguish between the unavoidable presence of melamine and unacceptable adulteration. Selected calibration domains were similar to validation domains.

The statistical analysis was performed using Microsoft Excel 2003.

2.4. Experimental Designs. Validation and calibration designs both consisted in 3 days ($k = 3$), 3 replicates ($n = 3$), and 3 levels ($m = 3$) of concentration. Altogether the number of trials was 27. These values were selected as a good compromise between the total number of analyses that can be achieved over one day and the cost of the validation study. Validation and calibration measurements were collected on the same days.

For calibration standards, a stock solution of melamine was prepared in methanol at 1.0 mg/mL and stored at 4°C. Three solutions were obtained by diluting appropriate amounts of stock solution with the chromatographic mobile phase (acetonitrile/acetate buffer pH 5.0, 95 : 5 (v/v)) to the final concentration levels of 0.05, 1.5, and 3.0 μg mL^{-1}.

In order to validate the method following the "total error" approach, validation samples were obtained by spiking, as blank matrix, a dietary supplement, found to be negative for melamine. Spiked samples were prepared with convenient

volumes of the intermediate stock solutions of melamine corresponding to three different spiking levels (0.05, 1.5, and 3.0 mg Kg^{-1}).

For each concentration level an amount of 1.0 g dietary supplement matrix was spiked. Sample preparation procedure consisted in adding 10 mL of ethanol to accurately weighed 1.0 g of matrix into a glass centrifuge tube. The tube was vortexed for 1 min to disperse the sample and centrifugated at 5000 g for 10 min at room temperature. The supernatant was discarded. Next 10 mL of acetonitrile/water 90 : 10 (v/v) was added to the tube, vortexed for 1 min, and centrifuged at 5000 g for 20 min at room temperature. The supernatant was recovered and evaporated in a SpeedVac system at 35°C. The dried extract was then reconstituted with 1 mL of the chromatographic mobile phase.

3. Results and Discussions

3.1. HPLC Method. Melamine is a polar compound with high water solubility and never remains on the reverse stationary phase in HPLC. Recently, methods related to hydrophilic interaction liquid chromatography (HILIC) have been developed and applied to melamine analysis to enhance its retention and facilitate its separation [19, 24, 49–58]. HILIC is now clearly established as an important chromatographic technique for the retention and separation of polar and hydrophilic compounds. Although it has been accepted that partitioning is the main retention mechanism, secondary interactions (such as dipole-dipole and ion-exchange) can play an important role in the separation, leading to changes in selectivity. The retention mechanism in HILIC is due to the fact that complex interactions occur between the polar analyte, solvent molecules of the mobile phase, the polar stationary phase, and residual silanol on silica gel. For this reason the HILIC columns can retain polar compounds that are not retained by RP chromatography.

Based on these considerations a Spherex 5OH diol phase was chosen. The mobile phase was acetonitrile/acetate buffer (pH 5.0) 95 : 5 (v/v). The pH value was selected at 5.0 in order to have a good absorption spectrum for melamine and, at same time, to preserve the stability of the diol phase. Taking into account the absorption spectra and the literature [53], detection of melamine was carried out at 220 nm.

3.2. Method Validation

3.2.1. Selectivity. Detection selectivity was assessed by the constancy of retention time of melamine and by monitoring the UV spectrum during the different analyses. The absence of interferences was checked on dietary supplement extract. No interferences were observed from this extract at the retention times of melamine, indicating that the proposed extraction procedure is effective, selective, and suitable for the determination of the target analyte (Figure 1).

3.2.2. Linearity. The linearity of the analytical procedure [59] was evaluated in the concentration range 0.05, 1.5, and 3.0 $\mu g\,mL^{-1}$, selected depending on melamine levels established from EFSA to distinguish between the unavoidable background presence of melamine (from food contact materials, pesticide use, etc.) and unacceptable adulteration [18]. The calibration curve was calculated using ordinary least-square linear regression. The data are shown in Table 1. The linearity was expressed by the coefficient of determination (r^2). The relationship was linear as the r^2 value was equal to 0.9999 and the equation was close to $y = x$.

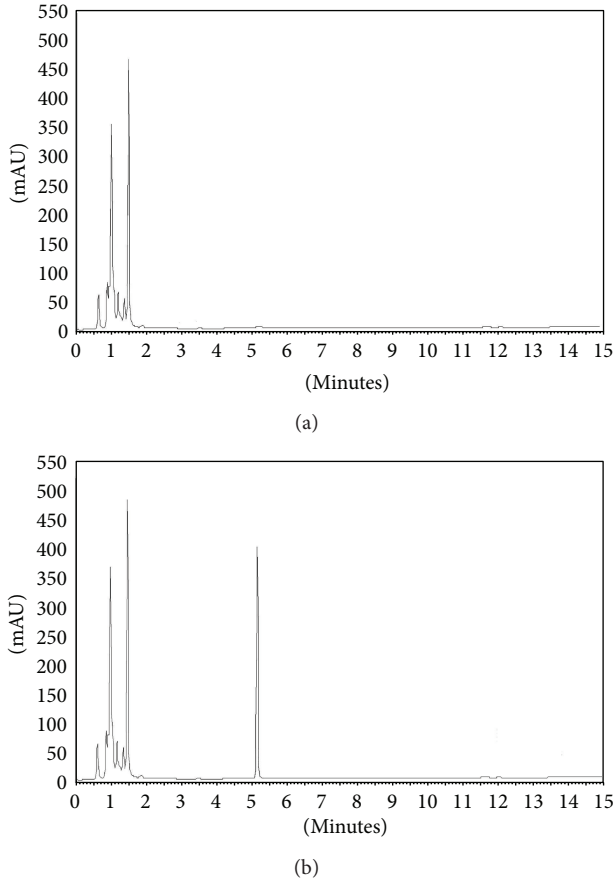

Figure 1: HPLC-DAD chromatogram (a) a blank dietary supplement and (b) of melamine (4.20 min) in validation sample (dietary supplement) spiked at 1.5 mg/Kg.

3.2.3. Trueness, Precision, Accuracy, and Uncertainty Assessment. The concentrations of the validation samples were back-calculated and were used to determine the relative bias, the repeatability, the intermediate precision, and the β-expectation tolerance intervals at the 95% probability level.

Trueness and precision give information, respectively, on systematic and random errors of the method. From data shown in Table 1, it can be concluded that trueness of the method was excellent, since the relative bias (%) at each concentration level [36, 40] was less than 1.7%, with good recoveries values demonstrating the high extraction efficiency of the method, and that a good precision, expressed as the relative standard deviations (RSD%) for repeatability and

Table 1: Accuracy profile validation results for melamine in dietary supplements.

Linearity ($k = 3, m = 3, n = 3$)		
Range ($\mu g\,mL^{-1}$)	0.05–3.0	
Slope	0.9659	
Intercept	0.0081	
r^2	0.9999	
Trueness ($mg\,kg^{-1}$) ($k = 3, m = 3, n = 3$)	Relative bias (%)	Recovery (%)
0.05	−1.4	98.6
1.5	−0.6	99.4
3.0	−1.7	98.3
Precision ($mg\,kg^{-1}$) ($k = 3, m = 3, n = 3$)	Repeatability (RSD %)	Intermediate precision (RSD %)
0.05	2.1	2.2
1.5	2.3	2.7
3.0	2.0	2.3
Accuracy ($mg\,kg^{-1}$) ($k = 3, m = 3, n = 3$)		β-expectation tolerance limits (%)
0.05		[−7.5; 4.7]
1.5		[−9.2; 7.9]
3.0		[−8.9; 5.5]
Uncertainty ($mg\,kg^{-1}$) ($k = 3, m = 3, n = 3$)		Relative expanded uncertainty (%)
0.05		4.7
1.5		5.8
3.0		4.9
Limits of quantitation (LOQ)		
Lower LOQ	Upper LOQ	
0.05	3.0	

k: number of series; m: number of amount levels; n: number of replicates.
RSD: relative standard deviation.

intermediate precision [38, 42], was obtained not exceeding 5%.

Accuracy takes into account the total error (sum of the systematic and random errors) of the test results [36, 38, 39]. It refers to the closeness of agreement between the test results and the acceptance reference value. As illustrated in Figure 2, the relative upper and lower β-expectation tolerance limits (%) (reported in Table 1) did not exceed the acceptance limits of total error for each concentration level [38], confirming that the proposed method was accurate. Furthermore the risk of having future assay results exceeding ±15% limits of the targeted amounts is strictly controlled.

Finally, the accuracy profile validation methodology allows, without any additional experiments, to obtain estimation of measurement uncertainty [35]. The expanded uncertainty was computed using a coverage factor of $k = 2$ [60, 61], representing an interval around the results where the unknown true value can be observed with a confidence level of 95%. Table 1 shows that relative expanded uncertainties were less than 6%, which means that the unknown true value is located at a maximum of ±6% around the measured result with a confidence of 95%.

A lower limit of quantitation (LOQ), corresponding to the lowest limit of validation domain ($0.05\,mg\,Kg^{-1}$), may have been determined but, due to the actual level to establish the fraudulent addiction of melamine, it was unnecessary to explore lower concentration levels.

4. Conclusions

This paper described a simple, fast, and selective HPLC-DAD method for the determination of melamine used to adulterate protein dietary supplements, based on HILIC technique. The method was validated by applying the accuracy profile approach. Compared to other traditional validation procedures, this strategy is a simple decision graphical tool based on the notion of the total error (bias and standard deviation) of the method allowing the immediate assessment of the method's validity. The accuracy profile of the proposed HPLC-DAD method guarantees that the method can

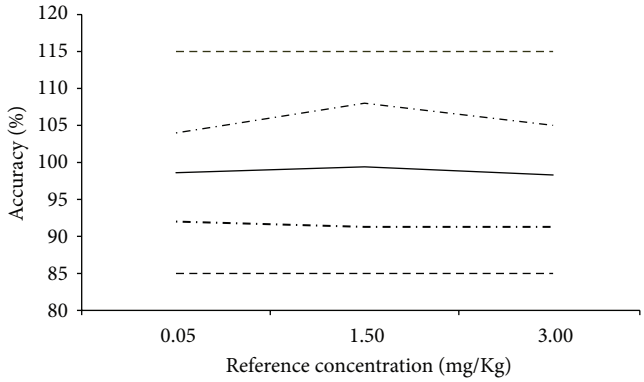

Figure 2: Accuracy profile obtained for the determination of melamine in dietary supplements using linear regression model. The continuous line is the recovery yield (%), the dotted lines are the ±15% acceptance limits, and the dashed lines are the upper and lower relative 95%expectation tolerance limits.

measure accurately melamine, for any protein supplement samples, over a range from 0.05 to 3.0 mg Kg^{-1}. Indeed, the usefulness, practicability, and accessibility of this approach simplify the in-house single laboratory validation, allowing not only to evaluate its own performance but also to assess the uncertainty of measurements without applying inter-laboratory studies.

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