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- electrospray mass spectrometric (HPLC/ESI-MS)
quantification**

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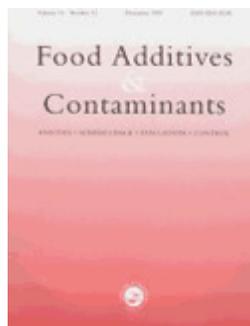
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Supercritical fluid extraction of 4(5)-methylimidazole (4MeI) and 2-acetyl-4(5)-(1,2,3,4)tetrahydrohydroxybutylimidazole (THI) from grounded coffee with liquid chromatographic - electrospray mass spectrometric (HPLC/ESI-MS) quantification

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2 1 **Supercritical fluid extraction (SFE) of 4(5)-methylimidazole (4MeI) and 2-acetyl-4(5)-**
3 **(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) from ground-coffee with liquid**
4 2 **chromatographic-electrospray mass spectrometric quantification (HPLC/ESI-MS)**
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7 4

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19 15
20 16

21 17 **Abstract**

22 18 Two polar analytes, 4(5)-methylimidazole (4MeI) and 2-acetyl-4(5)-(1,2,3,4-
23 19 tetrahydroxybutyl)-imidazole (THI) were extracted with supercritical CO₂ modified with
24 20 aqueous methanol. The method was applied to a roasted coffee powder with good recoveries.
25 21 Method efficiency was compared with that of solid phase extraction using SCX Disc
26 22 cartridges and validated for spiked solid matrix. The analytes were determined using isocratic
27 23 liquid chromatography – mass spectrometry (LC/MS) on an Atlantis HILIC Silica column
28 24 (150 x 2.1 mm, 3 μm) with 80 % methanol and 20 % 0.01 mol.l⁻¹ ammonium formate as
29 25 mobile phase. The limit of quantification was around 1.5 pg for 4MeI and 2.0 pg for THI. The
30 26 linearity of the calibration curves was satisfactory as indicated by correlation coefficients of >
31 27 0.999. The coefficient of variation for the intra-day and the inter-day precision was < 4% (n =
32 28 6). The accuracy was in the range 98 % - 101% and recovery was ≥ 98% and ≥ 99% for THI
33 29 and 4MeI, respectively. Several samples of Arabica coffee from various locations and
34 30 commercially available “shelf” coffee products (Arabica/Robusta mixtures) were analyzed to
35 31 test the method.
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33 **Keywords:** supercritical fluid extraction, solid phase extraction, 4(5)-methylimidazole, 2-
46 acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole, 4MeI, THI, LC/MS, roasted coffee
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35 Introduction

36 Coffee is well known as a stimulant of a central nervous system and as a diuretic. Its
37 world production is about 4 millions tons per annum. Small, evergreen woods of *Coffea*
38 *arabica*, *C. liberica* and *C. canephora* (*Rubiaceae*) are planted in many varieties in tropic
39 areas all over the world. They bear coffee seeds (*Semen Coffeae*) containing 0.3 – 2.5 % of
40 caffeine and traces of theophylline – important natural drugs - purine derivatives originated
41 from xanthine methylation (Tomko 1999, Reeve et al. 1993). Ripe coffee seeds are rid of the
42 pulp, endocarp and seed coat in so-called wet or dry way. Finally the selected grains are
43 roasted at 200 – 250 °C to get typical coffee aroma and taste.

44 During the roasting process, undesirable imidazole compounds – a neurotoxic 4(5)-
45 methylimidazole (4MeI) and an immunosuppressive 2-acetyl-4(5)-(1,2,3,4-
46 tetrahydroxybutyl)-imidazole (THI) – are formed by heating saccharides with nitrogenous
47 compounds. THI has a lymphopenic effect and impairs immunity of rats and mice by
48 modifying splenocyte function, in a histamine-like receptor site (Vávrová et al. 2003). THI
49 also restrains contact hypersensitivity. Mice fed with THI have less CD4+ and CD8+ T cells –
50 THI probably inhibits a contact hypersensitivity response by preventing the recruitment of
51 CD4+ T cells in the draining lymph nodes (Gugasyan et al. 1995).

52 It is impossible to avoid completely the THI and 4MeI formation during the coffee
53 roasting and other food industry processes, but the concentration of both compounds should
54 be kept as low as possible in foods and beverages. For example, the European Commission
55 specified the legal limit of 10 and 200 mg.kg⁻¹ for THI and 4MeI, respectively, for Class III
56 caramel colour.

57 Several procedures have been published for separate determination of either 4MeI or
58 THI in 1986 – 95 period. They have been based on LC/UV and LC/MS (Thomsen and
59 Willumsen 1995, Moravcová et al. 1992, Müller et al. 1998, Wagner et al. 1993, Lawrence
60 and Charbonneau 1987, Ding et al. 1991), capillary electrophoresis (Ong et al. 1994), and GC
61 with derivatisation (Kröplien 1986, Fernandes and Ferreira 1997). Quantification of 4(5)-
62 methylimidazole in coffee was performed via ion-pair extraction with bis-2-
63 ethylhexylphosphate (BEHP), isobutylchloroformate derivatisation and GC/MS (Casal et al.
64 2002). These methods are sensitive and precise, but limited to one or the other of the two
65 compounds. The first successful attempt to separate and determine 4MeI and THI in a single
66 run via LC/M) was published in 2003 (Klejdus et al. 2003). The same research group also
67 investigated solid phase extraction as an instant tool for simultaneous separation of both

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2 68 analytes from liquid matrices (Klejdus et al. 2006). Both analytes can be separated and
3
4 69 determined in a short time using a single-run analysis.

5 70 Solid matrices present another challenge in THI/4MeI separation. Roasted coffee can
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7 71 be, of course, turned to liquid “coffee drink” matrix. A set of spike experiments showed, that
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9 72 although the recovery of spiked analytes from liquid solutions is quantitative, spiking the
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11 73 standards to roasted coffee beans and subsequent treatment with hot water brought less
12
13 74 favourable results. SPE is able to extract all analytes from the liquid phase, but the hot water
14
15 75 soaking seemingly does not remove all spiked analytes from solid coffee matrix (Klejdus et
16
17 76 al. 2006).

18
19 77 Thus, supercritical fluid extraction was selected as a method for THI and 4MeI
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21 78 isolation from powdered solid coffee matrix. Due to low viscosity and high diffusivity of
22
23 79 supercritical fluid, analytes can be extracted from the matrix most effectively. Apart from
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25 80 that, analytes are carried out of the system during the dynamic extraction. The balance in the
26
27 81 extraction cell shifts and it causes faster analyte removal from the matrix. Extraction
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29 82 efficiency and precision was validated in a series of spiked sample experiments. Reminders
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31 83 of coffee samples after supercritical fluid extraction were soaked in hot water and extracted
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33 84 with SPE to verify SFE efficiency. Also, several different varieties and consumer coffee
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35 85 mixtures were tested to exclude possible matrix effect.

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37 86 Together with new isolation method, a new LC/MS method was developed for the
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39 87 simultaneous determination of THI and 4MeI. Method used in previous experiments (Klejdus
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41 88 et al. 2003, 2006) works well. But it is quite costly, as the pH of analyses is a limiting pH
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43 89 value of the MetaChem Polaris C-18A column applicability. The column is exposed to a
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45 90 series of serious stresses and after a limited number of analyses, separation performance is
46
47 91 decreased: retention times are shifted and peak width broadened. Because of that, brand new
48
49 92 method for AtlantisTM HILIC Silica column was developed for a mildly acidic mobile phase
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51 93 in pH range 5 - 6, with excellent precision and reproducibility.

52
53 94 A hydrophilic interaction chromatography (HILIC) is a variant of a normal phase
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55 95 chromatography, where polar stationary phase is used with a mobile phase, which contains
56
57 96 high concentration of organic solvent and low concentration of aqueous solvent. In HILIC,
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59 97 organic portion of mobile phase is a weak solvent, the aqueous portion is a strong solvent and
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61 98 the compound elution is in the order of increasing hydrophobicity. HILIC is also referred to as
62
63 99 “aqueous normal phase” or “reverse reversed phase”, since the elution order is similar to that
64
65 100 of normal phase and used solvents are similar to those of reversed phase chromatography.

1
2 101 HILIC columns retain highly polar analytes with volatile mobile phases that are ideal for
3 102 compound ionization by ESI – MS. This leads to good sensitivity and low limits of detection.
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6 104 **Materials and methods**

8 105 *Chemicals*

10 107 4(5)-methylimidazole (4MeI) was purchased from Sigma-Aldrich (St Louis, USA). The
11 108 standard of 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) was obtained from Dr.
12 109 D. V. Myers (Coca Cola Co., USA). HPLC grade methanol and ammonium formate were
13 110 from Merck (Darmstadt, Germany). Standards were prepared by dissolution in reverse-
14 111 osmosis Milli-Q water (Millipore, Bedford, USA) at a stock concentration of 10 µg.ml⁻¹. The
15 112 solutions were stable for at least one week when stored in dark at 4 °C. All solutions were
16 113 filtered through a 0.45 µm Teflon membrane filters (MetaChem, Torrance, USA) prior to
17 114 HPLC analysis. All other chemicals were of analytical grade.
18 115

23 116 *Samples and sample preparation*

25 117 The samples of coffee varieties were obtained from the Oxalis (import comp., Zlín, Czech
26 118 Republic). The „Brazilia-Santos“ was the well-known 100 % brazil Arabica. The „Haiti 5X“
27 119 contained extra large coffee beans from the Haiti region Pilate. The „Kenya“ originated from
28 120 the Mount Kenya plantations with altitude over 2000 m. The „Mexico Altura“ was a high
29 121 mountain Arabica harvested at mexican coffee plantations in altitude 1000 – 1600 m. The
30 122 „Boncampo“ was obtained from Migros-Genossenschafts-Bund (Zürich, Switzerland). It was
31 123 a commercially available coffee mixture containing Arabica from Central and South America
32 124 and also from India/Indonesia, and Robusta from Africa and Asia – the most complex mixture
33 125 available at the market. Except the Boncampo mixture (Arabica/Robusta), all samples were
34 126 pure Arabica. All samples were used as the whole beans and ground in an analytical grinder
35 127 IKA Yellow Line A10 (IKA Works Inc., Wilmington, USA) to assure similar size of particles
36 128 in all samples.

42 129 In supercritical fluid extraction, sample amount was 1 g. For the solid-phase extraction
43 130 (Klejduš et al 2006), 5 g of coffee was extracted with hot water (90 ml) at 90 °C for 5 min.
44 131 The mixture was then cooled and solids were filtered off. The clear filtrate was transferred
45 132 into a 100 ml volumetric flask and adjusted with water up to the mark. An aliquot (1000 µl)
46 133 was diluted with water (3000 µl) and extracted.
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134 *Supercritical fluid extraction*

135 The standard solutions of THI and MeI were prepared by mixing 1 mg of each compound in
136 100 ml of ultra-pure water. A given volume of THI/4MeI solution was injected into the
137 middle of a matrix bed (glass wool). As for real samples, 1.00 g of roasted coffee powder was
138 weighted into the extraction cell. The coffee samples spiked with the THI/4MeI standard
139 solutions were left to equilibrate for at least 10 min (5 days usually) at laboratory conditions.
140 The procedure avoided immediate dissolution of freshly spiked analytes in too soon added *in-*
141 *situ* modifier. An attempt was made to use higher amounts of *in-situ* modifier; but at higher
142 temperatures the solvent caused some pressure in the extraction cell, leaved through the
143 restrictor and caused slow extraction of ballast compounds, impeding the restrictor plugging.
144 When 200 μ l of the *in-situ* modifier was added to the 0.7 ml cartridge, it was immediately
145 sealed and closed in the extraction cell of a heating block to avoid evaporation of the *in-situ*
146 modifier.

147 The extraction cells were cleaned in ultra-pure H₂O in sonication bath and rinsed with
148 MeOH. Inner space of extraction cell of the heating block was also washed with water and
149 methanol, since not all experiments had quantitative recovery during the extraction conditions
150 optimisation. This eliminated or minimised cross-contamination dragged by the extraction
151 phase modifier during de-pressurization after the extraction step. Blank samples were
152 prepared by extracting the same amounts of the matrix. Neither the analytes were found in
153 blank matrices, nor THI/4MeI residues were found in the instrument after the extraction cell
154 heating block cleaning.

155 All extractions were carried out using an SE-I instrument (SEKO-K, Ltd., Brno, Czech
156 Republic) equipped with a valve for continuous modifier addition (VALCO, Switzerland,
157 loop 500 μ l) according to the method described by Ventura (Adam et al. 2000, Eisner et al.
158 2002). The extraction cell was pressurized at 50 to 150 °C up to 10 - 40 MPa using SFC/SFE
159 grade CO₂ (Siad, Brno, Czech Republic) with 10 % v/v of modifier mixture added via
160 VALCO valve to the instrument piston pump.

161 The modified supercritical fluid went through the extraction cartridge filled with the
162 sample for 45 min (dynamic extraction). The cell was de-pressurized through a fused silica
163 restrictor (12 cm, 50 μ m i.d., flow rate of 750 - 850 ml.min⁻¹, Supelco) into a liquid trap - a
164 glass vial containing 30 ml trapping solvent at the laboratory temperature. Much smaller
165 amount of the trapping solvent would be sufficient for the quantitative analyte trapping; but
166 due to 800 ml/min flow rate a 100 ml flask with a flexible foil seal was used as a trapping vial

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2 167 to prevent the solvent splashing out of a vial. Thus a larger volume was needed to achieve
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4 168 sufficient solvent column height.

5 169 Prior to the analyses, the extracts were evaporated to dryness in a rotary vacuum
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7 170 evaporator IKA RV 05-ST with a water bath HB 4 (all from IKA – Werke GmbH and Co.,
8 171 Staufen, KG, Germany), dissolved in 500 μl of ultra-pure water and injected directly into the
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10 172 HPLC/MS system (spiked samples) or filtered through the 0.45 μm membrane filter
11 173 (MetaChem) before the injection (real samples, spiked real samples) to protect the HPLC
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13 174 system.

14 175 15 176 *Solid-phase extraction*

16 177 Ansys SPEC SCX Disc 15mg/3ml (Ansys Technologies, Torrance, USA) SPE cartridges,
17 178 tried-and-true in previous analyses (Klejduš et al. 2003), were used for all solid-phase
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19 179 extractions. The cartridges were conditioned with 500 μl methanol and 500 μl water. Usually
20 180 3 ml samples with 20 μl 0.1 mol.l^{-1} HCl passed through the cartridge. Impurities were washed
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22 181 out with 1000 μl methanol. Retained imidazoles were eluted from discs with 2 ml of methanol
23 182 acidified with 5 mol.l^{-1} HCl v/v (3:1) and extracts were evaporated to dryness in the rotary
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25 183 vacuum evaporator at 39 °C. The residues were dissolved in 500 μl of Milli Q water and 10 μl
26 184 volumes were injected directly onto the reverse phase chromatographic column.

27 185 28 186 *High-performance liquid chromatography*

29 187 The HPLC chromatographic system HP 1100 (Hewlett Packard, Waldbronn, Germany)
30 188 equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler
31 189 (G1313A), a column thermostat (G1316A) and a diode array detector (model G1315A) was
32 190 coupled on-line to a mass selective HP MSD detector (G 1946A, Hewlett Packard, Palo Alto,
33 191 USA). The ChemStation software (Rev. A 07.01) controlled the whole liquid
34 192 chromatographic system. A hydrophilic interaction chromatographic column Atlantis HILIC
35 193 Silica (150 x 2.1 mm, 3 μm) was employed. The mobile phase was methanol (80 %) and 0.01
36 194 mol.l^{-1} ammonium formate in Milli-Q water (20 %). Isocratic mode was used for the
37 195 separation. The flow rate was 0.2 mL.min^{-1} and the temperature of the column oven was set at
38 196 25 °C. The column effluent was monitored with the diode array detector at 290 nm for THI
39 197 and at 215 nm for 4MeI.

40 198 41 199 *Mass spectrometry*

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2 200 The eluent from the liquid chromatograph was directly introduced into the quadruple mass
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4 201 spectrometer operated in the positive ESI mode. The nebulizer gas pressure was 50 psi, the
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6 202 drying gas was nitrogen at the 12 L.min⁻¹ and at the temperature of 350 °C. The capillary
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8 203 voltage was 1 500 V. The gain was 1. The spectra were recorded from m/z 50 to 250 for full
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10 204 scan mode. All experiments were performed at the fragmentor voltage 35 eV for molecular
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12 205 ions. For qualitative analysis, ion ratio experiments were performed for standard solutions and
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14 206 real samples at the fragmentor voltage 65 eV for parent ions and m/z 231/213, m/z 231/195
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16 207 and 231/153 for THI and at the fragmentor voltage 100 eV for the parent ion and m/z 83/56
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18 208 for 4MeI. The dwell time for each analyte was 199 ms. The mass spectrometer was calibrated
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20 209 with an ESI tuning solution obtained from Hewlett Packard (Palo Alto, CA, USA).

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22 210 A sequence 231 – 213 – 195 appears in the mass spectrum of both THI tautomers that
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24 211 corresponds to stepwise liberation of a single molecule of water from the side chain. The
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26 212 fragment 195 liberates ketene from the double bond of the acetyl group forming an ion 153 as
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28 213 can be seen in both spectra. The sequence is the same for both isomers. In addition the
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30 214 tautomer liberates another water molecule from the fragment 195 forming an ion 176.
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32 215 Because of the water liberated preferentially through six-member cyclic intermediate, it looks
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34 216 like tautomer depicted in Figure 1 (right side).
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37 218 *Accuracy, precision and recovery*

38 219 Accuracy, precision and recovery of the determination of imidazole analytes were evaluated
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40 220 with the powdered coffee beans spiked with THI/4MeI standards (concentrations varying
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42 221 from 0.5 µg.g⁻¹ to 3 µg.g⁻¹). Coefficients of variation % C.V. of intra-day assay were
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44 222 performed in 6 spiked extracts. Inter-day precision was determined by analysing six spiked
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46 223 extracts during a 6-day period. Analyte concentrations were calculated from calibration
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48 224 curves. Accuracy (in %) was evaluated by comparing the estimated concentration with the
49
50 225 known concentrations of the individual imidazoles.
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52 226

53 227 **Results and discussion**

54 228 *High-performance liquid chromatography – mass spectrometry*

55 229 A new LC/ESI/MS analytical method, using Atlantis HILIC Silica column and mildly acidic
56
57 230 medium (pH 5 – 6) was developed for unambiguous identification and determination of THI
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59 231 and 4MeI. Inherent pH and thermal instability of silica-based column, like MetaChem Polaris
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232 C-18A used in previous paper (Klejdus et al. 2003) significantly limits the range of
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2 234 chromatographic conditions, under which such columns can be used, especially under
3 235 reversed phase conditions. In the THI/4MeI analyses, the column was operated at edge
4 236 conditions of pH applicability that decreased its lifetime and led to retention times shift and
5 237 peak width broadening.

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7
8 238 The optimisation was carried out by flow injection analysis (FIA) injecting several
9 239 solutions containing the individual compounds at the concentration of $1 \text{ ng} \cdot \mu\text{l}^{-1}$. The analytes
10 240 produced protonated molecules $[\text{M}+\text{H}]^+$ at m/z 231 for THI and at m/z 83 for 4MeI. The
11 241 tandem mass spectrometry was therefore used in order to obtain additional structural
12 242 information by detecting diagnostic product-ions obtained by in-source collision-induced
13 243 dissociation (CID) of the precursor ion. Three qualifying fragment ions (m/z 231/213, m/z
14 244 231/195 and m/z 231/153) for THI and one qualifying fragment ion (m/z 83/56) for 4MeI
15 245 were used, according to the selected-ion monitoring (SIM) technique.
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21 247 *Validation of the LC/ESI-MS method*

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23 248 Several experimental parameters such as limit of detection (LOD), limit of quantification
24 249 (LOQ), linearity, recovery, stability and repeatability were evaluated for validation of the
25 250 HPLC/ESI-MS method. The separated HPLC zones were detected by the selected ion-
26 251 monitoring (SIM) mode (m/z 231/213, m/z 231/195 and m/z 231/153 for THI and m/z 83/56).
27 252 The limit of detection (LOD) was estimated to be around 1.5 pg for 4MeI and at 2.0 pg for
28 253 THI with calculated $S/N = 3$ (see Table 1), while the limit of quantification (LOQ) was 4.4 pg
29 254 for 4MeI and 6.3 ng ml^{-1} for THI, respectively ($S/N = 10$). Molecular protonated ions were
30 255 used for LOD and LOQ determinations. The calibration curves were linear in the
31 256 concentration ranges 1 - 70 ng ml^{-1} for 4MeI and 1 - 60 ng ml^{-1} for THI with correlation
32 257 coefficient $r > 0.9993$ for 4MeI and $r > 0.9996$ for THI, respectively. Very good results were
33 258 obtained for run-to-run repeatability of retention times, peak areas and concentrations of 4MeI
34 259 and THI (see Table 1). The data shown in Table 2 were achieved by injecting the standard
35 260 solution of 4MeI and THI to the column in six replicates, in order to test the intra-day
36 261 variation.
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43 262 The inter-day variation of the method was assessed by analysing of the standard
44 263 mixtures containing 1 and 3 $\mu\text{g} \cdot \text{g}^{-1}$ of both 4MeI and THI injected independently during 6
45 264 days in six replicates. The inter-day accuracy for the standard solution at the concentration 1
46 265 $\mu\text{g} \cdot \text{g}^{-1}$ was found to be 101.5 and 100.6 %, for 4MeI and THI, respectively, whereas that of
47 266 the standard solution at the concentration of 3 $\mu\text{g} \cdot \text{g}^{-1}$ was found to be 99.1 and 100.2 %,
48 267 respectively. The precision of the inter-day analysis, expressed as RSD%, was calculated as
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1
2 268 1.66 and 0.60 % for 4MeI and THI, respectively, in the 1 $\mu\text{g}\cdot\text{g}^{-1}$ standard solutions, and 1.57
3 269 and 0.7 %, respectively, in the 3 $\mu\text{g}\cdot\text{g}^{-1}$ standard solutions (see Table 2).

4
5 270 The recovery of the method was verified analysing roasted coffee beans samples after
6 271 addition of two known amounts of 4MeI and THI standard solution and subjected to the
7 272 previously described SPE extraction after equilibration (ageing for 5 days). The theoretical
8 273 concentrations of the spiking 4MeI and THI standards were 1 and 3 $\mu\text{g}\cdot\text{g}^{-1}$. The mean
9 274 recovery and the standard deviations for six replicates are given in Table 3.

10 275

11 276 ***Supercritical fluid extraction***

12 277 *Optimisation of extraction conditions for imidazoles*

13
14 278 Both analytes, THI and 4MeI, are quite polar compounds for an SFE context (see Fig. 2).
15 279 Quantitative extraction of polar molecules requires long extraction time or very high flow-rate
16 280 to ensure sufficient mass transfer. Also polarity of a supercritical fluid must be shifted by
17 281 means of a modifier addition, because pure CO_2 has solvation power close to hexane and is an
18 282 excellent solvent for non-polar compounds.

19 283 Several sets of spiked sample experiments were performed to find optimal extraction
20 284 conditions for both analytes. An instrument adaptation for high CO_2 flow-rate, developed for
21 285 isoflavone extraction (Klejdus et al. 2003) was used, providing the gaseous phase flow-rate of
22 286 c. 750 – 850 $\text{ml}\cdot\text{min}^{-1}$ and using liquid trapping into foil-sealed 100 ml vial with 30 ml of
23 287 trapping solvent. Trapping efficiency and expected stripping effect was further tested.
24 288 Fortunately, non-volatile analytes are trapped quantitatively and stripping effect was not
25 289 observed in the first 45 min of any extraction. RSDs of all spiked analyte experiment were in
26 290 the range 1,2 % – 4,7 %.

27 291 The best extraction conditions were examined in several sets of experiments. First,
28 292 recovery vs. extraction time was examined at 40 MPa and 150 °C (see Figure 3). As can be
29 293 seen the 55 % of THI and 74 % of 4MeI were extracted in the first 10 min and the 30 min
30 294 extraction was nearly quantitative (98.4 % and 98.1 % for THI and 4MeI, respectively).
31 295 Recoveries increased slightly up to 98.9 % and 99.8 % for THI and 4MeI in the 45th min,
32 296 respectively (see Figure 3). Fractions were taken also for 60, 75 and 90 min, but they did not
33 297 bring any measurable improvement of the results.

34 298 Study of extraction kinetics was repeated with a real sample of roasted coffee powder
35 299 due to expectation of matrix effects. Courses of extraction kinetic curves were similar. In fact
36 300 the real sample extractions were faster a little bit, partly due to the lower analyte content in
37 301 real samples and, mostly, thanks to better-tuned modifier composition.

1
2 302 With the optimal extraction time selected, extraction temperature and pressure were
3 303 optimised (see Figure 3). Series of extraction was performed at 40 MPa for different
4 304 temperatures and at 150 °C for different pressures. As for THI, the higher the temperature is,
5 305 the better (150 °C is an instrument limiting value, higher temperatures were not examined).
6 306 The decrease of extraction efficiency was observed for 4MeI at 75 and 100 °C. The highest
7 307 recovery was also obtained at 150 °C in this case. Recoveries vary from 86 % to 99 % for
8 308 THI and from 69 % to 89 % at 150 °C, according to extraction pressure applied (with
9 309 modifier composition best suitable for both analytes, see Figure 3). The best recovery values
10 310 were obtained at 40 MPa in both cases.

11 311 Ultra-pure water, methanol, ethanol, acetonitrile and their combinations were tested in
12 312 preliminary experiments to find the best modifier composition (2, 5 and 10 % (v/v) of each
13 313 were added on-line). An aqueous methanol with 10 % (v/v) of water gave the best results thus
14 314 the concentrations close to that amount were examined more carefully (see Figure 3). The
15 315 proper modifier composition for THI was aqueous methanol with MeOH/H₂O 5:1 (v/v) ratio
16 316 or with higher percentage of water (see Figure 3). Lower percentage of water in aqueous
17 317 methanol, i.e. MeOH/H₂O 9:1 (v/v), is favourable for 4MeI. The recovery of the extraction
18 318 decreased with the higher amount of water. However, the modifier composition MeOH/H₂O
19 319 5:1 (v/v) provided recoveries 101.3 % and 89.4 % for THI and 4MeI, respectively. These
20 320 values could be considered sufficient for their simultaneous extraction (quantitative recoveries
21 321 of polar analytes are difficult to achieve with SFE and nearly 90 % recovery at conditions
22 322 optimal for another compound seems to be quite satisfactory). THI is also extractable quite
23 323 well (with recovery 86.9 %) under the conditions optimised for the 4MeI quantitative
24 324 extraction (MeOH/H₂O 9:1 (v/v), recovery of 4MeI 98.9 %). The optimised composition
25 325 MeOH/H₂O 5:1 (v/v) was selected for the simultaneous extractions of THI and 4MeI for
26 326 further experiments due to its higher total recoveries. In experiments, where 4MeI is the more
27 327 important analyte, 9:1 (v/v) concentration will be more useful.

28 328 Another set of extractions was performed to find out the best volumes of on-line
29 329 modifier. The initial amount of the modifier added to the extraction cartridge was 200 µl. The
30 330 higher volumes caused troubles during the extraction cell heating and leaved the cell trough
31 331 the restrictor together with some ballast compounds. The concentration of a modifier in the
32 332 extraction fluid depends on the amount of a liquid modifier added to the extraction phase
33 333 during every filling of the extractor pump. Up to 1 ml of modifier can be added through the
34 334 Valco valve loop. That is c. 10 % of the piston micro-pump volume. The modifier percentage
35 335 is viable only for some substances and their combinations. Ultra-pure water, for example

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2 336 tends to tarry in the piston micro pump. It is accumulated there and decreases piston micro
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4 337 pump inner space. To avoid this, the modifier addition must be skipped for each n^{th} pump
5 338 filling (according to the modifier type and its volume). Thus the maximum concentration of a
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7 339 modifier in an extraction fluid is lower. However, the modifier concentration MeOH/H₂O 5:1
8 340 (v/v) can be added in 10 % (v/v) amount without any troubles and it is also best composition
9 341 for 4MeI (see Figure 3). For THI, 5 % (v/v) is sufficient and any higher concentration is
10 342 suitable.

12 343 Last but not least was the determination of a suitable trapping solvent (see Figure 3).
14 344 Methanol is not good for THI trapping (recovery 85.6 %); acetonitrile is an excellent solvent
15 345 for both analytes (99.4 % and 98.2 %, for THI and 4MeI, respectively). Ultra-pure water is
17 346 very good solvent for 4MeI (100.2 %), but poor for THI (38.7 %).

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2 347 Thus, spiked imidazoles are extractable very well with the modified supercritical fluids
3 348 with sufficient recoveries in an acceptable extraction time. Of course, all the optimised
4 349 parameters were not known at the beginning of this study. Most of the experiments were
5 350 repeated in a “second run” under the “optimised conditions”. In Figure 3, only the results of
6 351 the optimised experiments are given. Temperature optimisation, trapping solvent selection
7 352 and the study of extraction kinetics were performed separately for the modifier composition
8 353 MeOH/H₂O 5:1 (v/v) for THI and MeOH/H₂O 9:1 (v/v) for 4MeI. All the other studies were
9 354 performed together only with MeOH/H₂O 5:1 (v/v) modifier. The composition was used also
10 355 for all the real sample extractions. Recovery verification of SFE was also performed for the
11 356 both modifier concentrations to authenticate the best conditions for both compounds. The
12 357 values for 4MeI extracted with MeOH/H₂O 5:1 (v/v) modifier were also calculated and found
13 358 out to be satisfactory.
14 359

21 360 *Real samples extraction and method validation*

22 361 SFE is a matrix-dependable method, where every new procedure must be verified also by
23 362 application to a set of different real samples. The optimised extraction conditions were
24 363 applied to the samples of roasted coffee powder, containing THI and 4MeI. A solid phase
25 364 extraction was used as a comparative method for the neurotoxins determination (Klejduš et al.
26 365 2003, 2006). The both analytes were isolated and quantified in the concentrations high above
27 366 their detection limits. Despite the expected matrix effects the method seems to work well with
28 367 the acceptable reproducibility. The samples of fine-grained coffee (1.00 g) were homogeneous
29 368 enough to avoid measurable differences between individual SFE samples. Any differences in
30 369 their extractability from various matrices were not observed.

31 370 The efficiency of the supercritical fluid extraction was tested in two ways: via
32 371 comparison with validated SPE extraction and via post-SFE liquid extraction of the
33 372 “extracted” samples. Comparison of the results of SFE and SPE is presented in Table 4.
34 373 Noteworthy, the results of both extraction methods are in the good coincidence for THI,
35 374 although SPE was reported non-quantitative with spiked solid samples. For post-SFE tests,
36 375 five used samples of coffee matrix (c. 5 g) were combined in one beaker and “coffee” was
37 376 prepared in the same way like for the classical SPE. No traces of the analytes were found in
38 377 any test solution.

39 378 For validation purposes, the coffee samples were spiked with the standard solution
40 379 adding 0.5, 1, 2 and 3 µg of the analytes per 1 g of the sample (details given in experimental
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2 380 section). All the samples were extracted under the optimised conditions for both analytes. The
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4 381 values obtained for the more suitable modifier are given for each substance (see Table 5).

5 382 Slightly higher selectivity was achieved with SFE, but the conditions necessary for the
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7 383 quantitative extraction of the analytes are quite hard, and many ballast compounds were co-
8 384 extracted. Because of that, quick test of available clean-up procedures was performed. Finally,
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10 385 0.45 µm Teflon filter was selected for the extracts clean up, as it didn't hold part of analytes.
11 386 Although the goal to avoid any extract clean up was not achieved, viable method was found
12 387 that can also be usable for SPE extracts. Thus, both SFE and SPE can be used for the
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14 388 quantitative extraction of THI/4MeI from roasted coffee solid samples (SFE) and lixiviates
15 389 (SPE).

17 390 18 391 **Conclusions**

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20 392 The SFE method was optimised for the THI and 4-MeI separation from solid coffee
21 393 samples. Both analytes are quantitatively extracted and it is also possible to co-extract one
22 394 analyte together with the other with acceptable recovery (101.3 % THI and 89.4 % 4MeI or
23 395 98.9 % 4MeI and 86.9 % THI). Extraction time is moderate – 45 min – and both, the precision
24 396 and the recovery of analytical method are sufficient. The method is suitable for all matrices
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26 397 and the spiked analytes recovery is quantitative.

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28 398 Thus, an alternative method exists now for THI and 4MeI determination from real
29 399 coffee samples. Although the method lacks some merits of a standard “low flow-rate” SFE –
30 400 30 ml extracts must be pre-concentrated and even a clean-up procedure is necessary in the
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32 401 case of real samples – it is relatively fast, reliable and works well with spiked solid samples.

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34 402 An attempt was made to enhance environmental friendliness of the method by using
35 403 ultra-pure water as a trapping solvent, but acetonitrile proved to be the only quantitative
36 404 trapping solvent. THI trapping in water is insufficient.

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38 405 Still, the solid phase extraction represents, in fact, an instant separation, while SFE
39 406 takes at least 30 min for extraction and some extra time for pre-concentration. Our results
40 407 indicate, that SPE of real samples is as efficient as SFE. However, SFE was successfully
41 408 validated for spiked solid samples now. With Teflon filter clean up, both SFE and SPE are
42 409 viable methods. Perhaps, SFE present an advantage for small amount extractions and for
43 410 samples, from which quantitative lixiviation of analytes with common solvents would be
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45 411 difficult.

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47 412 Although imidazole concentrations in coffee are very low, coffee imidazoles are one
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49 413 part of a total food imidazole puzzle. They are formed when sugars are heated together with

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2 414 nitrogen containing compounds. Thus, all baked products could contain some imidazoles in
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4 415 their dark parts (Casal et al., 2002). In some cases, baking tins are sprinkled with flour or
5 416 desiccated coconut (although probably not because of this reason) and the burned
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7 417 flour/coconut layer, containing most of harmful compounds, should stay in the baking tin. In
8 418 addition to coffee, baked pastries, caramel sweets, and dark beers participate in the puzzle
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10 419 partly while cola-drinks quite seriously, because of both, larger concentrations of imidazoles
11 420 and lower respect of producers to standard concentration limits (40-times higher than limits
12 421 for imidazoles were found in several cola-drinks in our lab). Thus, each source of imidazoles
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14 422 should be carefully examined.)
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18 425 **Acknowledgements**

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21
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References

- Adam M, Ventura K, Jandera P, Churáček J. 2000. Combination of solid phase extraction with supercritical fluid extraction for the determination of contaminants in water. *Journal of Liquid Chromatography and Related Technologies* 23:1511-1522.
- Casal S, Fernandez JO, Oliveira MBPP, Ferreira MA. 2002. Gas chromatography – mass spectrometric quantification of 4(5)-methylimidazole in roasted coffee after ion-pair extraction. *Journal of Chromatography A* 976:285-291.
- Ding XD, Magiera DJ, Mazzeo JR, Edmar K, Krull IS. 1991. Determination of 2-acetyl-4-tetrahydroxybutylimidazole in ammonia caramel color by HPLC. *Journal of Agriculture Food Chemistry* 39:1954-1597.
- Eisner A, Ventura K, Adam M. 2002. Combination of solid phase extraction with supercritical fluid extraction for the determination of contaminants in water. *Scientific Papers, University of Pardubice, Serie A* 8:47-63.
- Fernandes JO, Ferreira MA. 1997. Gas chromatography – mass spectrometric determination of 4(5)-methylimidazole in ammonia caramel colour using ion-pair extraction and derivatisation with isobutylchloroformate. *Journal of Chromatography A* 786:299-308
- Gugasyan R, Losinno C, Mandel T. 1995. The effect of 2-acetyl-4-tetrahydroxybutylimidazole on lymphocyte subsets during a contact hypersensitivity response in the NOD mouse. *Immunological Letters* 46:221-227.
- Klejdus B, Moravcová J, Kubáň V. 2003. A Reversed-Phase High-Performance Liquid Chromatographic/Mass Spectrometric (RP-HPLC/MS-SIM) Method for Separation of 4-methylimidazole and 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole at pg levels. *Analytica Chimica Acta* 477:49-58.
- Klejdus B, Moravcová J, Lojková L, Vacek J, Kubáň V. 2006. Solid-Phase Extraction of 4(5)-methylimidazole (4MeI) and 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) from Foods and Beverages with Subsequent Liquid Chromatographic-Electrospray Mass Spectrometric Quantification. *Journal of Separation Science* 29:378-384.
- Kröplien U. Quantitative analysis of THI. 1986. *Journal of Chromatography* 362, 286-290.
- Lawrence JF, Charbonneau CF. 1987. Direct method for the determination of 2-acetyl-4-tetrahydroxybutylimidazole in caramel colors and beers by high performance liquid chromatography with absorbency detection. *Journal of Chromatography* 407:405-407.
- Moravcová J, Staněk J, Mílová B, Jarý J. 1992. Determination of 4-methylimidazole in couler. *Czech Journal of Food Sciences* 10:233-238.

- 1
2 461 Müller L, Langseth W, Solheim E, Sivertsen T. 1998. Ammoniated forage poisoning:
3 462 Isolation and characterization of alkyl-substituted imidazoles in ammoniated forage and in
4 463 milk. *Journal of Agriculture and Food Chemistry* 46:3172-3177.
5
6 464 Ong CP, Ng CL, Lee HK, Li SFY. 1994. Separation of imidazole and its derivatives by
7 465 capillary electrophoresis. *Journal of Chromatography A* 686:319-324.
8
9 466 Thomsen M, Willumsen D. 1995. Quantitative ion-pair extraction of 4(5)-methylimidazole
10 467 from Caramel colour and its determination by reverse phase ion pair liquid
11 468 chromatography. *Journal of Chromatography* 211:213-221.
12
13 469 Tomko J. 1999. *Farmakognózia*, Osveta, Martin.
14
15 470 Reeve VE, Boehm-Wilcox C, Bosnic M, Rozinova E. 1993. Suppressive effect of 2-acetyl-4-
16 471 tetrahydroxybutylimidazole on contact hypersensitivity in the SKH-HR hairless mouse.
17 472 *International Archives of Allergy and Immunology* 1102:101-106.
18
19 473 Vávrová J, Mareková-Řežáčová M, Vokurková D, Szkanderová S, Psutka J. 2003. Caffeine
20 474 induces a second wave of apoptosis after low dose-rate gamma radiation of HL-60 cells.
21 475 *Radiation and Environmental Biophysics* 42:193-9.
22
23 476 Wagner J, Jork H, Koglin E. 1993. HPTLC as a reference method in clinical-chemistry –
24 477 Online coupling with spectroscopic methods. *JPC - Journal of Planar Chromatography*
25 478 and *Modern TLC* 6:21-28.
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2 479 **CAPTIONS TO FIGURES**
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5 481 **Fig. 1** Probable chemical structures of THI tautomers
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8 483 **Fig. 2** HPLC/MS chromatograms (SIM mode for m/z 231 THI, for m/z 83 4MeI) of real
9 484 samples A, standards B. Peak identification 1 - THI, 2 - 4MeI. For chromatographic
10 485 conditions see text.
11 486

12 487 **Fig. 3** Optimisation of extraction parameters. All extraction performed from spike samples
13 488 under following conditions: T = 150 °C, p = 40 MPa, t = 45 min, c (modifier) = 10 %
14 489 (v/v), *in situ* modifier: 200 µl, trapping: 30 ml acetonitrile, ACF: modifier THI -
15 490 MeOH/H₂O 5:1 (v/v), 4MeI MeOH/H₂O 9:1(v/v), BDE: modifier MeOH/H₂O 5:1 (v/v)
16 491 for both analytes. RSDs were in the range 1,2 % - 4,7 %.
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Table 1: Retention time (R.T.), peak area (P.A.) and their repeatabilities (RSDs given in brackets, n = 6) and calibration data and LODs for 4MeI and THI obtained under optimised separation conditions using UV and MS detection (injection volume 5 μ l)

	4MeI		THI	
	UV ^a	SIM	UV ^a	SIM
R.T. (min) ^b	11.25(0.09) ^b		6.94(0.14) ^c	
P.A. ($\times 10^5$) ^c	4.45(1.12) ^d		2.23(2.24) ^e	
Concentration range (ng.ml ⁻¹)	40 – 650	1 – 70	22 – 850	1 - 60
Correlation coefficient (n = 6)	0.9998	0.9993	0.9999	0.9996
LOD (ng.ml ⁻¹)	14.5 ^f	0.1	8.2 ^g	0.2
LOQ (ng.ml ⁻¹)	48.3 ^f	0.4	27.4 ^g	0.6

^a cit. Moravcová et al. 1999, ^b range 6.94 – 6.95, ^c range 2.12 – 2.30, ^d range 11.23 – 11.26, ^e range 4.39 – 4.53, ^f 215 nm, ^g 290 nm

Table 2: HPLC-MS validation: determination of THI and 4MeI in real coffee extracts, standard solutions and coffee extracts spiked with known concentrations of analytes (n=6)

Compound	THI	4MeI	THI	4MeI	THI	4MeI	THI	4MeI
C [$\mu\text{g/g}$]	0.5		1		2		3	
Standard solutions of known analyte concentration								
C [$\mu\text{g/g}$]	0.505	0.498	0.993	0.991	1.973	2.021	3.048	3.030
SD [$\mu\text{g/g}$]	0.009	0.006	0.015	0.020	0.027	0.054	0.078	0.062
RSD [%]	1.76	1.19	1.51	2.04	1.38	2.69	2.55	2.05
R [%]	100.93	99.67	99.32	99.12	98.67	101.03	101.59	101.00
Ground roasted coffee extract spiked with known analyte concentration								
C [$\mu\text{g/g}$]	0.536	1.662	1.014	2.156	2.034	3.150	3.017	4.138
SD [$\mu\text{g/g}$]	0.005	0.019	0.012	0.024	0.010	0.005	0.032	0.026
RSD [%]	0.91	1.15	1.18	1.13	0.47	0.16	1.05	0.63
R [%]	100.48	101.06	98.14	100.50	100.04	100.17	99.44	99.83
Real sample (coffee Mexico)								
C [$\mu\text{g/g}$]	0.034	1.145						
SD [$\mu\text{g/g}$]	0.0005	0.0073						
RSD [%]	1.57	0.64						

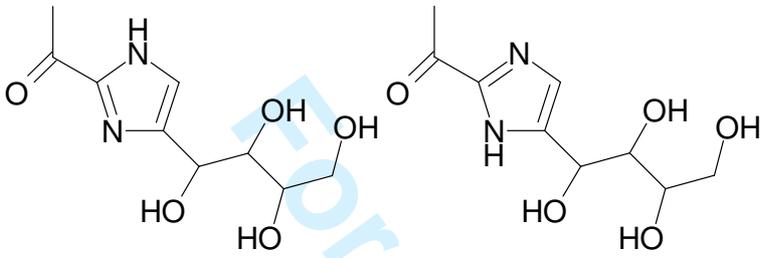
Table 4: Quantification of imidazoles in real sample (ground roasted coffee).

COFFEE	THI [$\mu\text{g/g}$] (RSD [%])		4MeI [$\mu\text{g/g}$] (RSD [%])	
	SFE (solid sample)	SPE (liquid coffee)	SFE (solid sample)	SPE (liquid coffee)
Brazil	0.0165 (4.29)	0.0154 (1.72)	1.34 (2.41)	0.60 (3.22)
Kenya	0.0025 (3.82)	0.0021 (2.25)	0.77 (3.65)	0.41 (2.59)
Haiti	0.0262 (3.04)	0.0250 (2.61)	1.45 (4.47)	0.77 (1.66)
Mexico	0.0337 (2.32)	0.0332 (1.63)	1.11 (3.70)	0.39 (2.48)
Boncampo	0.0088 (2.66)	0.0086 (2.08)	1.01 (4.29)	0.35 (2.70)

Table 5: SFE validation: the extraction of THI and 4MeI from real coffee sample, sea sand model matrix and real coffee sample spiked with known concentrations of analytes (n=5)

Compound	THI				4MeI			
	0.05	0.10	0.15	0.20	1	2	3	4
C [$\mu\text{g/g}$]	0.0509	0.103	0.147	0.199	0.992	2.038	2.949	4.088
SD [$\mu\text{g/g}$]	0.0019	0.005	0.005	0.009	0.047	0.105	0.132	0.230
RSD [%]	3.78	4.57	3.35	4.73	4.75	5.14	4.48	5.62
R [%]	101.71	102.55	98.19	99.30	99.17	101.92	98.30	102.19
Sea sand (p.a.) matrix spiked with known analyte concentration								
C [$\mu\text{g/g}$]	0.085	0.131	0.184	0.231	2.076	3.033	4.065	4.975
SD [$\mu\text{g/g}$]	0.004	0.006	0.008	0.011	0.064	0.096	0.176	0.195
RSD [%]	4.71	4.81	4.43	4.90	3.09	3.18	4.32	3.91
R [%]	101.97	97.78	100.07	100.23	98.61	97.51	98.92	97.36
Ground roasted coffee matrix spiked with known analyte concentration								
C [$\mu\text{g/g}$]	0.034	1.110						
SD [$\mu\text{g/g}$]	0.001	0.041						
RSD [%]	2.32	3.70						
Real sample (coffee Mexico)								
C [$\mu\text{g/g}$]	0.034	1.110						
SD [$\mu\text{g/g}$]	0.001	0.041						
RSD [%]	2.32	3.70						

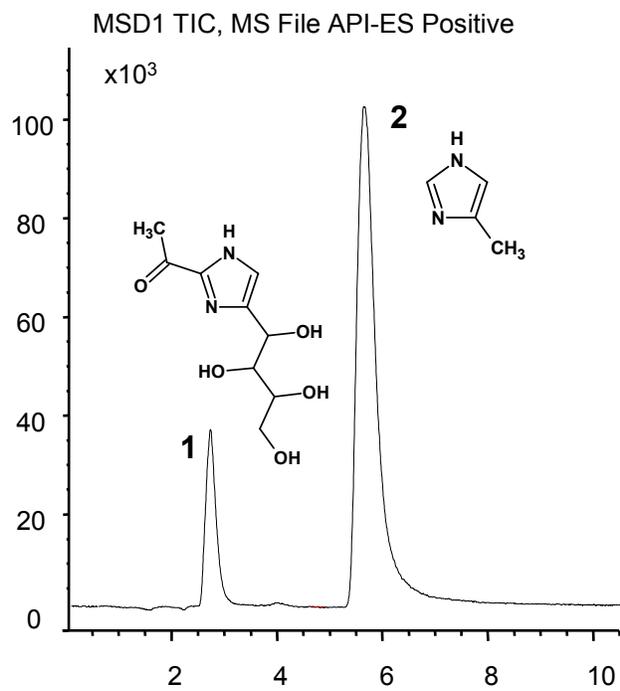
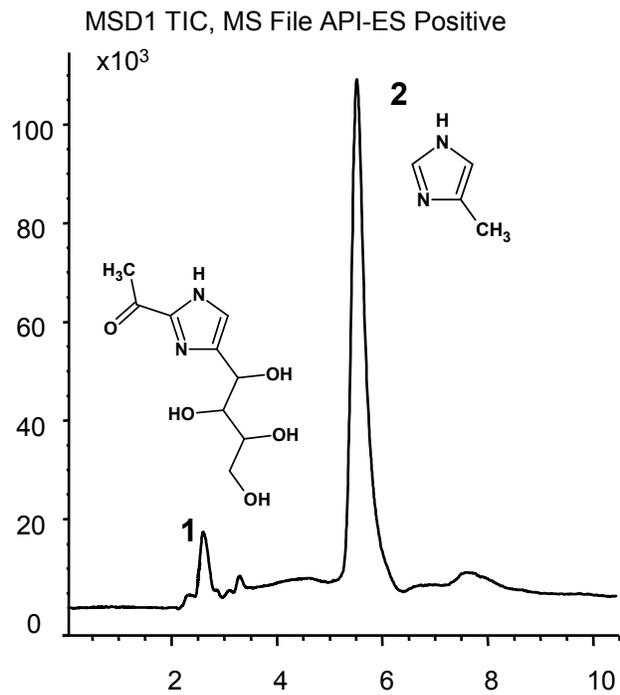
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