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DART-MS/MS screening for the determination of 1,3-dimethylamylamine and undeclared stimulants in seized dietary supplements from Brazil

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ABSTRACT

1,3-dimethylamylamine (DMAA) is an alkylamine with stimulating properties that has been used predominantly as an additive in dietary supplements. DMAA is mostly consumed by professional athletes, and several doping cases reported since 2008 led to its prohibition by the World Anti-Doping Agency (WADA) in 2010. Adverse effects have indicated DMAA toxicity, and there is few data regarding its safety, so it was banned by regulatory agencies from Brazil and the United States. Ambient ionization methods such as Direct Analysis in Real Time Tandem Mass Spectrometry (DART-MS/MS) are an alternative for dietary supplements analysis, because they enable the analysis of samples at atmospheric pressure in a very short time and with only minimal sample preparation. Therefore, the aim of this work was to develop a methodology by DART-MS/MS to detect the presence of DMAA, ephedrine, synephrine, caffeine, sibutramine, and methylphenidate in 108 dietary supplements seized by the Brazilian Federal Police (BFP). The results show that DART-MS/MS screening was successfully employed to simultaneously detect the six substances in casework samples with rapid and reliable results and with minimum sample preparation. DMAA was present in 20% of the seized dietary supplements, being more prevalent along with sibutramine and caffeine. Out of the 108 samples, almost 50% were positive for sibutramine and 10% for methylphenidate. It appears that even after prohibition, dietary supplements and weight-loss products containing DMAA are still being commercialized.

1. Introduction

Dietary supplements and herbal dietary supplements are very popular worldwide, showing an increase in consumption in recent decades [1–4]. 1,3-dimethylamylamine (DMAA) is considered to be an alkylamine-type stimulant, characterized by the presence of a primary amine attached to a short carbon chain [5]. Pharmacologically, DMAA is classified as an α-1-adrenergic agonist [6]. Known popularly as methylhexanamine, 2-amino-4-methylhexane and geranamine, DMAA began to be marketed in North America in the 1970s under the name "Forthane" and was used as a nasal decongestant because of its vasoconstricting properties [6–8]. In 2006, DMAA reappeared on the market as a constituent of dietary supplements, with stimulating properties. Over the years, DMAA has been used predominantly as an additive in dietary supplements [9,10].

Since 2008, dozens of athletes have been excluded of sports activities by the World Anti-Doping Agency (WADA) due to the abuse of DMAA. [11]. The continuous use of laced sports supplements by professional athletes encouraged WADA to ban DMAA in 2010 [12]. After cases of deaths and toxicity events [10,13], and no sufficient data indicating its safety and efficacy, DMAA was banned in the United States [14] and in other countries such as Canada and Brazil in recent years [15,16]. In 2014, Foley and contributors [17] reported eight cases of military personnel (men and women) who consumed DMAA and had liver damage. Even after its prohibition; by WADA [18] the analyses carried out at the XXII Winter Olympic and XI Paralympic games; revealed the presence of DMAA along with other controlled or monitored substances, such as pseudoephedrine [19]. According to the latest

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reports published by WADA, DMAA is still the most widely used banned-stimulant among athletes [20–22].

Considering the prohibition of DMAA, the US Food and Drug Administration (FDA) issued an alert to manufacturers, banning its addition to dietary supplements, emphasizing the dangers arising from consumption of DMAA in dietary supplements. However, despite the alerts, in April 2013 the FDA received 86 notifications related to health problems and deaths resulting from the use of supplements containing DMAA [14]. The National Agency of Sanitary Surveillance (ANVISA) suspended its distribution, disclosure,

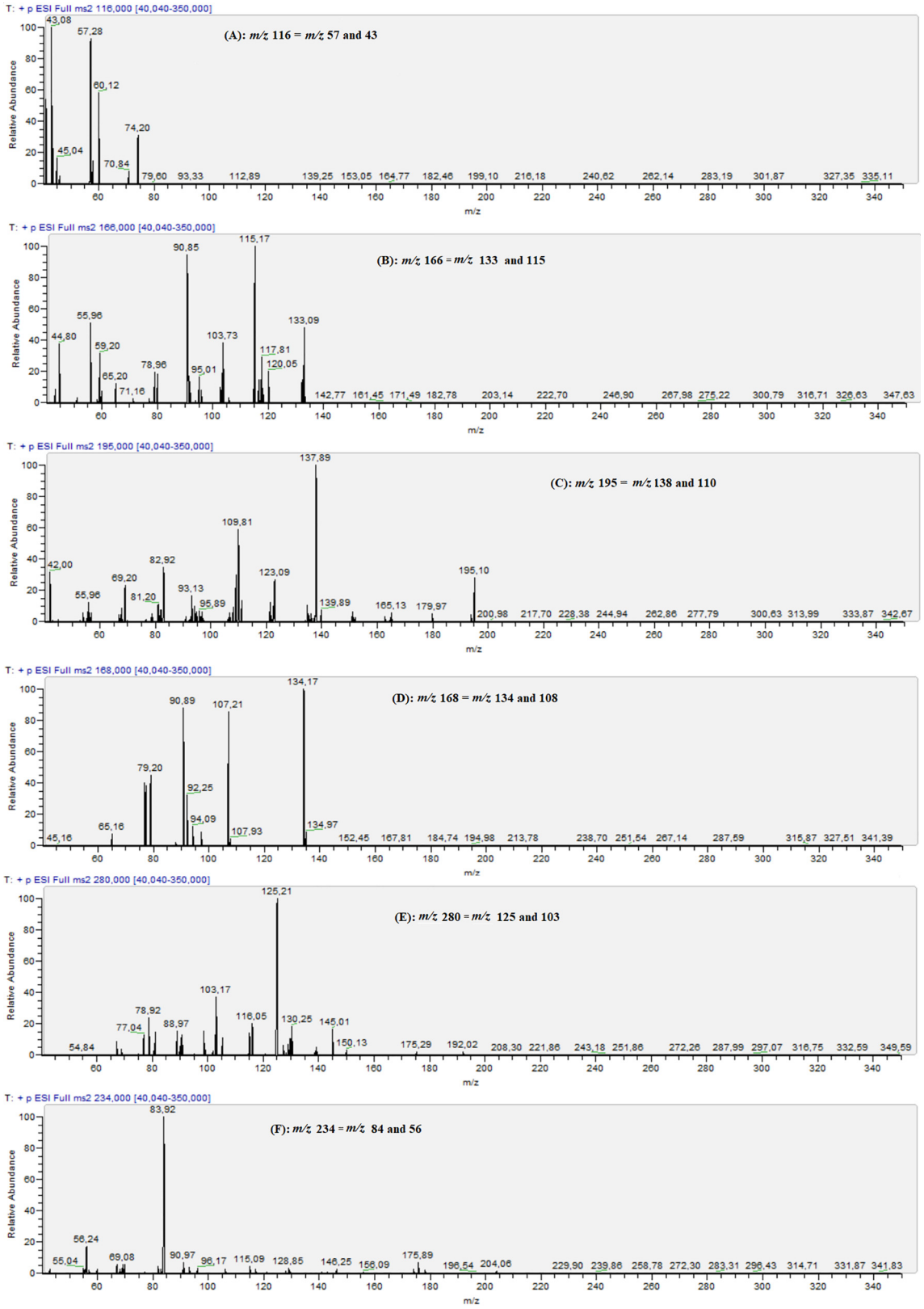

Fig. 1. Product ions observed postcollision energy optimization. (A) DMAA, (B) ephedrine, (C) caffeine, (D) synephrine, (E) sibutramine and (F) methylphenidate.

trade and use in Brazil. ANVISA also included DMAA in the List F2 – List of Psychotropic Substances of outlawed use in annex I of Portaria SVS/MS nº 344/98, according to RDC nº. 37 of July 2, 2012 [16]. In Brazil, studies conducted with dietary supplements seized by the Brazilian Federal Police (BFP) show that compounds containing DMAA continue to be clandestinely marketed and smuggled [2,23].

A good alternative to determine the adulteration of dietary supplements, identifying unlabeled or forbidden substances, is using Direct Analysis in Real Time Tandem Mass Spectrometry (DART-MS/MS) [24–26]. DART-MS/MS is capable of rapid analysis of samples at atmospheric pressure with only minimum sample preparation [24,27]. This technique is currently being used for different purposes, such as the determination of plants ingredients, pharmaceutical products and drugs of abuse [24,28]. Thus, DART-MS/MS can be considered a good screening test to analyze emerging drugs by identifying a large spectrum of substances simultaneously, through a combination of ambient ionization and tandem mass spectrometry [26,29].

This study presents a fast screening method by DART-MS/MS to detect the presence of DMAA and other stimulants found in dietary supplements such as ephedrine, synephrine, caffeine, sibutramine and methylphenidate in 108 samples of dietary supplements seized by the BFP. A select number of casework samples that tested positive with this DART-MS/MS method were later confirmed using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).

2. Material and methods

2.1. Chemicals and materials

HPLC optima grade methanol and LC-MS grade water was purchased from Fisher Scientific (Pittsburgh, Pennsylvania). 1S, 2R (+) ephedrine hydrochloride (1.0 mg/mL), methylphenidate hydrochloride (100 μg/mL) and sibutramine hydrochloride (1.0 mg/mL) in methanol were purchased from Cerilliant Corporation (Round Rock, Texas). Methylhexanamine (DMAA), caffeine and synephrine hydrochloride 10 mg, were purchased from Sigma Aldrich (St Louis, Missouri); JT Baker (Phillipsburg, New Jersey) and Apex Bio Technology (Fannin St, Houston), respectively. Capillary tubes, 1.5–1.8 × 90 mm, Pyrex, were purchased from Corning Life Sciences (Tewksbury, Massachusetts).

2.2. Dietary supplements

Dietary supplements (capsules with solid contents, capsules with liquid contents and tablets) were purchased at a local retailer located in Morgantown, West Virginia to develop the analytical method. Also, 108 Brazilian samples were seized according to the criteria established by BFP and constitute a group of the samples that were described by Neves and Caldas [2]. The seized dietary supplements included solid capsules, liquid capsules and tablets.

2.3. Sample preparation

The analytical standards used for the mass spectrometer optimization parameters and method development were diluted in methanol to a concentration of 1 ppm. The solid tablets were tested by holding the tablet directly between the ionization source and the inlet of the mass spectrometer. Extracts of the tablets were also analyzed on glass capillary tubes by first diluting 1/200 of the median weight of three capsules/tablets with 8 mL of methanol and 2 mL of Milli-Q water, depositing 2 μL of the extracted solution into a capillary tube for sampling. Liquid capsules were diluted using only 10 mL of methanol due to their oily content. After dilution, solutions were vortexed for 1 min and 2 μL of sample was pipetted into a glass capillary tube and analyzed.

2.4. Analytical procedure

2.4.1. Instrumentation

DART analyses were performed using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer. The DART source was attached to a standard Vapur® interface from IonSense (Saugus, Massachusetts) and fitted to the mass spectrometer using a custom-built 3D-printed flange. The DART ion source was operated with helium gas at 300 °C, at a flow rate of 2 L/min, and a grid voltage of 530 V. Xcalibur 2.0.7 software was used for data processing. Ultra-high purity helium (DART gas) and argon (collision gas) were purchased from Matheson TRIGAS (Fairmont, West Virginia). The capillary tube temperature for the mass spectrometer was set at 350 °C. The data acquisition consisted of 3 s (s) for sample analysis and 20 or 30 s for initial and final background, totaling less than 1 min per analysis. The precursor and product ions were determined using full scan mode from *m/z* 40 to *m/z* 350 and the precursor to product ion transitions were monitored using selected reaction monitoring (SRM) mode using compound-specific transitions. The values monitored for each compound were: DMAA = *m/z* [M + H] 116 → 57 and *m/z* 116 → 43; Caffeine = *m/z* [M + H] 195 → 138 and *m/z* 195 → 110; Ephedrine = *m/z* [M + H] 166 → 133 and *m/z* 166 → 115; Methylphenidate = *m/z* [M + H] 234 → 84 and *m/z* 234 → 56; Sibutramine = *m/z* [M + H] 280 → 125 and *m/z* 280 → 103; Synephrine [M + H] *m/z* 168 → 134 and *m/z* 168 → 108. The decision to use two transitions per compound maximized the number of data points acquired during the relatively short signal durations of the DART ion source. However, the use of two transitions per compound may not offer the highest degree of specificity for DART-MS/MS, and until all the samples are confirmed using an independent method, like LC-MS/MS, the possibility of some false positives cannot be ruled out.

2.4.2. DART-MS/MS optimization parameters

The values of collision energy (CE), gas temperature (250 °C, 300 °C, 350 °C, 400 °C and 450 °C) and scan times (0.20 s, 0.10 s, 0.05 s, 0.02 s and 0.01 s) were optimized for DMAA, ephedrine, synephrine, caffeine, sibutramine and methylphenidate. The optimum values were chosen considering a signal to noise ratio of three (S/N = 3) (Fig. 1). All the optimization experiments were analyzed in triplicate with the standards solutions prepared as described at Section 2.3.

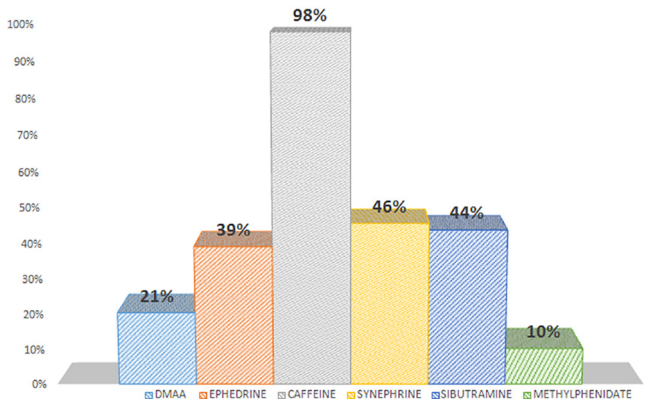

Fig. 2. Incidence of stimulants in 108 samples of dietary supplements after DART-MS/MS analysis.

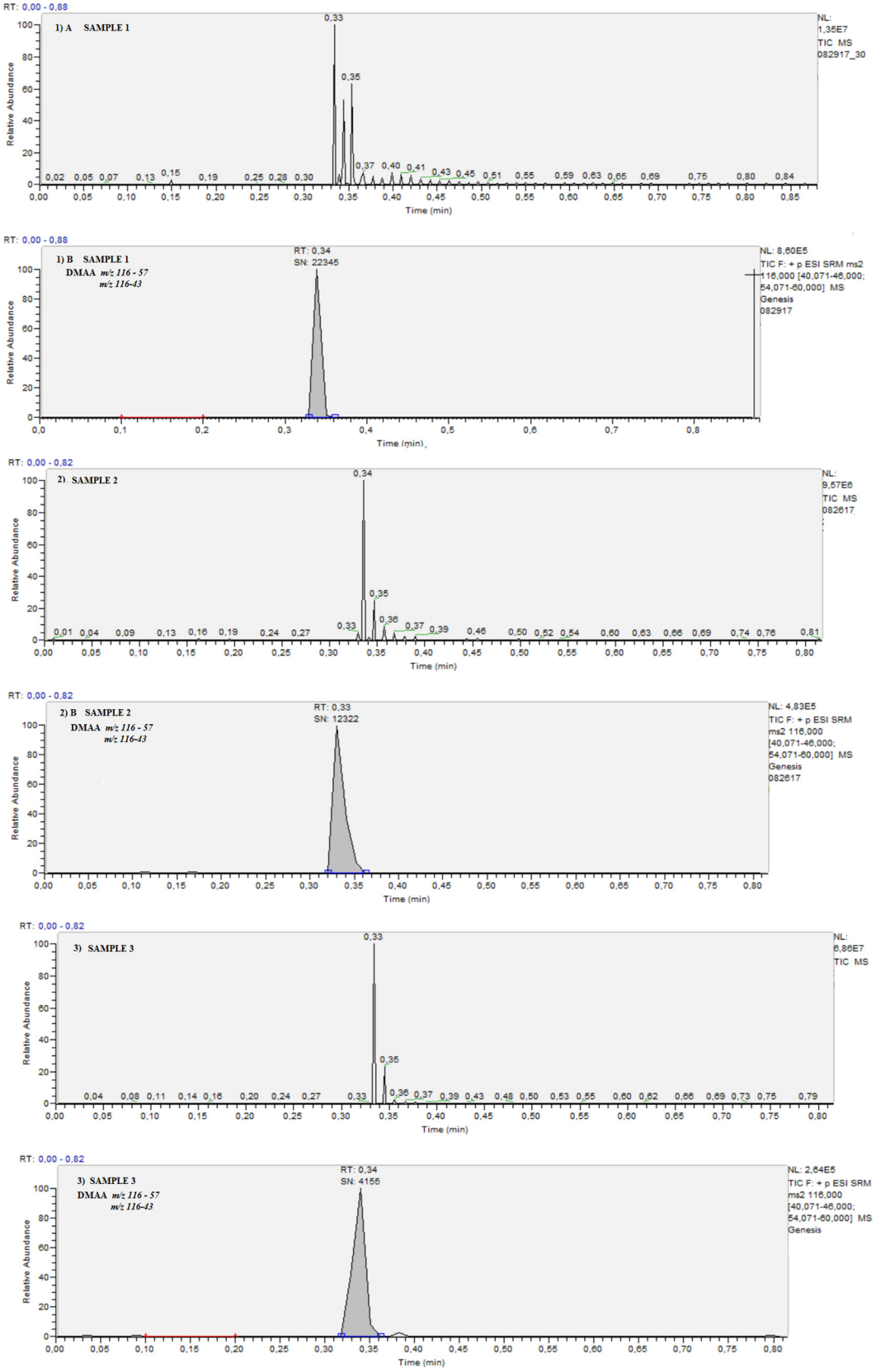

Fig. 3. DART-MS/MS chromatograms and SRM spectra of samples with positive results for DMAA. Sample 1 (1A and 1B), Sample 2 (2A and 2B) and Sample 3 (3A and 3B).

3. Results and discussion

To ensure the use of the best conditions for detection of all selected compounds in seized dietary supplements by DART-MS/MS, an optimization of collision energy (CE), gas temperature, and scan times was performed. The CE determines the amount of internal energy gained through collisions with the argon collision gas. CE optimization is important and very common in MS/MS methods, as optimized CE results for the specific target molecule can increase product ion intensities [30,31]. The optimum CE was obtained for each analyte considering the two product ions with the most stable relative abundances observed in the MS/MS spectra (Fig. 1) using the product ion scan mode. The final values for each standard were: DMAA = 18 V; Caffeine = 30 V; Ephedrine = 35 V; Methylphenidate and Synephrine = 37 V and Sibutramine = 40 V.

The DART gas temperature and flow rate are also important parameters affecting the desorption and ionization process of the substances, thus influencing the sensitivity of detection [32]. Gas temperatures were tested from 250 °C to 450 °C. Low intensities were observed at 250 °C for the six substances analyzed, indicating poor desolvation or desorption. When the temperature was raised to 300 °C, significantly better results were obtained. However, at temperatures greater than 300 °C, lower signal intensities were observed for DMAA and ephedrine, probably due the degradation of the molecules [33,34]. 300 °C provided the best results for the six analytes, considering our multianalyte method development, so this temperature was selected for the remainder of the study. After the gas temperature optimization, the capillary temperature was raised to 350 °C, to ensure that the compounds would not condense inside the capillary transfer tube entrance of the mass spectrometer.

Scan time or dwell time is the time spent acquiring a specific SRM transition during each cycle. A very short dwell time can be used for each analyte; however, a longer dwell time is desirable for better signal/noise and sensitivity. Scan times of 0.20 s, 0.10 s, 0.05 s, 0.02 s, and 0.01 s were tested. The optimum scan time was determined to be 0.05 s since it allowed for the detection of each compound with a low standard deviation regarding the signal-to-noise ratio.

After optimization, the method was tested on commercial dietary supplements acquired at a local retailer store located in Morgantown, West Virginia. Direct analysis of the solid tablets/capsules allowed for the identification of analytes without the need for sample preparation. The samples were also diluted as described in Section 2.3. The results of the direct analysis were then compared to the results of diluted samples to ensure that the dilution was suitable for the identification of the target substances. However, after running the first few solid samples, prolonged carryover was observed for certain direct analysis of solid samples. To prevent the need to clean the transfer optics in the atmospheric sampling interface, we therefore decided to continue the analysis on diluted samples only. Blank samples of methanol were run between the analyses.

The limit of detection was established using the standard definition of S/N = 3. However, analytes cannot be accurately quantified near the threshold levels because the limit of quantitation is considerably higher [35,36]. The minimum relative abundance for all analytes was on the order of 1×10^4, except for caffeine, which was usually very concentrated when it was present and has a higher background level in the DART-MS/MS method. The lower limit of detection (LLOD) was between 25 and 50 pg/μL for the six substances, which is more than sufficient to detect the six analytes at their relevant levels in the dietary supplements.

The 108 samples of dietary supplements seized by the BFP were categorized according to different types of matrices: solid capsules (65 samples), liquid capsules (33) and tablets (10). After dilution, samples were analyzed to assess presence of DMAA, caffeine, ephedrine, synephrine, sibutramine and methylphenidate. DMAA was present in 20% of the samples (Fig. 2) and the majority of positive results were found in solid capsules (25%), but positive results were also obtained from liquid capsules (Fig. 3). For tablet form samples, only one test sample showed presence of DMAA, synephrine, ephedrine, sibutramine and caffeine. Regarding the positive samples, when DMAA was present, it was always present with other stimulants. For example, DMAA was found with caffeine in 95% of the samples. DMAA was found with synephrine, sibutramine and ephedrine in almost 30% of the samples and methylphenidate in 5% of the samples (Fig. 4). These results agree with previous data reported that found DMAA along with other stimulants as well as caffeine, synephrine and sibutramine [2,37], a very common combination in these products mainly due their thermogenic activity and appetite suppressant properties [37–39].

The results presented in our study supports that DMAA presence is still highly troublesome [19,22]. For example, based on the last two WADA doping monitoring list, DMAA is one of the most widely used stimulant among athletes [20,22]. According to the anti-doping report from 2013, DMAA was the most commonly found stimulant detected in doping cases [20]. The adverse effects of DMAA were already described and include tachycardia, nausea, vomiting, dizziness, headache, chest pain and in some cases, death, which led to its prohibition in Brazil and United States, in 2012 and 2013 respectively [13,17,40,41]. However, our results show that DMAA can still be found in the dietary supplements, either declared or undeclared, because it is still clandestinely commercialized and smuggled in Brazil [2] and other countries [42].

DMAA can also be declared in the formulations as “geranamine”, due to its possible natural origin from the *Geraniaceae* family [43,44]. The presence of DMAA was initially attributed to the essential oils from *Pelargonium* sp., in 1996 [45]. After this initial report, several research groups conducted independent studies debating over the synthetic or natural origin of DMAA. The conclusions from these research efforts discarded the natural presence of DMAA in the essentials oil [6,43,44,46–48]. Furthermore, it is important to emphasize that DMAA was proscribed by ANVISA, which included DMAA at the F2 List of Psychotropic substances of outlawed use, the same category as ecstasy (3,4-methylenedioxymethampheta mine) and LSD (lysergic acid diethylamide); DMAA and its commerce is therefore completely illegal in Brazil [16].

Methylphenidate is the preferred drug commonly prescribed to Brazilian patients diagnosed with attention deficit hyperactivity disorder (ADHD). Because of its stimulating properties, there are also reports of its recreational use, generating concern to the authorities [49,50]. Moreover, MPH is one of the most frequently detected stimulants in doping test according to the last WADA

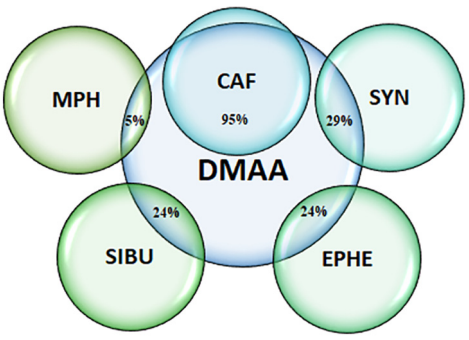

Fig. 4. Correlation between positive samples of DMAA along with other stimulants.

reported data [20,22]. From our dataset of tested specimens, we highlight the trace occurrence of methylphenidate (MPH) in 10% of the samples analyzed (Fig. 2). Solid capsules were the tested samples with the most part of positive results for MPH (Fig. 5). Additionally, the seized samples that showed positive results for MPH also presented other stimulants, being the most prevalent caffeine, followed by sibutramine and synephrine. The fact that MPH appeared as one of the most widely use stimulants among athletes, might be due to its addition in some dietary supplements, rather than being consumed as a medication, deserving attention of the authorities.

Sibutramine was detected in 44% of the formulations (Fig. 2). Solid and liquid capsules were the most prevalent samples with a positive result (Fig. 6). Likewise, other stimulants were detected alongside sibutramine, specially, synephrine, ephedrine and caffeine. The known toxicological effects for sibutramine are cerebrovascular accidents, myocardial infarction and psychiatric disorders, and so, its consumption should be firmly restricted and followed by medical monitoring [51–53]. However, in accordance with our data and other reports, sibutramine is one of the most common substances added as a undeclared compound to dietary supplements and weight loss compounds [2,54,55]. This represent a major health risk, especially if consumed with caffeine, ephedrine and synephrine due to their synergistic effects [51,56]. Sibutramine is considered a psychotropic anorectic drug, and is the oldest allowed weight loss medicine in Brazil; it is being

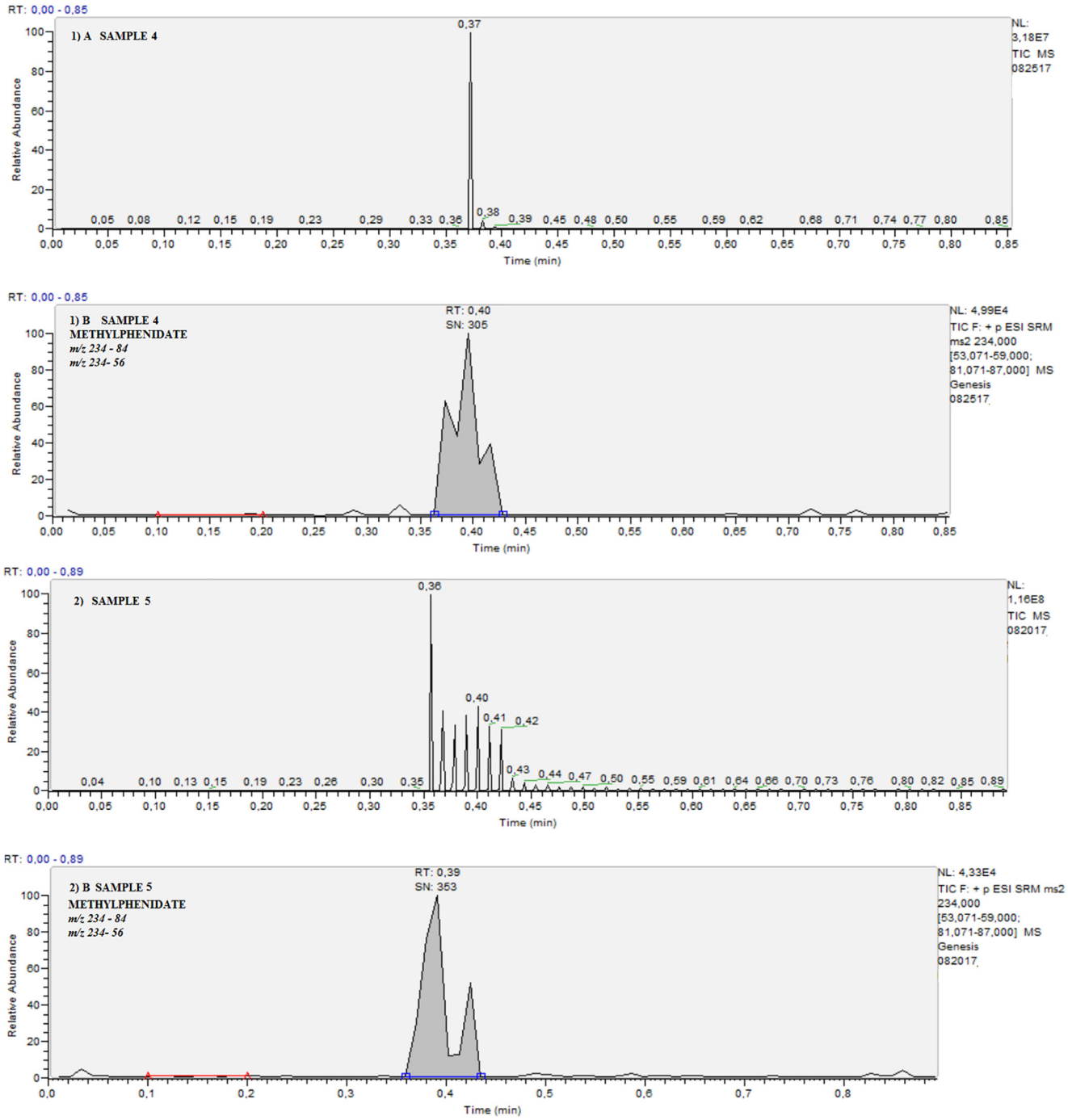

Fig. 5. DART-MS/MS chromatograms and SRM spectra of samples with positive results for methylphenidate. Sample 3 (1A and 1B) and Sample 4 (2A and 2B).

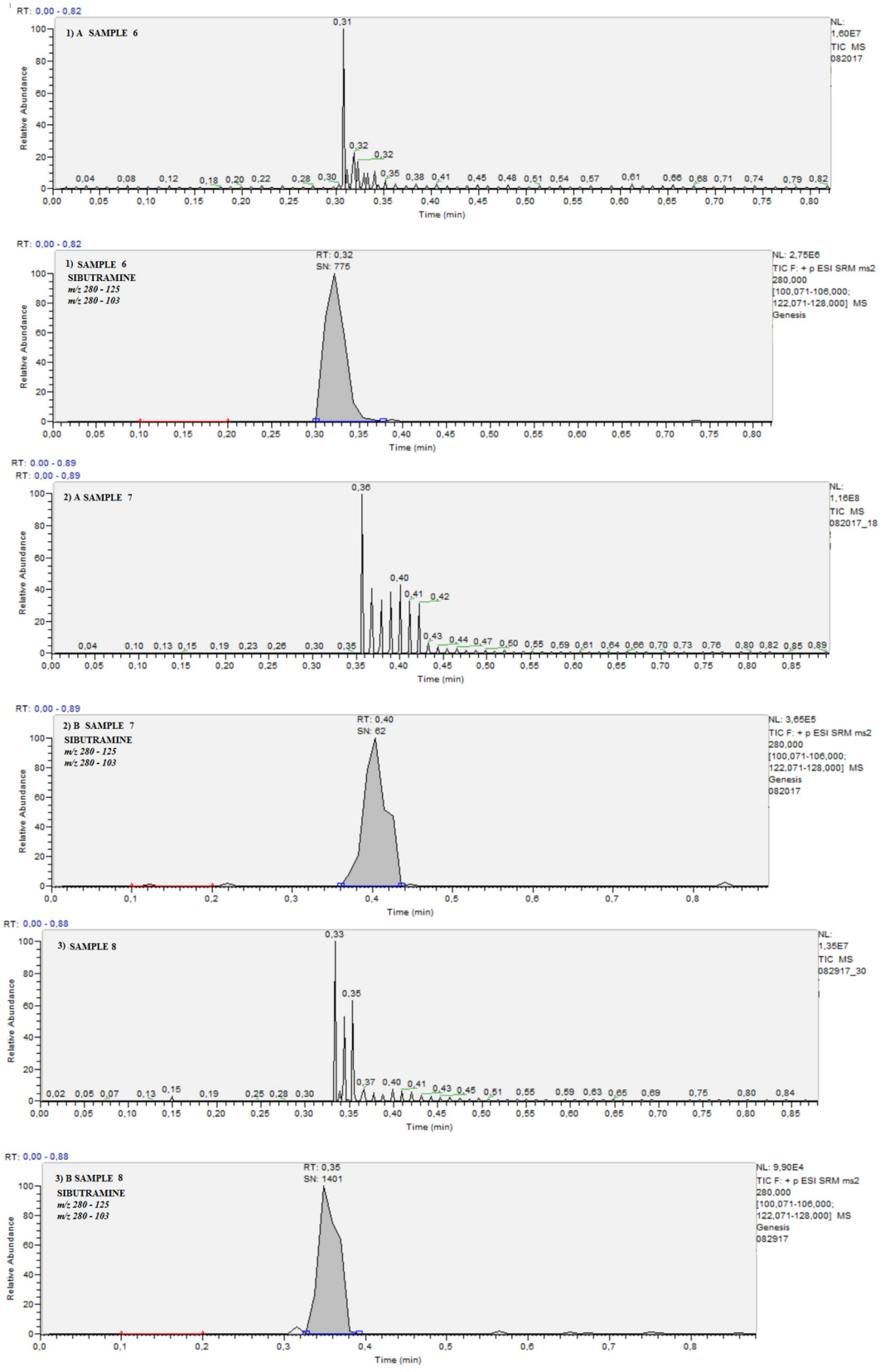

Fig. 6. DART-MS/MS chromatograms and SRM spectra of samples with positive results for sibutramine. Sample 6 (1A and 1B), Sample 7 (2A and 2B) and Sample 8 (3A and B).

commercialized since 1998. Control of sibutramine was reinforced since 2011, it was submitted for special prescription for its commercialization [57], and recently updated by the Brazilian authorities under the RDC 133 of 2016 [58].

Synephrine and ephedrine were also found in a significant portion of the seized samples, at rates of 46% and 39%, respectively (Fig. 2). Liquid capsules were the most prevalent (Fig. 7), along with tablets, considering both compounds. Ephedrine is a

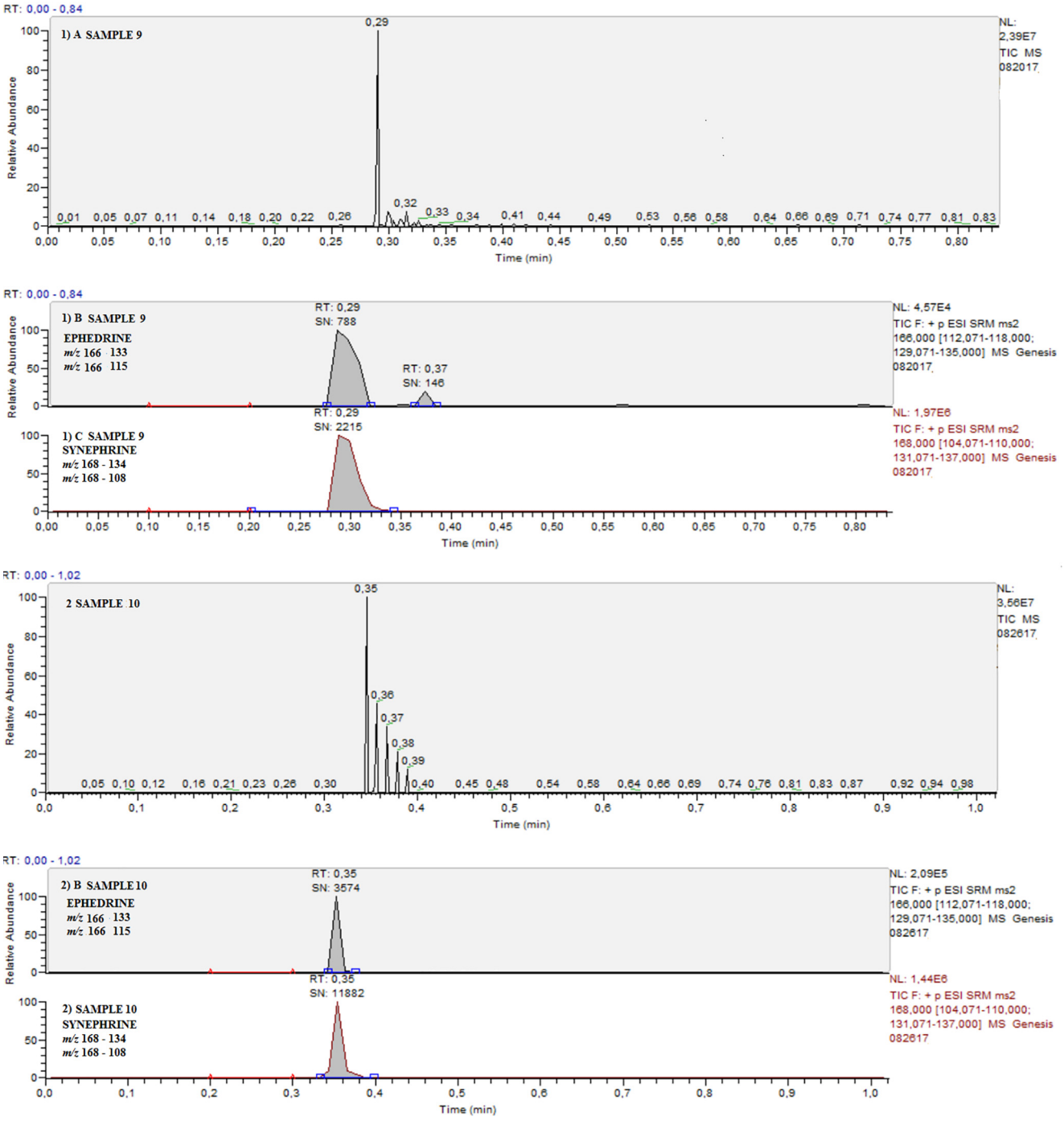

Fig. 7. DART-MS/MS chromatograms and SRM spectra of samples with positive results for ephedrine and synephrine. Sample 9 (1A, 1B and 1C) and Sample 10 (2A, 2B and 2C).

sympathomimetic amine belonging to *Ephedra* genus that is used in weight loss compounds and dietary supplements, having thermogenic and stimulant properties [59,60]. Its adverse effects are manifested mostly to as cardiac symptoms, with consequences such as stroke, myocardial infarction and sudden death [51,61,62]. Ephedrine consumption was restricted by WADA in 2007 [63], and replaced by synephrine over the years in the formulations of dietary supplements and weight loss products. However, considering the results and reported data, ephedrine can still be found in these products, and is predominantly associated with other stimulants such as caffeine [39,64,65]. Synephrine is the most active substance naturally found in *Citrus aurantium*. The addition of synephrine to dietary supplements and weight loss products is very common due to its stimulant and weight-loss properties [38,64]. Synephrine consumption has been regulated by WADA since 2009 [66]. We detected synephrine in 49 samples (~45%), which agrees with previous data reported, confirming that its addition is a common practice, particularly together with other stimulants such as ephedrine, sibutramine and caffeine [39,56,64,65].

Caffeine was the most prevalent substance, present in 105 analyzed samples (Fig. 2), in agreement with data already reported by Neves and Caldas [2]. During the method development with the commercial samples, a carryover due the high concentration of caf-

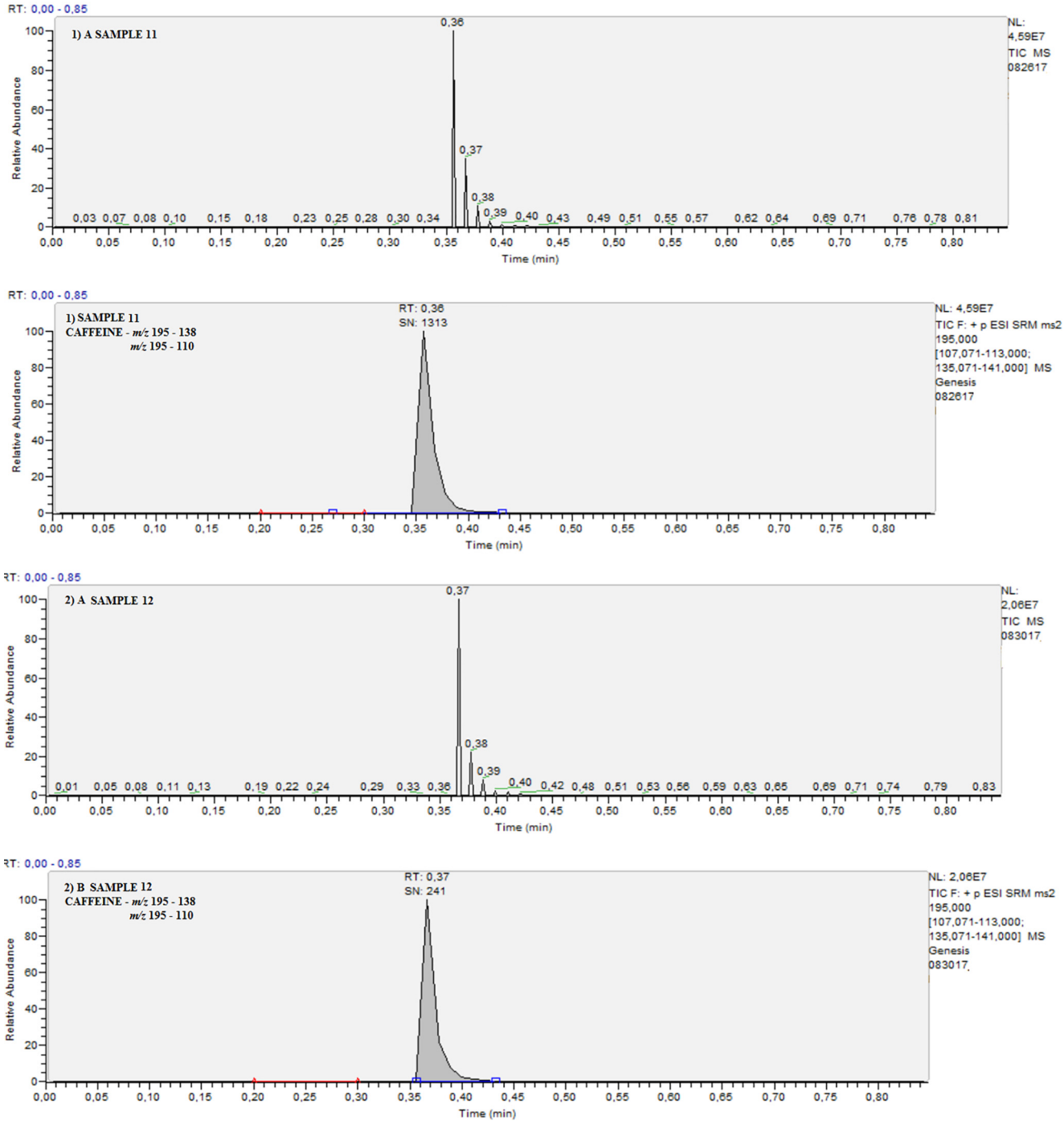

Fig. 8. DART-MS/MS chromatograms and SRM spectra of samples with positive results caffeine. Sample 11 (1A and 1B) and Sample 12 (2A and 2B).

feine reported in these products was detected. Thus, it was decided that a higher threshold value for caffeine of 1×10^6, instead of 1×10^4, was required in addition to considering the signal to noise ratio of three for positive results. Caffeine is the most common stimulant known [59] and its consumption is allowed by WADA, in established concentrations [18]. Nevertheless, caffeine can be toxic when consumed in high doses and particularly in association with other stimulants [59], which is very common in dietary supplements according to these results (Fig. 8) and data already published [2,56,67].

The 108 seized samples analyzed showed the presence of DMAA in most of the solid capsules, and approximately 20% of the liquid capsules and tablets. The DART-MS/MS screening results of were validated using a confirmatory method by multiple reaction monitoring through an HPLC-MS/MS triple quadrupole mass spectrometer. Considering these results and other reported [42], it can be assumed that clandestine commercialization of DMAA is still occurring. Also, it was noted that all the six stimulants analyzed were frequently found in the seized samples, and occasionally, with five of the compounds together in different combinations (Fig. 9). Therefore, the consumption of stimulants simultaneously represents a major health risk for the consumer, leading to toxicity and even death [8,17,41,51,56]. Fast and simple methods are required for routine screening analysis of adulterants, which are

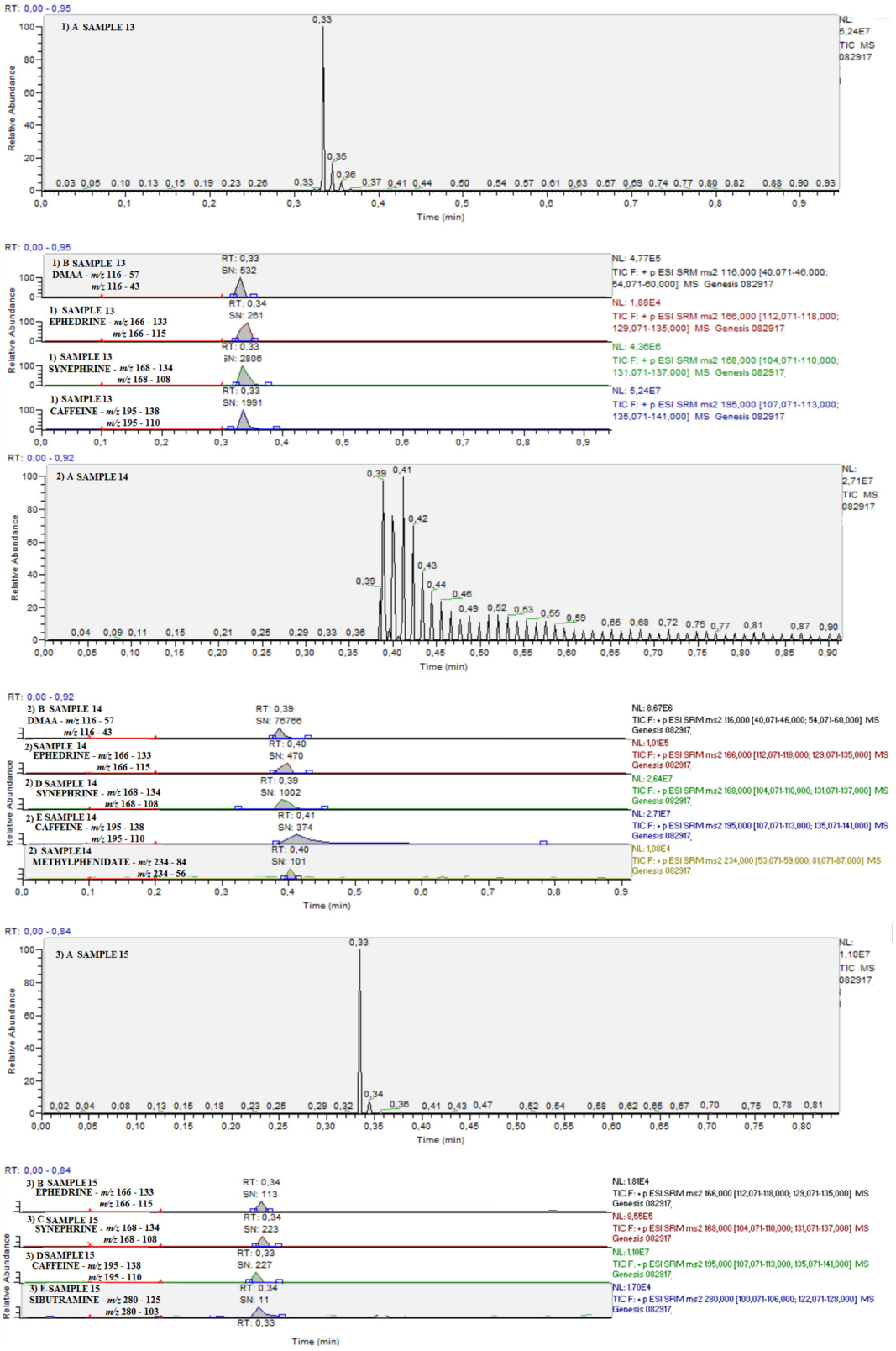

Fig. 9. DART-MS/MS chromatograms and SRM spectra of samples with positive results for DMAA, ephedrine, synephrine, caffeine, sibutramine and methylphenidate. Sample 13 (1A, 1B, 1C, 1D and 1E) Sample 14 (2A, 2B, 2C, 2D, 2E and 2F) and Sample 15 (3A, 3B, 3C, 3D and 3E).

present on numerous dietary supplement samples, therefore, ambient ionization methods simplify screening analyses by eliminating the need for clean-up steps and provide mass spectral data without the sample preparation required by the chromatography based methods [26,27,29].

4. Conclusions

The DART-MS/MS approach used in this study was able to rapidly detect six different stimulants in dietary supplements. DART-MS/MS detected DMAA in 20% of the seized dietary

supplements, and DMAA was usually found together with other stimulants such as sibutramine, which is used for appetite suppressant. Sibutramine and methylphenidate—which is used for attention deficit hyperactivity disorder—were found in almost 50% and 10% of the samples, respectively that contained DMAA. These findings highlight the concern about the composition of clandestine dietary supplements, which represent a serious health risk for the users, due to the simultaneous consumption of anorectics and stimulants, which certainly deserves attention of regulatory agencies.

Acknowledgments

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