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Application of three-dimensional liquid chromatography for quantification of 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole (THI) in caramel colours

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Multidimensional chromatography is a widely used technique to quantify specific compounds in complex samples. A three-dimensional liquid chromatography with UV detection was developed to separate 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole, an undesirable neoformed by-product found in class III caramel colours. The first column was a classical reversed-phase column (C18 column); the second one was a cation-exchange column operated with acidic mobile phase; the last column was a specific reversed-phase column (porous graphitic carbon). This method is validated to quantify this compound in caramel colours in 45 min in the 5–50 mg kg^{-1} concentration range. The limit of quantification is found at 5 mg kg^{-1}. The total error is less than 20% at 5 mg kg^{-1} and less than 10% between 10 and 50 mg kg^{-1}. This method is used routinely to quantify THI in class III caramel colours and to check compliance with caramel regulations.

Introduction

Class III caramel colour is produced by heating carbohydrates with ammonia and is used to colour products like beer or vinegar. The caramelization reaction of this class of caramel is close to the Maillard reaction and the end product is a complex mixture of low molecular mass molecules responsible for the flavour of caramel[1,2] and high molecular mass molecules responsible for the typical colour of caramel.[3] Upon heating, 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole (noted as THI), a minor unwanted by-product, can be formed. This molecule was reported as an immunosuppressive compound by decreasing the number of blood lymphocytes linked to vitamin B6 metabolism.[4] The European Commission put the legal limit of THI at 10 mg kg^{-1} or less in class III caramel colour (on an equivalent colour basis of 0.1 A at 610 nm at 1 g L^{-1}) (Commission Regulation No. 231/2012).

In the publication on the re-evaluation of caramel colours in 2011, the EFSA concluded that the current exposure estimate for THI is not of concern.[5]

Several procedures have been recently published for the analysis of THI in class III caramel colours by liquid chromatography coupled with mass spectrometry.[6–13] They generally used reversed phase column operated with aqueous mobile phases. The quantification with a less expensive and not very selective detector as diode array UV absorbance detector needs a time-consuming sample preparation step[14] or the use of an efficient chromatographic separation.[15,16] To fully automate the method and reduce the analysis cost, the multidimensional chromatography was chosen after a simple dilution of caramel in water.

A previous article described two liquid chromatography methods with UV detection for quantifying THI in class III caramel colours.[17] They both required a dilution of caramel in water and used a heart-cutting two-dimensional liquid chromatography with a reversed-phase or a cation-exchange chromatography in the first dimension and a specific reversed-phase stationary phase in the second column (porous graphitic carbon). These two methods were validated by the accuracy profiles approach with an acceptance limit fixed to ±20% and a proportion of measurements inside the acceptance limit fixed to 90%.

As shown in Fig. 6 and 7 on these published methods,[17] the resolution between the THI peak and its neighbours is only around 1. After dozens of analysis, the columns aging involves a decrease of the resolution, thus the accuracy of the THI quantification decreases.

A new method has been developed in order to keep a resolution with return to baseline analysis after analysis. The use of a heart-cutting three-dimensional liquid chromatography by coupling the three columns of the previously published method[17] has been tested with the aim to improve the resolution and the robustness of the method. One of the major disadvantages of multidimensional chromatography is the dilution of the target molecule[18] because the total dilution

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factor is the product of the dilution factor of each dimension.[19] To decrease this factor, we must have a focusing step between each dimension.[19] It requires that the previously dimension mobile phase was a weak eluent in the next dimension and the elution strength of the next dimension eluent was much higher.[18,19]

Materials and methods

Chemicals

HPLC-grade methanol and acetonitrile as well as ammonia (min 25%), ammonium formiate (purity 97%) and formic acid (purity 95–97%) were supplied by VWR (West Chester, PA, USA). The standard of THI was obtained from ITCA (International Technical Caramel Association, Washington DC, USA). 18.2 MΩ deionized water was filtered through an Elga Purelab Option R 7 purification pack (Elga, Bucks, U.K.). Class III caramel colours were obtained from Nigay S.A.S. (Feurs, France).

Equipment

LC experiments were performed on an Ultimate 3000 x2 dual liquid chromatography system (Thermo, Waltham, MA, USA), which includes two ternary low-pressure microgradient pumps, an autosampler, a thermal compartment with two 10-port 2-position valves, and a photodiode array detector. Instrument control, data acquisition, and compilation of results were made using Thermo Chromeleon software, V6.8.

LC-LC-LC method

Caramel samples (liquid or powder) are diluted ten-fold in distilled water before injection.

The detection wavelength is fixed to the maximum absorbance of THI *i.e.* 287 nm.[17] The temperature of the column oven was set at 40 °C.

The injection volume is fixed to 50 μL.

The separation is made in multidimensional mode with three chromatographic columns and two 10-port 2-position valves (Fig. 1).

In the first step (Fig. 1A), the pump 1 is used to elute compounds in the first column, a classical C18 column (Acclaim 120 C18, 250 mm × 4.6 mm, 5 μm; Thermo). The mobile phase is a mixture of methanol and water (10/90 v/v) (Table 1). The eluent during the first minutes is sent to the waste. The pump 2 is used to equilibrate the second column, a strong cation

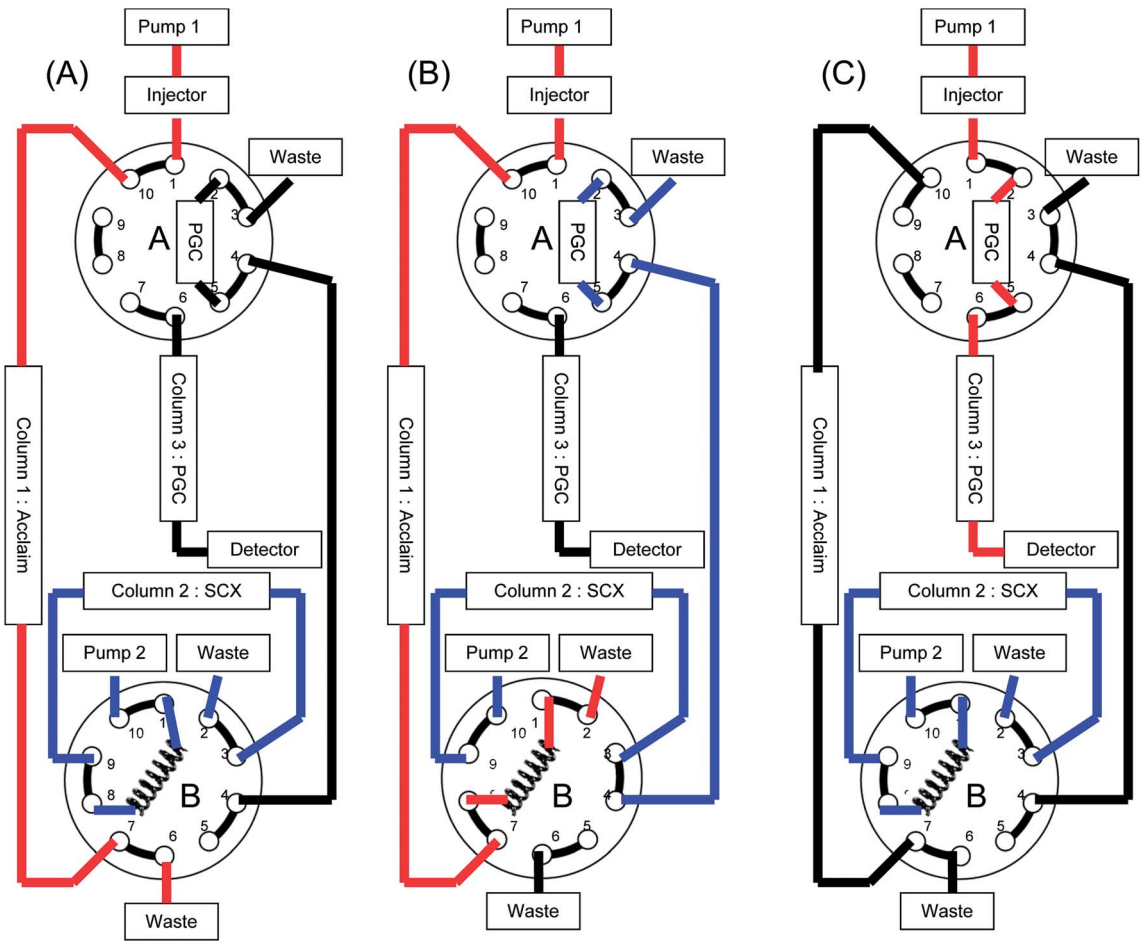

Fig. 1 Experimental device for heart-cutting three-dimensional liquid chromatography. (A) separation on column 1 and column 2; (B) heart-cut at the outlet of column 1 and column 2; (C) separation on column 3.

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Table 1 Valve position and chromatographic conditions used in the three-dimensional liquid chromatography procedure

	Valve position		Column 1: Acclaim C18			Column 2: PL-SCX			Column 3: Hypercarb PGC			
Time	A	B	Mobile phase	Flow rate	Outlet	Mobile phase	Flow rate	Outlet	Mobile phase	Flow rate	Outlet	Comments
0–4 min	1–10	1–10	Water–methanol 90–10	1 mL min^{-1}	Waste	10 mM formiate ammonium adjusted to pH 2.5 with formic acid	0.3 mL min^{-1}	Waste	—	—	Detector	Column 1: elution Column 2: equilibration Column 3: —
4–5 min	1–10	1–2	Water–methanol 90–10	1 mL min^{-1}	1 mL loop	10 mM formiate ammonium adjusted to pH 2.5 with formic acid	0.3 mL min^{-1}	Waste by PGC guard column	—	—	Detector	Column 1: recovery of THI Column 2: equilibration Column 3: —
5–15 min	1–10	1–10	Methanol	1 mL min^{-1}	Waste	10 mM formiate ammonium adjusted to pH 2.5 with formic acid	0.3 mL min^{-1}	Waste	—	—	Detector	Column 1: wash Column 2: elution Column 3: —
15–17 min	1–10	1–2	Water–methanol 90–10	1 mL min^{-1}	Waste	10 mM formiate ammonium adjusted to pH 2.5 with formic acid	0.3 mL min^{-1}	PGC guard column	—	—	Detector	Column 1: equilibration Column 2: recovery of THI Column 3: —
17–20 min	1–10	1–10	Water–methanol 90–10	1 mL min^{-1}	Waste	100 mM formiate ammonium adjusted to pH 10 with ammonia	0.5 mL min^{-1}	Waste	—	—	Detector	Column 1: equilibration Column 2: wash Column 3: —
20–30 min	1–2	1–10	—	—	Waste	100 mM formiate ammonium adjusted to pH 10 with ammonia	0.5 mL min^{-1}	Waste	Water–acetonitrile 97–3 to water–acetonitrile 90–10	1 mL min^{-1}	Detector	Column 1: — Column 2: wash Column 3: elution
30–38 min	1–2	1–10	—	—	Waste	100 mM formiate ammonium adjusted to pH 10 with ammonia–methanol 50–50	0.5 mL min^{-1}	Waste	Methanol	1 mL min^{-1}	Detector	Column 1: — Column 2: wash Column 3: wash
38–45 min	1–2	1–10	—	—	Waste	10 mM formiate ammonium adjusted to pH 2.5 with formic acid	0.3 mL min^{-1}	Waste	Water–acetonitrile 97–3	1 mL min^{-1}	Detector	Column 1: — Column 2: equilibration Column 3: equilibration

exchange column (PL-SCX, 150 mm × 4.6 mm, 8 μm; Agilent, Lake Forest, CA, USA) with 10 mmol L^{-1} formiate ammonium adjusted to pH 2.5 with formic acid as mobile phase.

In the second step (Fig. 1B), when THI started to elute from the first column, the valve B is switched from 1–10 position to the 1–2 position. The column 1 eluent is then trapped into a 1 mL loop. When THI is finished eluting (approximately 1 min after the start), the valve B is switched to the initial position (Fig. 1A). In this third step, the first column is washed by methanol delivered by the pump 1. The pump 2 retrieves the eluent trapped in the loop and the mixture is separated in the second column with 10 mmol L^{-1} formiate ammonium adjusted to pH 2.5 with formic acid as mobile phase (Table 1).

When THI started to elute from the second column, the valve B is switched again to the 1–2 position (Fig. 1B) thereby recovering THI in the porous graphitic carbon (PGC) guard column (Hypercarb, 10 mm × 4 mm, 5 μm; Thermo). At the end of the THI elution, the valve B is switched to the initial position (Fig. 1A). The pump 2 is used to wash the second column and to equilibrate with the eluting mobile phase (Table 1).

In the last step, 20 min after the injection and when the first column is totally washed and equilibrated for the next analysis, the valve A is switched from the 1–10 position to the 1–2 position (Fig. 1C). The eluent trapped in the guard column is eluted by the pump 1 into the third column, a PGC column (Hypercarb, 100 mm × 4.6 mm, 5 μm; Thermo). The mobile phase is a mixture of acetonitrile–water in gradient mode, from 3/97 v/v at 20 min to 10/90 v/v at 30 min. Then, the PGC column is washed by methanol and equilibrated with acetonitrile–water 3/97 v/v (Table 1). The total analysis time was 45 min.

To determine the elution time of THI at the end of the first and the second column, the detector is directly connected at the end of each column.

Validation

The new method is validated by the accuracy profiles, developed by the "Société Française des Sciences et Techniques Pharmaceutiques".[20–23] This strategy is a decision-making graphical tool and allows us to access the accuracy (precision + trueness) as well as to estimate the uncertainty at each level of concentration. The acceptance limit is fixed to ±20% and β-expectation tolerance interval (proportion of measures inside the acceptance limits) to 90%. Method validation is performed from two repetitions of the calibration standard analysis and three repetitions of the validation standard (spiked caramel colours) analysis, during three days with three caramels (different references with different initial level of THI) and four spiked concentrations around the legal limit (5, 10, 20 and 50 mg kg^{-1}).

Results and discussion

Method development

Heart-cutting three-dimensional liquid chromatography requires three sufficiently different mechanisms of separation to improve the separation from one dimension to another but the mobile phase used in each dimension must be compatible with the next column and the next mobile phase.

As the caramel is soluble in water, reversed-phase separation is the easiest to implement mechanisms. The wide choice of column in reversed-phase separation allows us to find stationary phases with high differences in selectivity. The Porous Graphitic Carbon (PGC) column is a interesting reversed-phase column with a different retention mechanism of a classical C18 column.[17,24–27] Moreover, the cationic form of THI in acid condition ($pK_a = 3.5$) allows the use of cation exchange separation. The same solvents as the previously published method[17] is used for each column (Table 1). They are all aqueous mobile phases so the problem of solvent incompatibility is avoided.

The Ultimate 3000 has two ternary pumps. One pump must be used with the two reversed-phase columns, the first (C18) and the last one (PGC). After elution of THI from the first column and during the separation in the second one, the pump 1 must wash and reequilibrate the first column (Table 1). After that, this pump is used for the separation in the third column. The pump 2 is always dedicated to the cation exchange column.

This protocol is easily programmed with new HPLC systems with the presence of valves in the oven and the control of these valves with software.

Fig. 2 shows chromatograms of each dimension of a THI standard (50 mg kg^{-1}), a sample of caramel and the same sample of caramel spiked with 25 mg kg^{-1} of THI. The first column separation (Fig. 2A) is not efficient enough to separate THI from all other compounds in caramel. THI ($t_R = 4.4$ min) is completely obscured by the elution of many other compounds during the same time. The portion between 4 and 5 min is transferred to the second dimension column. Fig. 2B shows the chromatograms of the same samples at the outlet of the second dimension column. The separation seems better because the overall magnitude of the absorbance signal decreases from the first to the second column (4000 to 600 mAU). But THI ($t_R = 16$ min) is still not completely separated. The fraction between 15 and 17 min is transferred to the third column. Fig. 2C shows the chromatograms at the outlet of the third column. This chromatogram is much clearer than the first and the second dimension columns. Finally, THI ($t_R = 31.5$ min) is completely separated to all other compounds in caramel colours. There is no peak near THI peak and quantification errors will be lower than for the two methods of heart-cutting two-dimensional liquid chromatography.[17]

Focusing step

The focusing of the analyte at the head of the column is important to keep a low peak width and improve the sensitivity of the detection. The most retentive stationary phase is used in the last column to get the best focus of the analyte.

To start to focus THI at the outlet of the second column, a guard column with the same stationary phase as the third column is used instead of the recovery loop between the second and the third dimension. The mobile phase of the second column (100% aqueous) has low eluent strength on the carbon

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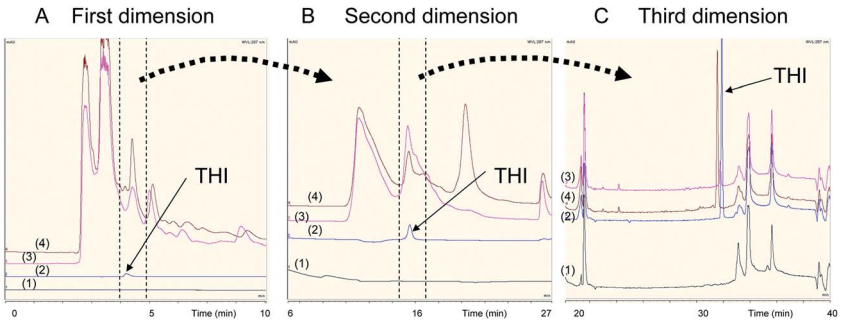

Fig. 2 Chromatogram at the end of the first column (A), second column (B) and third column (C). Detection: absorbance at 287 nm. Elution condition: see Table 1. (1) Blank; (2) THI standard at 50 mg kg^{-1}; (3) class III caramel colour; (4) class III caramel colour spiked with 25 mg kg^{-1} of THI.

stationary phase (guard column and third column).[17] Therefore THI is retained on the PGC guard column so its dilution at the end of the second column is cancelled. When switching the valve A, THI and its coeluted compounds were back-flushed into the third column with a more eluent mobile phase (acetonitrile–water 3–97). With these settings, the peak width at half-height of THI on the PGC column after the first two dimensions is equal to that of THI on the PGC column alone (0.20 and 0.19 min respectively).

Validation

The performance of the method is evaluated by the trueness (bias), the precision (standard deviation) and the accuracy profiles in the 5–50 mg kg^{-1} concentration range (Table 2).

The bias is lower than 3%; the intermediate precision is found at 8% at 5 mg kg^{-1} and is lower than 5% between 10 and 50 mg kg^{-1}. Thus, the accuracy of the method obtained with a β-expectation tolerance interval fixed at 90% is lower than 20% at 5 mg kg^{-1} and lower than 10% between 10 and 50 mg kg^{-1}.

Accuracy profiles of the LC-LC-LC method (Fig. 3) are widely included in the confidence interval (±20%) in the 5–50 mg kg^{-1} concentration range. Therefore, the limit of quantification (LOQ) was found to be 5 mg kg^{-1}, the lowest tested concentration. This method is thus validated to quantify THI in caramel colours around the legal limit (10 mg kg^{-1}).

Discussion

The performance of the method is compared with the results obtained in the previous publication.[17] The bias is lower in the LC-LC-LC method than in the two LC-LC methods; the intermediate precision is lower for the 10–50 mg kg^{-1} concentration range and slightly higher for the 5 mg kg^{-1} concentration. The accuracy in the LC-LC-LC method is much lower than in the two LC-LC methods notably thanks to the lower ratio between within-day variance and between-day variance. The quantification of THI in class III caramel colours is thus more accurate with the three-dimensional liquid chromatography than the two-dimensional ones.

Compared to methods developed by LC/MS/MS,[6–13] the LC-LC-LC method is longer (45 min per sample against 10–30 min) and the LOQ is higher (5 mg kg^{-1} against around 0.1 mg kg^{-1} in caramel colours). This LOQ is nevertheless sufficient to check

Table 2 Validation results of the method with three column (LC-LC-LC) and comparison with the two methods with two columns (LC-LC) previously published

	Spiked concentration (mg kg^{-1})	LC-LC C18-PGC[a] (%)	LC-LC SCX-PGC[b] (%)	LC-LC-LC (%)
Bias (difference between calculated value and true value)	5	7.2	10.3	2.0
	10	6.6	2.0	2.5
	20	4.4	−2.0	−2.6
	50	2.3	−0.8	−2.6
RSD intermediate precision (standard deviation of three days and three different samples repeatability)	5	4.1	6.8	8.0
	10	4.8	4.6	3.1
	20	4.4	3.6	3.4
	50	2.3	2.9	1.9
Accuracy (uncertainty obtained from the trueness and the precision)	5	[−5.2; 19.6]	[−10.5; 31.2]	[−14.3; 18.2]
	10	[−6.8; 20.0]	[−14.3; 18.3]	[−3.6; 8.7]
	20	[−11.3; 20.0]	[−19.5; 15.5]	[−9.3; 4.0]
	50	[−6.0; 10.6]	[−11.2; 9.6]	[−6.3; 1.2]

[a] LC-LC C18-PGC is a heart-cutting two-dimensional liquid chromatography with a C18 phase in the first column and a carbon phase in the second column. [b] LC-LC SCX-PGC is a heart-cutting two-dimensional liquid chromatography with a strong cation exchange phase in the first column and a carbon phase in the second column.

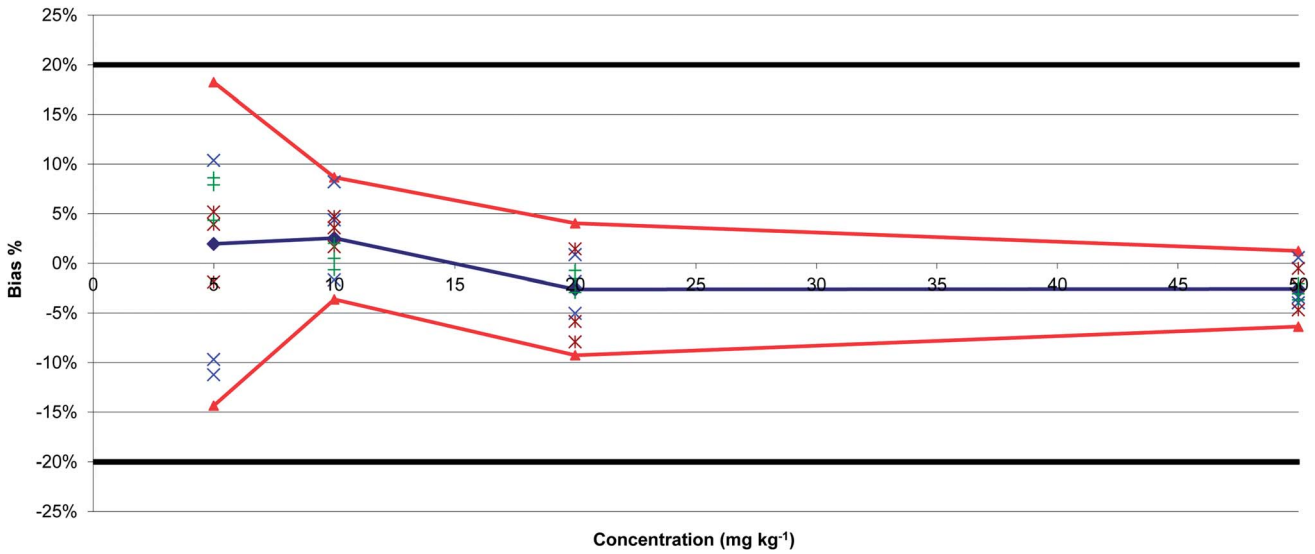

Fig. 3 Accuracy profile representation for the LC-LC-LC method: bias is represented in blue; accuracy is illustrated in red; acceptability limits are illustrated in black; the nine results obtained for each concentration are represented by crosses (3 days with 3 repetitions per day).

the conformity of caramel colours but not to quantify THI in final products (foods and beverages).

On the other hand, the sample preparation of the LC-LC-LC method is either the same (simple dilution in distilled water[8,11] or acetonitrile[10]) or simpler (solid phase extraction,[6,12,13] clarification with the method of Carrez[9]) than in published LC/MS/MS methods thanks to the separation power of multidimensional chromatography. The price of the device is also lower (UV detector is less expensive than MS detector).

Conclusions

This methodology shows the advantages of the heart-cutting multidimensional liquid chromatography to analyse a specific compound in complex matrices. This fully automated method needs very simple sample preparation (only a dilution in water) and an inexpensive detector. THI can be quantified in class III caramel colours between 5 and 50 mg kg^{-1} with 90% of measurements inside the acceptance limit fixed to ±20% (validation by accuracy profiles). These results show that this method can be used to check the conformity of caramel colours. This LC-LC-LC method is compared with two previously published methods using LC-LC technique. The quality of the separation is better (better resolution between the THI peak and its neighboring peaks) and the quantification accuracy is improved for the same concentration range. After analysis of more than one hundred of samples, no problem of decreasing quantification accuracy appeared and the new method is more robust.

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