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Morphine and codeine in racing horse feed: is there reason for concern?

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Opiates such as morphine and codeine are substances often misused to improve the performance of racing horses during competitions and are therefore on the International Federation for Equestrian Sports' list of prohibited substances. However, a positive antidoping test may be due to the consumption of feed (mainly lucerne or oats) contaminated by opium poppy containing the alkaloids morphine and codeine. In order to determine whether a positive antidoping test is the result of an intentional abuse of opiates or consumption of feed contaminated by poppies, we optimised conditions for the extraction of morphine and codeine from dehydrated lucerne and developed and validated a gas chromatography-mass spectrometry (GC-MS) method for the simultaneous determination of both analytes. The most efficient extraction of morphine and codeine from dehydrated lucerne was achieved using a citrate buffer pH4 followed by solid phase extraction. The method showed satisfactory linearity (R^2>0.9980) in the tested concentration range (85–1600 ng/g), as well as good precision (RSD<4 %), accuracy (>95 %), and sensitivity (limit of detection 22 and 25 ng/g for morphine and codeine, respectively). The proposed method was used for analysing a sample of dehydrated lucerne having measurable content of morphine (1510 ng/g) and codeine (327 ng/g) that can cause positive results of opiate blood or urine testing up to 4 hours after feeding the horse with less than 500 g of dehydrated lucerne. The use of this analytical method should enable the exclusion of horse feed as the cause of positive antidoping tests.

KEY WORDS: antidoping; GC-MS; lucerne; method development; opiates

The goal of antidoping controls is to determine if a horse has been treated or fed with illegal substances during competitions. For this purpose, the International Federation for Equestrian Sports established a list of prohibited substances and prescribed a procedure to implement anti-doping testing that includes the collection of blood and urine samples from horses. Testing can take place at any event, in any discipline, at any time (1). By improving analytical techniques, the smallest quantities of consumed prohibited substances can be discovered in urine samples collected after a race (2). Opiates such as morphine are used as analgesics and pre-anaesthetic drugs in humans, while in horses they stimulate the central nervous system, amplify locomotor activity, reduce pain, and increase endurance (3). Morphine was occasionally detected in post-race urine samples at concentrations less than 50 μg/L and in most cases, it was not present in the plasma samples. Such low morphine concentrations are likely related to environmental morphine contamination rather than pharmacologically effective doses of morphine (4). Considering the low oral bioavailability and urinary concentration of morphine after a 250 μg dose (0.5 % of the intravenous dose of morphine that does not cause a locomotor effect in horses), the permissible concentration of morphine in horse urine was set at 100 μg/L (4).

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As previously stated, apart from intentional opiate abuse to achieve better results in horse races, a positive antidoping test may be the result of accidental horse feed contamination with prohibited substances present in the environment. Morphine and codeine are alkaloid opioids of natural origin and are the main ingredient of opium poppy (*Papaver somniferum* L., Papaveraceae). Unlike capsules, opium poppy seeds contain almost none of these alkaloids. However, opiate contamination of seeds is common due to bad harvesting practices and damage caused by insects (5).

There are several ways by which horses can unintentionally consume poppy and thus be exposed to prohibited substances. Baking by-products, pastries, and muffins that contain poppy seeds are occasionally used in preparing feed or are given to horses as treats (2). Lucerne or alfalfa (*Medicago sativa* L.) is a forage crop and an excellent source of energy, protein, and minerals in the horse diet. In order to provide additional nutrients, it is often used in the form of pellets made from dehydrated lucerne (6). Considering that opium poppies are frequently found growing in the same areas and can contaminate lucerne with morphine and codeine (7), a reliable analytical method for the determination of these alkaloids in dehydrated pelleted lucerne is of the utmost importance. To our knowledge, such a method has not yet been reported. Therefore, the aims of this study were to optimise the extraction conditions

of morphine and codeine from dehydrated lucerne and develop and validate an analytical method for the simultaneous determination of both analytes using gas chromatography coupled with mass spectrometry (GC-MS). The development of this analytical method should ultimately enable the exclusion of horse feed as a possible cause of positive antidoping tests.

Attempts to improve competitive results in sports illegally, both in humans and in animals, are on the rise. The presence of any opiate at any concentration in post-race urine samples is cause for concern, as these drugs can be given to horses in illicit attempts to improve results. Opiates can be detected in urine samples only immediately after the race because, as previous researches have demonstrated, animals metabolise and eliminate drugs faster than humans (8, 9). By reviewing the relevant literature, we have not found any analytical method that has been developed and validated for the analysis of opiates that could be present as contaminants in horse feed. Chromatographic methods such as GC-MS, high performance liquid chromatography-diode array detection (HPLC-DAD) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) were developed only for the analysis of opiates in poppy seeds and straws (10–13). Therefore, our main goals were to optimise the conditions for extraction of morphine and codeine from dehydrated lucerne and quantify both analytes in a single chromatographic run by a validated GC-MS method.

MATERIALS AND METHODS

Chemicals and reagents

A standard solution of morphine, codeine, and 6-MAM (6-monoacetylmorphine, internal standard), with a concentration of 1 g/L in methanol, were purchased from Lipomed (Arlesheim, Switzerland). Working standard solutions of morphine and codeine (2 mg/L) and 6-MAM (3 mg/L) were prepared by dilution with methanol.

Methanol, ammonia, and ethyl acetate were purchased from Merck (Darmstadt, Germany), while sodium acetate, dichloromethane, n-propanol, glacial acetic acid (99.5 %), and potassium hydroxide were from Kemika (Zagreb, Croatia). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1 % trimethylchlorosilane (TMCS) was purchased from Restek (Bellefonte, PA, USA), and citric acid from Sigma (St. Louis, MO, USA). Ultrapure water for SPE-column preparation and washing was prepared using a Mili-Q water purification system (Millipore, Bedford, MA, USA).

Samples

Samples of dehydrated lucerne (samples 1 and 2) in the form of pellets were obtained from feed producers and stored in a dark place until the beginning of analysis. In order to obtain a homogeneous representative sample, pellets were crushed and then sifted through a sieve. The resulting powder was transferred into plastic tubes and stored in a dark place at room temperature until analysis.

Optimised extraction conditions

For analysis, 1 g of the lucerne sample was weighed into a 15 mL glass test tube with a ground stopper and mixed with 10 mL of 0.1 mol/L citrate buffer pH=4 and 100 μL of the internal standard 6-MAM at 3000 ng/mL.

In order to create a calibration curve within the range of 85–1600 ng/g, a blank lucerne sample (without measurable levels of morphine and codeine) was spiked with appropriate aliquots of analytical standards (morphine, codeine, and 6-MAM). The mass fraction of the internal standard was 300 ng/g in all samples.

The extraction procedure was adjusted according to a previously published procedure for determining the mass concentration of opiates in human urine (14) and poppy seeds (13).

The samples in citrate buffer were vortexed for 1 minute and the extract was filtered through filter paper. An aliquot of 3 mL of the extract was adjusted to pH=6 with 40 μL of 10 mol/L potassium hydroxide and passed through a solid phase extraction (SPE) column (Bond Elut LCR-Certify, Agilent Technologies, Santa Clara, CA, USA) pre-conditioned with 2 mL of methanol and 2 mL of water. After passing the sample, the SPE column was washed with 2 mL of water, 1 mL of 0.1 mol/L acetate buffer pH=4, and 2 mL of methanol and then dried under high vacuum for 2 minutes. The analytes were eluted from the sorbent with 2 mL of elution mixture (dichloromethane:*n*-propanol:ammonia=80:20:2, v/v/v). The eluates were collected in 3 mL glass tubes and evaporated to dryness under a stream of nitrogen. Then, 0.5 mL of ethyl acetate was added to the dry residue and evaporated again. Afterwards, 50 μL of BSTFA + 1 % TMCS was added to the dry residue and the sample was placed in a thermostat for derivatisation at 70 °C for 30 minutes. After cooling, 1 μL of each sample was analysed using a GC-MS.

Optimisation of the extraction conditions

To optimise the extraction conditions, a sample of dehydrated lucerne with a measurable level of morphine and codeine was used. Extraction efficiency using citrate buffer was tested by 1) vortexing the samples for 1 and 2 minutes; 2) vortexing the samples for 10 seconds and then heating in a thermostat for 20 minutes at 50 °C; and 3) vortexing the samples for 10 seconds and then placing the samples in an ultrasonic bath at room temperature for 20 minutes. The effect of different volumes of citrate buffer (10, 15, and 20 mL) on extraction efficiency was also examined. In addition to citrate buffer, methanol with 0.1 % acetic acid was also tested as an extraction agent. A total of 10 mL of methanol and 10 μL of 99 % acetic acid were added to the 1 g of lucerne samples. The samples were then filtered, evaporated at 40 °C, and dissolved in 10 mL of citrate buffer and were treated in the same manner as other samples (separation of aliquots, pH adjustment, and SPE).

Gas chromatography-mass spectrometry (GC-MS)

After derivatisation, 1 μL of the sample was introduced into the injector of the GC-MS system. The analyses were performed on a Varian 3400 CX gas chromatograph coupled to a Saturn 4D ion trap mass spectrometer (Walnut Creek, CA, USA). The analytes were separated on a HP-5MS capillary column [30 m length, internal diameter: 0.25 mm, film thickness: 0.25 μm, stationary phase: 5 % phenyl- and 95 % methylpolysiloxane (J&W Agilent Technologies, Santa Clara, CA, USA)]. Helium with a flow rate of 1 mL/min was used as the mobile phase. The initial temperature of the septum-equipped programmable injector (SPI) was 40 °C, and after 0.1 min the injector was heated to 280 °C at a rate of 200 °C per minute. At the beginning, the temperature in the gas chromatography oven was maintained at 50 °C for 1 minute, and then increased to 250 °C, heating at 50 °C per minute, and up to 280 °C, heating at 10 °C per minute. The analysis lasted 12 minutes. The transfer line temperature was set at 280 °C. Mass spectra were recorded in the range m/z 50–500 at a rate of 2 scans per second. The MS detector operated in electron impact ionisation mode (70 eV). Three ions were monitored for each trimethylsilyl (TMS) derivative: m/z 429, 287, 324 for morphine-2TMS, m/z 371, 234, 343 for codeine-TMS, and m/z 399, 340, 73 for 6-MAM-TMS. For quantification, ions m/z 429 for morphine-2TMS, m/z 371 for codeine-TMS, and m/z 399 for 6-MAM-TMS were used.

Analytical validation

The calibration curve was prepared using the optimised experimental parameters in the concentration range of 85–1600 ng/g. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using a signal-to noise ratio of 3:1 and 10:1, respectively. Precision and accuracy were evaluated by analysing the blank lucerne samples in which the standards of codeine and morphine were added to obtain mass fractions of 100 ng/g and 800 ng/g. Six replicates at each concentration were tested.

Statistical analysis

The statistical analysis of the results was carried out using the Dell™ Statistica™ 13.2 software (StatSoft, Tulsa, OK, USA). All measurements were performed in triplicate and results regarding optimisation of the extraction were expressed as mean ± standard deviation (SD).

RESULTS

Optimisation of the extraction conditions

Within this study, the extraction conditions of morphine and codeine from dehydrated lucerne were optimised followed by the validation of GC-MS method. Different extraction conditions were tested and the areas under the peaks of the analyte in the ion chromatogram were compared. The largest peak area for the target ion of particular analyte corresponded to the most efficient extraction.

In the first experiment, the effect of vortexing samples for 1 and 2 minutes was tested using 1 g of sample of dehydrated lucerne with a measurable level of morphine and codeine and 10 mL of citrate buffer pH4. As the extraction was equally effective for both extraction times, 1 min extraction time was used in further experiments to save time.

In the next experiment, the influence of temperature and ultrasound on the extraction of morphine and codeine from lucerne using citrate buffer was tested on samples at room temperature after vortexing for 1 minute, on samples that were heated for 20 minutes at 50 °C after vortexing for 10 seconds, and samples that were placed in an ultrasound bath for 20 minutes after vortexing for 10 seconds. Figure 1 shows the peak areas under different extraction conditions using citrate buffer. Peak areas were similar for morphine and codeine in all three types of extraction. However, due to the poor

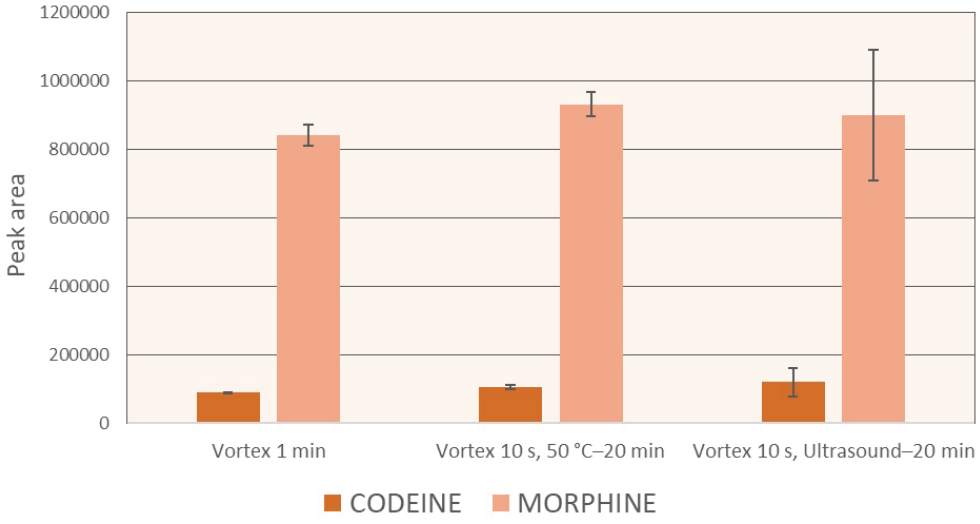

Figure 1 Effect of different extraction conditions on the peak area. Citrate buffer (0.1 mol/L, pH=4, V=10 mL) was used for extraction. Samples [dehydrated lucerne (sample 1) with a mass fraction of 327 ng/g codeine and 1510 ng/g morphine] were prepared in triplicate. Results were expressed as mean ± standard deviation

Table 1 Limit of detection and quantification, accuracy, and precision for determining the mass fraction of morphine and codeine in dehydrated lucerne samples (n=6)

Analyte	ω (ng/g)	Precision (RSD %)	Accuracy (%)	Limit of detection (ng/g)	Limit of quantification (ng/g)
Morphine	100	2.0	95.5	22	73
	800	0.6	98.9		
Codeine	100	3.9	96.2	25	83
	800	1.1	99.7		

n = number of replicates at each concentration level; ω – mass fraction; RSD – relative standard deviation

precision of extraction when heating the sample (RSD>6 %, in term of peak area for triplicate measurements) and extraction with ultrasound (RSD>20 %), extraction at room temperature with vortexing for 1 minute (RSD<4 %) was used in further experiments.

In addition to citrate buffer, acidified methanol was tested as an extraction solvent. The use of methanol with the addition of 0.1% acetic acid as an extraction agent resulted in a large baseline noise in chromatogram and a small peak area for both analytes, and therefore citrate buffer was chosen as an extraction solvent.

The extraction efficiency with regard to the solvent volume was tested on citrate buffer at volumes of 10, 15, and 20 mL. As the amount of solvent did not affect the extraction efficiency, the smallest volume (10 mL) was used to reduce costs and save the environment.

Analytical performance

Figure 2 shows the total and selected ion chromatograms of the lucerne blank sample to which the standard was added so that the mass fraction of morphine and codeine was 400 ng/g, obtained by SPE and analysis conditions described earlier. The proposed GC-MS conditions enabled an efficient separation of the targeted analytes from the interferences. Background noise had no influence on the interpretation of the results.

A calibration curve was made in the concentration range of 85–1600 ng/g for both analytes, with a coefficients of determination (R^2)>0.9980 and 1 for codeine and morphine, respectively, which confirmed linearity in the tested concentration range. Table 1 shows the LOD, precision, and accuracy for morphine and codeine in samples of lucerne. The LODs for morphine and codeine were 22 ng/g and 25 ng/g, respectively. Accuracy values greater than 95 % were obtained for six replicates at two concentration levels. RSD<4 % indicated satisfactory precision.

The proposed method was used to analyse two samples of dehydrated lucerne obtained from horse feed manufacturer. Both analytes were detected in sample 1, while sample 2 did not contain detectable mass fractions of either morphine or codeine. Figure 3 shows the chromatograms of lucerne sample 1 with a morphine and codeine mass fraction of 1510 ng/g and 327 ng/g, respectively.

DISCUSSION

According to the results obtained, a horse would need to consume only 485 g of lucerne to test positive in an anti-doping test. The literature indicates that the consumption of 10 g of poppy seeds containing 732 μg of morphine gives a detectable concentration of morphine in the serum and urine of horses up to 4 hours after consuming poppy seeds (15). Given that the 1 g of lucerne that we analysed (sample 1) contained 1.51 μg of morphine, 485 g of lucerne would contain 732 μg of morphine, which is enough for a positive test. It should also be considered that codeine is metabolised in the liver into morphine, so less than half a kg of lucerne can give a positive result for opiate testing.

Kollias-Baker and Sams (15) reported that contamination of horse feed with poppy seeds can result in measurable concentrations of morphine in blood or urine test samples collected before or after competition. Since opium poppies are sometimes found in gardens, these plants are likely the source of lucerne contamination with morphine. In opium poppy seeds, among other alkaloids, morphine (<0.1–620 μg/g) and codeine (<0.1–348 μg/g) were detected in a wide range indicating that their amount depends on the geographical location, climate conditions, harvesting time, and technology (10, 16, 17).

In this study, different extraction conditions were tested. Given that extraction on a vortex for one minute at room temperature was the simplest and fastest and gave very good precision, this procedure was used in further experiments. Extraction with citrate buffer after 20 minutes in an ultrasonic bath and 20 minutes in an incubation oven at 50 °C gave much lower precision (large standard deviation between triplicates). Namely, by heating the sample and using ultrasonic extraction, an emulsion was created, which probably made reproducible extraction more difficult. Given that the morphine in opium poppy (which was mixed with lucerne) is bound to meconic acid, to obtain free morphine the sample needs to be acidified. Choe et al. (18) used 0.1 N hydrochloric acid to obtain free morphine, whereas Sproll et al. (16) showed in preliminary experiment that methanol containing 0.1 % acetic acid is the best medium for extracting morphine and codeine from poppy seeds. Keeping in line with the literature, the effectiveness of methanol extraction was tested after the acidification of the methanolic sample with acetic

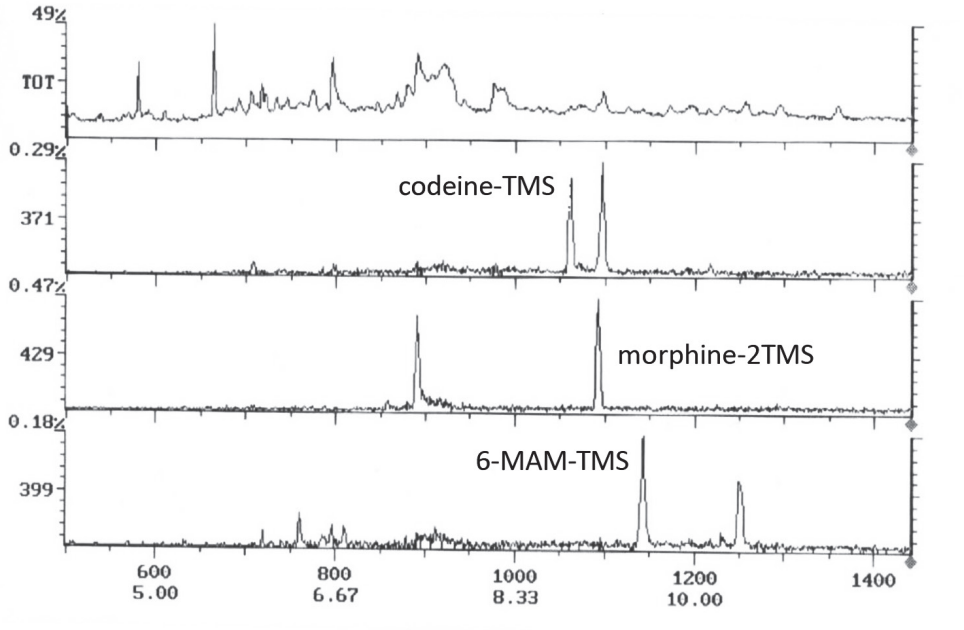

Figure 2 Total and selected ion chromatograms of a lucerne sample spiked with a standard to contain 400 ng/g of morphine and codeine. TMS–trimethylsilyl; 6-MAM–6-monoacetylmorphine, internal standard

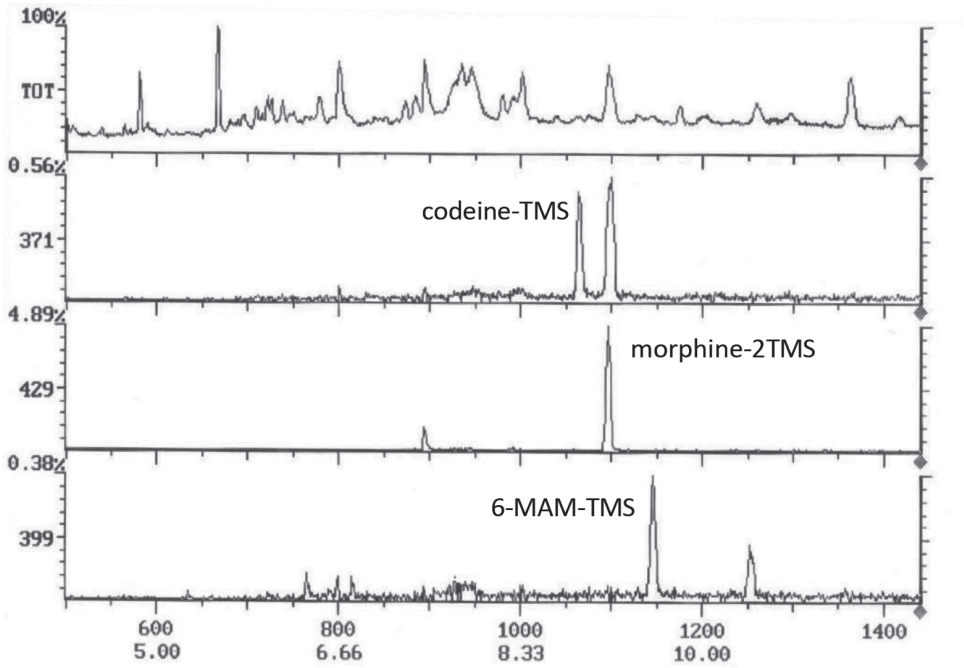

Figure 3 Total and selected ion chromatograms of dehydrated lucerne (sample 1) with a mass fraction of 327 ng/g codeine and 1510 ng/g morphine. TMS–trimethylsilyl; 6-MAM–6-monoacetylmorphine, internal standard

acid. Extraction of morphine and codeine from poppy seeds with citrate buffer pH=4 according to the procedure used by Skender et al. (13) yielded low chromatographic baseline noise as well as a large peak area, while extraction using acidified methanol would require further purification of the sample in order to achieve satisfactory sensitivity.

The applied gas chromatographic conditions enabled an effective separation of analytes from each other, as well as from other unidentified peaks. The low detection limits for morphine (22 ng/g) and codeine (25 ng/g) indicated the method's high sensitivity.

CONCLUSION

In this study, the extraction conditions of morphine and codeine from dehydrated lucerne were optimised, and a method was developed and validated for their simultaneous quantitative determination using GC-MS. Extraction conditions such as temperature and type of extraction and solvent had a significant influence on the results of analysis of morphine and codeine in dehydrated lucerne samples. The developed method is fast, sensitive, precise, and accurate, and its application makes it possible to rule out horse feed as a possible cause of a positive antidoping test.

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Conflicts of interest

The authors declare no conflict of interest.

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Morfin i kodein u hrani za trkaće konje: ima li razloga za zabrinutost?

Opijati poput morfina i kodeina često se zloupotrebljavaju kako bi se poboljšala izvedba trkaćih konja tijekom natjecanja te se stoga nalaze na listi nedopuštenih sredstava Međunarodne konjičke federacije. Međutim, pozitivan antidopinški test može biti posljedica konzumiranja hrane (uglavnom lucerne ili zobi) onečišćene opijumskim makom koji sadrži morfin i kodein. Da bi se utvrdilo je li pozitivan antidopinški test posljedica namjerne zlouporabe opijata ili konzumiranja hrane kontaminirane makom, optimizirani su uvjeti ekstrakcije morfina i kodeina iz dehidrirane lucerne te je razvijena i validirana plinskokromatografska metoda uz detekciju spektrometrijom masa za istovremeno određivanje sadržaja oba ispitivana analita u dehidriranoj lucerni. Najučinkovitija ekstrakcija oba analita iz dehidrirane lucerne postignuta je korištenjem citratnog pufera pH=4 uz dodatno pročišćavanje ekstrakcijom na čvrstom nosaču. Metoda je pokazala zadovoljavajuću linearnost (R^2>0,9980) u ispitivanom koncentracijskom rasponu (85–1600 ng/g), kao i dobru preciznost (RSD<4 %), točnost (>95 %) i osjetljivost (granica detekcije=22 ng/g za morfin i 25 ng/g za kodein). Predloženom metodom analiziran je uzorak dehidrirane lucerne koji je sadržavao 1510 ng/g morfina i 327 ng/g kodeina što može rezultirati pozitivnim nalazom opijata u krvi i mokraći do 4 sata nakon što je konj nahranjen s manje od 500 g dehidiriane lucerne. Razvojem i validacijom ove analitičke metode omogućeno je isključivanje hrane za konje kao uzrok pozitivnog antidopinškog testa.

KLJUČNE RIJEČI: antidoping; GC-MS; lucerna; opijati; razvoj metode