A sensitive LC-ESI-MS/MS method for isomer separation and quantitative determination of 51 pyrrolizidine alkaloids and two tropane alkaloids in cow’s milk

Electronic Supplementary Material (ESM)

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Table S1 Overview of previously published extraction and clean-up procedures, and (LC-)MS/MS methods applied for the determination of pyrrolizidine alkaloids (PA), pyrrolizidine alkaloid N-oxides (PANO) and tropane alkaloids (TA) in milk and milk products

| Analytes | Sample volume [mL or g] | Extraction and clean-up | Mobile phase | Stationary phase | LC-MS/MS instrumentation | Recovery [%] | LOQ [µg/kg or µg/L] | Ref. |
|----------|-------------------------|-------------------------|--------------|-----------------|--------------------------|-------------|-------------------|------|
| 22 PA    | 14 PANO                 | 1) Freeze-out (-20 °C, >4 h)   | A: H₂O with 0.1% acetic acid   | Acquity BEH C18 (100 x 2.1 mm, 1.7 μm) | UHPLC-QqQ-MS/MS | n/a        | 0.05 - 0.2       | [1]  |
|          |                         | 2) LLE (methanol with 0.1% formic acid) | B: Acetonitrile |                 |                           |             |                   |      |
|          |                         | 3) Freeze-out (-20 °C, >4 h)   |               |                 |                           |             |                   |      |
|          |                         | 4) Centrifugation (1,950 × g, 4 °C, 10 min) |               |                 |                           |             |                   |      |
|          |                         | 5) Evaporation, reconstitution (H₂O with 0.1% acetic acid) |               |                 |                           |             |                   |      |
|          |                         | 6) Centrifugation (1950 × g, 10 min) and filtration (0.45 μm) |               |                 |                           |             |                   |      |
| 10 PA    | 4 PANO                  | 1) LLE (0.05 mol/L H₂SO₄, vortex mixing, 30 s) | A: H₂O with 0.05% formic acid and acetonitrile (95/5, v/v) | Kinetex PFP (150 x 2.1 mm, 2.6 μm) | (U)HPLC-QqQ-MS/MS | 65 - 125   | 3.5               | [2]  |
|          |                         | 2) Centrifugation (3,421 × g, 10 min) | B: Acetonitrile |                 |                           |             |                   |      |
|          |                         | 3) Volume adjustment in flask |               |                 |                           |             |                   |      |
|          |                         | 4) SPE (SCX, Strata X-C) |               |                 |                           |             |                   |      |
|          |                         | 5) Evaporation, reconstitution (methanol) |               |                 |                           |             |                   |      |
|          |                         |                          |               |                 |                           |             |                   |      |
| 10 PA    | 4 PANO                  | 1) LLE (acetonitrile with 1% acetic acid, vortex mixing, 30 s) | A: H₂O with 0.05% formic acid and acetonitrile (95/5, v/v) | Kinetex PFP (150 x 2.1 mm, 2.6 μm) | (U)HPLC-QqQ-MS/MS | (a) 17 - 73, (b) 18 - 74 | 3.5 | [2]  |
|          |                         | 2) Addition of sodium acetate, anhydrous magnesium sulphate and acetonitrile with 1% acetic acid and shaken (1 min) | B: Acetonitrile |                 |                           |             |                   |      |
|          |                         | 3) Centrifugation (3,421 × g, 10 min) |               |                 |                           |             |                   |      |
|          |                         | 4) Addition of primary and secondary exchange material, carbon and anhydrous magnesium sulphate (dSPE, vortex mixing, 1 min) |               |                 |                           |             |                   |      |
|          |                         | 5) Centrifugation (3,421 × g, 10 min) |               |                 |                           |             |                   |      |
|          |                         | 6) (a) Evaporation, reconstitution (methanol) and syringe filtration (0.22 μm, PTFE) |               |                 |                           |             |                   |      |
|          |                         | (b) Only syringe filtration (0.22 μm, PTFE) |               |                 |                           |             |                   |      |
| 9 PA     | 10                      | 1) LLE (chloroform-methanol, 1:1, v/v, sonication, 40 min) | A: H₂O with 0.1% formic acid | HECTOR-M C18 (150 x 4.6 mm, 3.0 μm) | HPLC-QqQ-MS/MS (ESI+) | 83 - 100   | 0.23 - 1.76       | [3]  |
|          |                         | 2) Filtration, evaporation and reconstitution (methanol) | B: Acetonitrile |                 |                           |             |                   |      |
|          |                         | 3) Freeze-out (-24 °C, 30 min) and filtration |               |                 |                           |             |                   |      |
|          |                         | 4) Reconstitution (methanol) of the precipitated lipids |               |                 |                           |             |                   |      |
|          |                         | 5) Freeze-out (-24 °C, 30 min) and filtration |               |                 |                           |             |                   |      |
|          |                         | 6) Concentration of the extract (rotary evaporation) |               |                 |                           |             |                   |      |
|          |                         | 7) SPE (SCX, Strata X-C) |               |                 |                           |             |                   |      |
|          |                         | 8) Evaporation, reconstitution (methanol) |               |                 |                           |             |                   |      |
**Table S1 (continued)**

| Analytes  | Sample volume (mL or g) | Extraction and clean-up | Mobile phase | Stationary phase | LC-MS/MS instrumentation | Recovery [%] | LOQ [µg/kg or µg/L] | Ref. |
|-----------|-------------------------|-------------------------|--------------|-----------------|--------------------------|--------------|----------------------|------|
| 10 PA 6 PANO | 20 | 1) LLE (37% HCl and hexane, 60 °C, 60 min)  
2) Centrifugation (13,000 × g, 5 min) and syringe filtration | A: H₂O with 5 mmol/L ammonium acetate and 0.05% acetic acid  
B: Acetonitrile | Kinetex XB-C18 (100 x 4.6 mm, 2.6 µm) | UHPLC-QqQ-MS/MS (ESI+) | 89 - 114 | 0.003 - 0.033 | [4] |
| 19 PA 16 PANO | 3 | 1) LLE (0.2% formic acid and hexane, shaking, 30 min)  
2) Centrifugation (3,500 g, 15 min)  
3) Basification (ammonia (25%), pH 9 - 10)  
4) Centrifugation (3,500 × g, 15 min)  
5) SPE (C18, Strata X)  
6) Evaporation, reconstitution (methanol/H₂O, 10/90, v/v) and filtration (0.45 µm, PTFE) | A: H₂O with 6.5 mmol/L ammonium hydroxide  
B: Acetonitrile with 1.2 mmol/L ammonium hydroxide | Acquity BEH C18 (150 x 2.1 mm, 1.7 µm) | UHPLC-Qtrap-MS/MS (ESI+) | 45 - 107 | 0.1 | [5], [6], [7] |
| 15 PA 13 PANO | 2 | 1) LLE (0.05 mol/L H₂SO₄, sonication, 15 min)  
2) Centrifugation (8,500 rpm, RT, 10 min)  
3) Neutralisation (aqueous ammonia, pH 6 - 7)  
4) Freeze-out (-80 °C, >18 h)  
5) Centrifugation (8,500 rpm, 10 °C, 10 min)  
6) Syringe filtration (0.45 µm, RC)  
7) SPE (C18, Supleco DSC-C18, 2-step elution)  
8) Evaporation, reconstitution (H₂O with 0.5 mmol/L ammonium formate and 2 mmol/L formic acid) and syringe filtration (0.2 µm, RC) | A: Acetonitrile/H₂O (80/20, v/v) with 0.5 mmol/L ammonium formate and 2 mmol/L formic acid  
B: H₂O with 0.5 mmol/L ammonium formate and 2 mmol/L formic acid | Aquity CSH C18 (150 x 2.1 mm, 1.7 µm) | UHPLC-Qtrap-MS/MS (ESI+) | 57 - 120 | 0.010 - 0.087 | [8] |
| 6 PA | 5 | 1) Dilution (0.5% formic acid)  
2) Centrifugation (10,000 rpm, 10 min, twice)  
3) pH adjustment to 10 - 11  
4) LLE (dichloromethane, shaking, twice)  
5) Evaporation of combined dichloromethane phase (N₂) and reconstitution (methanol/H₂O, 50/50, v/v) | / | / | DART-IT-MS (positive mode) | 89.3 - 112.1 | 1.83 - 2.82 | [9] |
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### Table S1 (continued)

| Analytes | Sample volume (mL or g) | Extraction and clean-up                                                                 | Mobile phase                                      | Stationary phase | LC-MS/MS instrumentation          | Recovery [%] | LOQ [µg/kg or µg/L] | Ref. |
|----------|-------------------------|-----------------------------------------------------------------------------------------|--------------------------------------------------|------------------|-----------------------------------|--------------|---------------------|------|
| 2 TA     | 2                       | 1) LLE (0.5 mol/L EDTA, acetonitrile with 0.5% trifluoroacetic acid, vortex mixing)   | A: H₂O with 0.1% formic acid and 10 mmol/L ammonium formate | Kinetex EVO C18 | HPLC-QqQ-MS/MS (ESI+)             | 81 - 97      | 2 - 5               | [10] |
| (2 QA)   |                         | 2) Addition of magnesium sulphate, sodium chloride, sodium citrate trihydrate, sodium citrate trihydrate (vortex mixing, 5 min) | B: methanol                                      |                  |                                   |              |                     |      |
|          |                         | 3) Centrifugation (2,600 ×g, RT, 10 min)                                                |                                                  |                  |                                   |              |                     |      |
|          |                         | 4) Addition of C18 sorbent material and magnesium sulfate to supernatant (dSPE, vortex mixing, 5 min) |                                                  |                  |                                   |              |                     |      |
|          |                         | 5) Centrifugation (2,600 ×g, RT, 10 min)                                                |                                                  |                  |                                   |              |                     |      |
|          |                         | 6) Evaporation, reconstitution (methanol), centrifugation (10,840 g) and syringe filtration (0.2 µm) |                                                  |                  |                                   |              |                     |      |
| 2 TA     | 2                       | 1) LLE (methanol with 1% formic acid, vortex mixing, 1 min)                              | A: H₂O with 0.1% formic acid                      | Luna Phenyl-Hexyl | HPLC-Qtrap-MS/MS (ESI+)            | 96 - 99      | 0.075               | [11] |
|          |                         | 2) Centrifugation (4,200 ×g, 4 °C)                                                      | B: Methanol                                      | (150 x 2 mm, 5.0 µm) |                                   |              |                     |      |
|          |                         | 3) Freeze-out (-20 °C, >12 h)                                                           |                                                  |                  |                                   |              |                     |      |
|          |                         | 4) Centrifugation (18,000 ×g, 10 min, -10 °C)                                           |                                                  |                  |                                   |              |                     |      |

Abbreviations: DART direct analysis in real time, dSPE dispersive solid phase extraction, ESI+ electrospray ionization positive mode, IT ion trap, LLE liquid-liquid extraction, LOQ limit of quantification, MS mass spectrometry, MS/MS tandem mass spectrometry, PFP pentafluorophenyl, PTFE polytetrafluorethylen, QA quinolizidine alkaloids, QqQ triple quadrupole, Qtrap quadrupole-linear ion trap mass spectrometer, RC regenerated cellulose, Ref. references, SCX strong cation exchange, SPE solid phase extraction, UHPLC ultra high performance liquid chromatography
### Analytical and Bioanalytical Chemistry

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#### Table S2 Additional information on milk samples analysed for method development, validation and field sample testing

| Sample Name          | Labelling       | Sample Type | Source of Supply     | Bavarian Province of Origin | Production | Sampling Date [dd.mm.yy] | Expiration Date [dd.mm.yy] |
|----------------------|-----------------|-------------|----------------------|----------------------------|------------|--------------------------|----------------------------|
| Blank milk           |                 | Raw milk    | Milk filling station  | Upper Bavaria              | Organic    | 16.06.20                 | -                          |
| Blank milk           |                 | Raw milk    | Milk filling station  | Upper Bavaria              | Organic    | 07.10.20                 | -                          |
| Raw milk 1           |                 | Raw milk    | Milk filling station  | Upper Franconia            | Conventional | 31.03.21            | -                          |
| Raw milk 2           |                 | Raw milk    | Milk filling station  | Upper Franconia            | Conventional | 03.04.21            | -                          |
| Raw milk 3           |                 | Raw milk    | Milk filling station  | Upper Franconia            | Organic    | 03.04.21                 | -                          |
| Raw milk 4           |                 | Raw milk    | Milk filling station  | Upper Franconia            | Conventional | 03.04.21            | -                          |
| Raw milk 5           |                 | Raw milk    | Milk filling station  | Swabia                     | Conventional | 29.04.21            | -                          |
| Pasteurized milk 1   | Hay milk        | Pasteurised milk | Regional marketer | Upper Bavaria              | Organic    | 10.05.21                 | 03.05.21                   |
| Pasteurized milk 2   | Hay milk        | Pasteurised milk | Regional marketer | Upper Bavaria              | Organic    | 10.05.21                 | 01.05.21                   |
| Pasteurized milk 3   | Fresh organic alpine milk | Pasteurised milk | Regional marketer | Upper Bavaria              | Organic    | 10.05.21                 | 04.05.21                   |
| Pasteurized milk 4   | Fresh milk      | Pasteurised milk | Regional marketer | Upper Bavaria              | Organic    | 10.05.21                 | -                          |
| Pasteurized milk 5   | Hay milk        | Pasteurised milk | Regional marketer | Upper Bavaria              | Conventional | 20.07.21            | 24.07.21                   |
| Raw milk 6           |                 | Raw milk    | Milk filling station  | Upper Bavaria              | Conventional | 28.09.21            | -                          |
| Raw milk 7           |                 | Raw milk    | Milk filling station  | Upper Bavaria              | Conventional | 28.09.21            | -                          |
| Raw milk 8           |                 | Raw milk    | Milk filling station  | Upper Bavaria              | Conventional | 27.09.21            | -                          |
| Raw milk 9           |                 | Raw milk    | Milk filling station  | Upper Bavaria              | Conventional | 02.10.21            | -                          |
| Raw milk 10          |                 | Raw milk    | Milk filling station  | Upper Bavaria              | Conventional | 03.10.21            | -                          |
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Fig. S1 Chromatographic separation of a 5 ng/mL standard solution of 56 pyrrolizidine alkaloids and two tropane alkaloids under alkaline (solvent A: 10 mmol/L ammonium carbonate in water, solvent B: acetonitrile, a) and acidic conditions (solvent A: water, solvent B: acetonitrile/water (95/5, v/v.), both containing 5 mmol/L ammonium formate and 26.5 mmol/L formic acid, b) solvent conditions using a 150 x 2.1 mm KinetexTM 5 µm EVO C18 column. For Abbreviations, see Table 1
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Fig. S2 Multiple reaction monitoring (MRM) chromatograms of isomeric pyrrolizidine alkaloids indicine, intermedine, lycopsamine, echniatine and rinderine and their corresponding N-oxides under acidic solvent conditions using a 150 x 2.1 mm KinetexTM 5 µm EVO C18 column (a + b) and alkaline solvent conditions using a 150 x 2.1 mm KinetexTM 5 µm EVO C18 column (c + d) and alkaline solvent conditions using a 100 x 2.1 mm KinetexTM 2.6 µm EVO C18 (e + f). Individual mass transitions are illustrated with different colours.
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Fig. S3 Multiple reaction monitoring (MRM) chromatograms of isomeric pyrrolizidine alkaloids integerrimine, senecionine and senecivernine and their corresponding N-oxides under acidic solvent conditions using a 150 x 2.1 mm KinetexTM 5 µm EVO C18 column (a + b) and alkaline solvent conditions using a 150 x 2.1 mm KinetexTM 5 µm EVO C18 column (c + d) and alkaline solvent conditions using a 100 x 2.1 mm KinetexTM 2.6 µm EVO C18 (e +f). Individual mass transitions are illustrated with different colours.
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Table S3 Recovery, calculated using calibration standards in methanol/water (10/90, v/v), and precision, expressed as the relative standard deviation (RSD), of sample extraction and clean-up procedures using C18 cartridges with n-hexane (n=3) and polymer cation exchange (PCX) cartridges with and without n-hexane (n=4). Replicates were spiked to a concentration of 12.3 ng/mL in the final measuring solution.

For Abbreviations see Tab. 1

| Analyte | PCX material + n-hexane | PCX material - n-hexane | C18 material + n-hexane |
|---------|-------------------------|-------------------------|-------------------------|
|         | Recovery [%] | RSD [%] | Recovery [%] | RSD [%] | Recovery [%] | RSD [%] |
| AcIm    | 66.6        | 3.7     | 67.6        | 0.9     | 53.3        | 6.4     |
| AcImN   | 0.5         | 11.8    | 0.6         | 25.1    | 55.0        | 7.3     |
| AcLy    | 64.8        | 3.6     | 65.3        | 2.0     | 53.6        | 5.2     |
| AcLyN   | 0.6         | 5.3     | 0.7         | 16.6    | 99.7        | 6.9     |
| At      | 66.6        | 1.9     | 69.3        | 1.6     | 83.6        | 5.1     |
| Ec      | 61.2        | 1.8     | 57.9        | 1.6     | 87.0        | 4.8     |
| EcN     | 64.8        | 2.5     | 58.5        | 4.1     | 63.8        | 3.0     |
| Em      | 68.8        | 4.6     | 71.9        | 0.6     | 76.2        | 15.3    |
| EmN     | 72.0        | 3.1     | 70.7        | 1.3     | 72.7        | 5.7     |
| En      | 69.9        | 0.9     | 65.1        | 2.1     | 73.2        | 6.9     |
| EnN     | 70.7        | 3.1     | 65.4        | 3.6     | 58.4        | 4.3     |
| Eu      | 68.9        | 1.8     | 65.6        | 2.3     | 42.4        | 2.7     |
| EuN     | 62.1        | 3.7     | 62.8        | 6.5     | 78.6        | 6.3     |
| Hs      | 74.3        | 2.1     | 78.5        | 1.1     | 7.4         | 6.1     |
| HsN     | 74.4        | 2.7     | 73.9        | 1.7     | 46.9        | 3.7     |
| Ht      | 58.9        | 3.0     | 59.3        | 3.1     | 72.4        | 1.8     |
| HtN     | 76.7        | 2.2     | 74.5        | 3.5     | 63.4        | 3.0     |
| Ic      | 73.7        | 3.2     | 67.0        | 1.9     | 52.7        | 4.5     |
| IcN     | 92.0        | 2.6     | 88.8        | 2.8     | 68.5        | 5.2     |
| Ig      | 65.4        | 1.9     | 66.2        | 1.6     | 63.7        | 11.9    |
| IgN     | 70.1        | 2.2     | 64.0        | 1.1     | 61.3        | 4.1     |
| Im      | 74.4        | 2.5     | 67.8        | 1.5     | 53.2        | 4.4     |
| ImN     | 107.6       | 2.7     | 106.0       | 2.7     | 60.6        | 4.3     |
| Jb      | 71.4        | 2.0     | 69.3        | 2.1     | 52.5        | 10.3    |
| JbN     | 70.5        | 3.6     | 62.7        | 3.0     | 30.9        | 4.0     |
| JI      | 60.9        | 5.0     | 55.7        | 2.7     | 31.0        | 2.8     |
| JIN     | 58.9        | 4.5     | 52.6        | 5.7     | 62.9        | 3.1     |
| Jn      | 43.6        | 2.9     | 44.0        | 3.7     | 64.8        | 18.1    |
| Lc      | 8.5         | 4.5     | 8.8         | 5.5     | 50.8        | 4.6     |
| LcN     | 73.1        | 1.6     | 74.8        | 1.1     | 61.4        | 5.3     |
| Ly      | 75.3        | 3.1     | 68.8        | 1.5     | 61.0        | 5.9     |
| LyN     | 87.8        | 2.6     | 85.4        | 1.8     | 62.5        | 4.3     |
| Mc      | 66.7        | 2.6     | 58.2        | 2.8     | 51.9        | 2.7     |
| McN     | 57.5        | 5.7     | 51.7        | 5.0     | 69.2        | 1.5     |
| Mk      | 49.2        | 2.2     | 50.6        | 2.6     | 67.2        | 9.5     |
| MkN     | 7.6         | 7.4     | 8.7         | 29.6    | 48.9        | 62.4    |
| Mx      | 52.9        | 3.0     | 48.9        | 1.9     | 53.8        | 7.0     |
| MxN     | 126.8       | 2.3     | 106.1       | 3.5     | 40.5        | 4.1     |
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Table S3 (continued)

| Analyte | PCX material + n-hexane | PCX material - n-hexane | C18 material + n-hexane |
|---------|-------------------------|-------------------------|-------------------------|
|         | Recovery [%] | RSD [%] | Recovery [%] | RSD [%] | Recovery [%] | RSD [%] |
| Rl      | 41.3       | 2.2     | 41.4       | 2.1     | 63.1       | 6.2     |
| RlN     | 21.5       | 1.1     | 19.6       | 8.8     | 39.8       | 2.4     |
| Rr      | 73.1       | 2.8     | 67.2       | 1.5     | 52.5       | 6.0     |
| RrN     | 72.9       | 2.3     | 67.1       | 3.5     | 69.1       | 3.2     |
| Rs      | 57.3       | 1.4     | 56.9       | 1.6     | 48.5       | 5.2     |
| RsN     | 68.6       | 4.1     | 59.1       | 3.1     | 49.8       | 1.1     |
| Sc      | 63.6       | 3.7     | 65.6       | 1.8     | 25.6       | 9.4     |
| ScN     | 59.9       | 3.7     | 64.2       | 3.5     | 58.2       | 1.4     |
| Sco     | 62.9       | 2.3     | 64.7       | 0.7     | 53.6       | 7.5     |
| Sc      | 58.0       | 3.2     | 60.2       | 0.9     | 32.2       | 3.0     |
| Sk      | 73.0       | 2.4     | 68.3       | 0.6     | 45.1       | 5.5     |
| Sl      | 39.4       | 1.6     | 36.3       | 3.9     | 38.4       | 7.0     |
| SlN     | 60.4       | 6.8     | 52.6       | 4.5     | 37.5       | 3.2     |
| Sp      | 58.0       | 3.1     | 58.8       | 1.1     | 29.3       | 5.7     |
| SpN     | 32.3       | 4.8     | 32.3       | 4.4     | 16.5       | 8.0     |
| Sv      | 60.2       | 5.9     | 63.3       | 1.1     | 32.2       | 4.3     |
| SvN     | 69.1       | 4.1     | 64.2       | 1.6     | 12.4       | 7.4     |
| Td      | 59.9       | 2.9     | 59.9       | 2.8     | 17.5       | 5.6     |
| Us      | 59.0       | 1.6     | 59.9       | 0.5     | 44.6       | 4.7     |
| UsN     | 73.7       | 4.3     | 64.4       | 2.7     | 50.7       | 6.5     |
| Minimum | 0.5        | 0.9     | 0.6        | 0.5     | 7.4        | 1.1     |
| Mean    | 61.7       | 3.3     | 59.3       | 3.7     | 53.0       | 6.5     |
| Median  | 65.1       | 2.9     | 64.1       | 2.2     | 53.4       | 5.2     |
| Maximum | 126.8      | 11.8    | 106.1      | 29.6    | 99.7       | 62.4    |
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Fig. S4 Chromatograms of retrosine quantifier (a) and qualifier (b) mass transitions obtained from raw milk sample 2 (1. Replicate)
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Fig. S5 Chromatograms of lycopsamine quantifier (a) and qualifier (b) mass transitions obtained from raw milk sample 9 (1. Replicate)
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Fig. S6 Chromatograms of erucifoline quantifier (a) and qualifier (b) mass transitions obtained from pasteurised milk sample 3 (1. Replicate)
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Fig. S7 Chromatograms of lycopsamine quantifier (a) and qualifier (b) mass transitions obtained from pasteurised milk sample 3 (1. Replicate)
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Fig. S8 Chromatograms of senkirkine quantifier (a) and qualifier (b) mass transitions obtained from pasteurised milk sample 3 (1. Replicate)
Table S4 Results of the confirmation analysis. Calculated content in each spiked replicate and theoretical spiked pyrrolizidine alkaloid amount.

| Analyte    | Calculated Concentration [µg/L] | Spiked Amount [µg/L] |
|------------|---------------------------------|----------------------|
|            | Replicate 1 | Replicate 2 | Replicate 3 |
| Eruçifoline| 0.010       | 0.008<sup>a</sup> | 0.011       | 0.010       |
| Lycopsamine| 0.017       | 0.016       | 0.016       | 0.015       |
| Retrorsine | 0.015       | n. d.       | 0.017       | 0.020       |
| Senkirkine | 0.014       | 0.018       | 0.019       | 0.020       |

n.d., not detected
<sup>a</sup> calculated value below the LOD
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References

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