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Determination of bixin and norbixin in meat using liquid chromatography and photodiode array detection

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The development of an analytical method that enables routine analysis of annatto dye, specifically bixin and norbixin, in meat tissue is described. Liquid-solid extraction was carried out using acetonitrile. Analysis was by HPLC with photodiode array detection using two fixed wavelengths (458 and 486 nm). The possibilities of ion trap mass spectrometry (MS) were also assessed. Method performance characteristics, according to Commission Decision 2002/657/EC, were determined, with recoveries between 99 and 102% and calibration curves being linear in the 0.5–10 mg kg^{-1} range. The limit of quantification was 0.5 mg kg^{-1}.

Keywords: annatto; bixin; norbixin; meat; liquid chromatography; photodiode array

Introduction

As colour is often a key consumer perception in food preference and acceptability, natural and synthetic colorants have been extensively used to colour food, drugs and cosmetics (Bittencourt et al. 2005). Due to restrictions on the use of these synthetic colorants, there has been a growing interest within the food industry towards the use of natural colorants.

Annatto extracts (E160b) are orange/red natural carotenoid dyes obtained from the seeds of the tropical shrub *Bixa orellana* L. and are now widely used by the food industry to colour many food products, including high fat dairy products such as butter and cheese, cereals, snack foods, seasonings, creamers, ice creams, flour, sugar confectionary, soft drinks and fish (Lancaster and Lawrence 1996, Scotter et al. 1994, 1995, 2002). The principal coloring component of annatto seeds is *cis*-bixin, which is soluble in most polar organic solvents to which it imparts an orange colour, but which is largely insoluble in vegetable oil (Scotter et al. 1994, 1995, 2002). *Cis*-bixin is the monomethyl ester of *cis*-norbixin (Figure 1) (Scotter et al. 1994). The amounts of the active pigments, bixin and norbixin, in annatto can vary from less than 1% to over 85%, dependent on the type of annatto extract, e.g. water, vegetable oil, solvent (Tennant and O'Callaghan, 2005; WHO 2007) and are affected by degradation agents, such as light, temperature, air, anti- and pro-oxidants and pH (Najar et al. 1988). The degradation products are of commercial significance since, by controlling the degree of the degradation, the orange/red to yellow colour balance of an annatto formulation can be adjusted (Scotter 1995).

Notwithstanding the widespread use of annatto pigments as food colorants, practically no information exists on their toxicological properties for animals and humans. Toxicity was mostly determined in animals using commercial annatto preparations containing undetermined amounts of bixin and norbixin (Engelberth-Holm and Iversen, 1955, Van Esch et al. 1959). Toxicological data on annatto pigments are limited, possibly because food additives derived from natural sources have been exempt from certification (Hallagan et al. 1995). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) estimated the ADI for annatto as 0–2.5 mg kg^{-1} body weight day^{-1} (for a preparation containing 2.6% cartenoids expressed as bixin) and 0–0.065 mg kg^{-1} body weight day^{-1} expressed as the pure pigment (WHO 2007).

In recent years, there has been a growing concern about the presence of these compounds in edible matrices of animal origin. Consequently, the use of colorants in foodstuffs has been regulated by the European Union (Council Directive EC/94/36). Depending on the colorant (e.g. annatto or E160b) and the matrix (e.g. fish tissue and dairy products), maximum residue levels (MRLs) have been proposed. Annatto extracts are listed amongst those colorants

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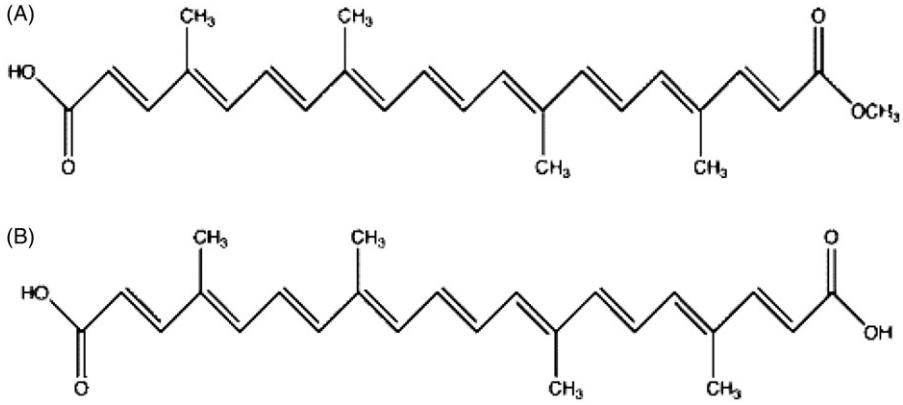

Figure 1. Chemical structure of bixin (MW 395) (A) and norbixin (MW 378) (B).

that may be used separately or in combination in certain foods up to the maximum levels specified (on a ready-for-consumption basis) (Scotter et al. 2002). Other edible matrices, e.g. molluscs, crustaceans, meat tissue, poultry and game, as well as their preparations may not contain added annatto.

To date, most analytical studies on annatto have been concerned with dairy products and high fat foods, where annatto has a well-established usage, such as cheese and dairy spreads (Scotter et al. 2002). Very few studies have documented the possible detection of annatto or bixin and norbixin in meat tissue. For the determination of annatto and, in particular, bixin and norbixin, several detection techniques, such as ultraviolet (UV), photo-diode array (PDA) and ion trap mass spectrometry (MS), in combination with liquid chromatography (LC), were evaluated. Our study aimed at developing a routine residue method for the detection of annatto (bixin and norbixin) in meat using liquid chromatography. The method was also validated in accordance with the Commission Decision 2002/657/EC.

Experimental

Chemicals

Acetonitrile (AcN), ethanol (EtOH) and acetic acid were of HPLC grade and supplied by VWR (Merck, Darmstadt, Germany). HPLC grade water was obtained from Across Organics (Fairlawn, NI, USA).

Standards

Bixin and norbixin powders were provided by LGC Promochem (Molsheim, France) with a purity of 88.5 and 97.1%, respectively. Annatto was purchased from Chromadex, Inc (Santa Ana, CA, USA) and had a norbixin content of 14–16%. Stock standard solutions of 200 ng μl^{-1} were prepared in ethanol. Individual and composite working standards were prepared by appropriate dilution of the standard stock solutions in 0.1% acetic acid in acetonitrile/H_2O (80 : 20, v/v). All standard and working solutions were stored in the dark at −20°C to minimize degradation and isomerization.

Extraction and clean-up

A 1-g aliquot of homogenized meat tissue was extracted with 2 ml acetonitrile (AcN). For validation, gammon sausage was used and bixin, norbixin or annatto were spiked in the AcN. After vigorously vortex-mixing and centrifugation (5 min at 5500 rpm, 4°C, 3726 *g*) (Sorvall RC 5C plus; Goffin Meyvis, Belgium), 120 µl of the supernatant with addition of 30 µl 0.1% aqueous acetic acid was used for chromatographic analysis, with an injection volume of 60 µl.

Chromatographic instrumentation

Analysis was carried out using a high performance liquid chromatography (HPLC) apparatus comprising an 1100 series quaternary gradient pump and autosampler (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using a Symmetry™ C_{18} column (5 µm particle size, 150 × 2.1 mm; Waters, Milford, MA, USA). The mobile phase consisted of a mixture of acetonitrile (A) and 0.1% aqueous formic acid (B). A linear gradient was run: 80% A : 20% B, increasing to 95% A : 5% B over 10 min, increasing to 100% A over 5 min (hold 5 min) and, finally, back to initial conditions 80% A : 20% B (hold 10 min). Total run time was 30 min at a mobile-phase flow of 0.3 ml min^{-1}.

When detection was performed using photodiode-array detection, a TSP Spectra System UV6000 LP (Thermofinnigan, San José, CA, USA) with a deuterium lamp was used with a detection range 300–580 nm (bandwidth: 1 nm). Two fixed wavelengths, 458 and 486 nm (bandwidth: 9 nm), were also tested.

Mass spectrometric (MS) detection was carried out with a Thermofinnigan LCQ DECA ion trap with electrospray ionisation (ESI) interface using the above described chromatographic parameters. Multiple mass spectrometry (MS^2) spectra were acquired. The MS method consisted of segments, the first (0–4 min) in negative ionisation mode, with a full scan MS scan event and an MS^2 scan event (parent ion: 379 MW; collision energy: 25 eV). The second segment (4–10 min), in positive ionisation mode, consisted also of a full scan MS scan event and an MS^2 scan event (parent ion: 395 MW; collision energy: 30 eV). Isolation width was set at 2.0 and activation Q at 0.250.

Data analysis

Chromatograms and spectra were recorded and processed using Xcalibur 1.2. software (Thermofinnigan, San José, CA, USA). All data were further analyzed using Microsoft® Excel (Microsoft Corporation, Seattle, WA, USA).

Quality assurance

Before and after sample analysis, a standard mixture (10 ng on column) of the targeted colour agent was injected to check the operation conditions of the LC–PDA devices. For quantitative analysis, calibration standards were spiked at three levels in the sample matrix: 0.5, 1 and 5 mg kg^{-1}. When using this analytical method for routine analysis, quality control was carried out by analysis of blank and spiked comparable sample matrices together with every series of samples. At regular times, secondary quality control, using blind fortified samples, was carried out within the routine analysis.

Validation procedure

Method validation for the detection of annatto, bixin and norbixin in meat tissue was carried out according to Commission Decision 2002/657/EC, concerning detection of residues of veterinary drug substances in matrices of animal origin. The examined validation parameters for the complete analytical method were selectivity/specificity, linearity, precision, trueness/recovery, applicability/ruggedness/stability and decision limit (CCα) and detection (CCβ) capability.

For this, blank sausage meat samples were fortified with a standard solution of a mixture of the dyes at the level of 0.5, 0.75, 1, 2 and 4 mg kg^{-1}, unless otherwise stated.

Results and discussion

Method performance

To date, analytical approaches for the detection of annatto, or bixin and norbixin, have been described for different matrices, but not for meat. Thus, the aim of this study was to develop an analytical approach for the detection of annatto and, in particular, bixin and norbixin in meat tissue. Two detection techniques, photodiode array (PDA) and ion trap mass spectrometry (MS), were evaluated.

With the developed method, the two compounds of annatto, norbixin and bixin, were separated and simultaneously detected by liquid chromatography (LC) using either photodiode array or ion trap mass spectrometry with electrospray ionisation (ESI). Using PDA detection, the main peaks obtained from the chromatographic separation of bixin and norbixin were scanned between 300 and 580 nm and were similar to those reported by Scotter (1995). In particular, two fixed wavelengths were used: 458 and 468 nm. In Figure 2, UV and MS^2 chromatograms and spectra for 10 ng bixin, norbixin and annatto on the column are shown. The PDA spectra of norbixin and bixin are identical, with absorption maxima at 458 and 486 nm, respectively. Moreover, a typical increase in absorption intensity was observed around 430 nm for both compounds. It was also observed that annatto mainly consists of norbixin, though both α- and β-isomers of bixin and norbixin were present, consistent with the report of Lancaster and Lawrence (1996).

Multiple mass spectrometry (MS^2) was used to obtain structural information on the compounds corresponding to each chromatographic peak. Bixin and norbixin produced the $[M\text{-}H]^+$ ion in full scan MS. This molecular ion, more specifically m/z 379 and 395 for norbixin and bixin, respectively, was used as the precursor ion for MS^2. As can be seen in Figure 2, the MS^2 product ions of norbixin (at 379) are 253, 291, 310 and 335, whereas the main product ions of bixin (at 395) are 317, 335, 345, 363 and 377. Using MS^2, detection is possible from a concentration level of 1 mg kg^{-1}, as the presence of several ions in the spectrum of bixin makes interpretation difficult due to matrix interference. With PDA, the limit of quantification is 0.5 mg kg^{-1}; therefore, PDA detection was preferred for use in routine analysis.

Full in-house method validation for bixin and norbixin in meat

Identification

For method validation and interpretation of the results, the criteria described in Commission Decision 2002/657/EC were used. Targeted dye compounds in meat samples were identified based on comparison of their relative retention time (±2.5%), which is the ratio of the retention time of the analyte to that of the internal standard with a standard or fortified sample. When detection was carried out with PDA, the absorption maxima (±2 nm) in the spectrum of the

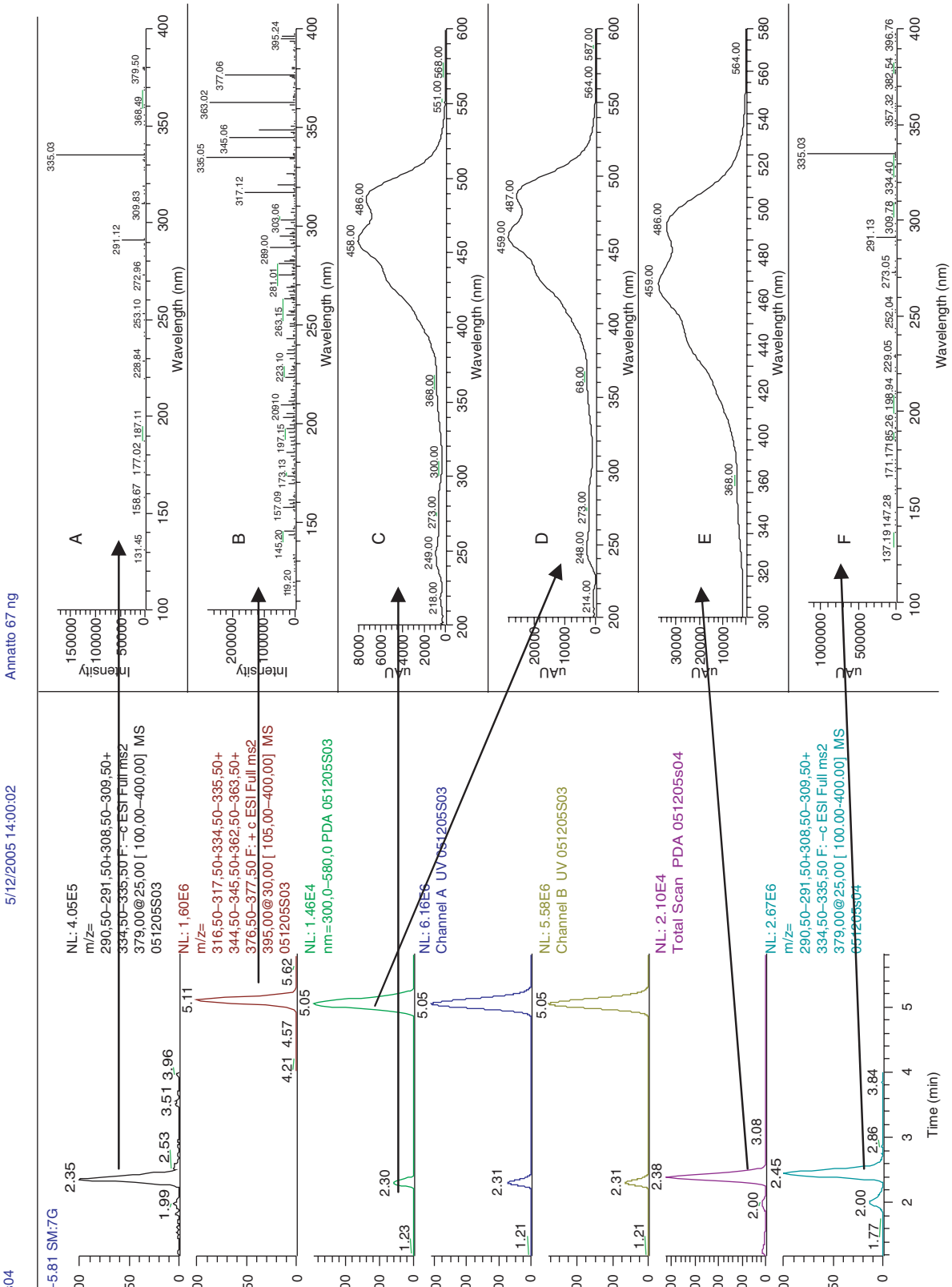

Figure 2. Chromatograms and MS^2 spectra of norbixin (A) and bixin (B), PDA spectrum of norbixin (C) and bixin (D), PDA spectrum of annatto (E) and MS^2 spectrum of annatto (norbixin) (F).

analyte were taken into account. Using mass spectrometric detection, the ion ratio of the precursor/product ions in the obtained spectrum was used when the corresponding peak in the chromatogram had a signal-to-noise ratio of at least 3:1. In samples, the presence of bixin, norbixin and annatto was confirmed if the retention time, the maximum absorption wavelength and the shape of the UV spectrum of the unknown were in accordance with a fortified sample or a standard mixture. Comparison of the UV spectra of a blank sample fortified at a level of 10 mg kg^{-1} for norbixin/bixin and at a level of 67 mg kg^{-1} for annatto (=10 mg kg^{-1} norbixin) is shown in Figure 3.

Specificity/selectivity

As one of the general requirements of EC/2002/657, namely the investigation of interference of matrix components, specificity/selectivity was evaluated through the analysis of blank samples fortified separately with the considered compounds at national action level (AL). This AL was based on the acceptable daily intake (ADI) for bixin and norbixin, with a concentration level of 0.5 mg kg^{-1} being postulated. The specificity of the chromatographic method was proven since no significant interfering peaks with a signal-to-noise ratio of 3 or more were observed at the retention times of the targeted compounds by analysis of fortified samples. As described previously, selectivity was proven to conform to 2002/657/EC, since analytes were identified on the basis of their relative retention time and the ion ratio of the precursor and product ions in the obtained spectrum.

Linearity

The linearity of the method was evaluated using repeated analysis of five calibration points at concentrations of 0.5, 0.75, 1, 2 and 4 mg kg^{-1}. Correlation coefficients were between 0.93 and 1.

Trueness/precision

Because no certified reference material (CRM) was available, trueness of the measurements was assessed

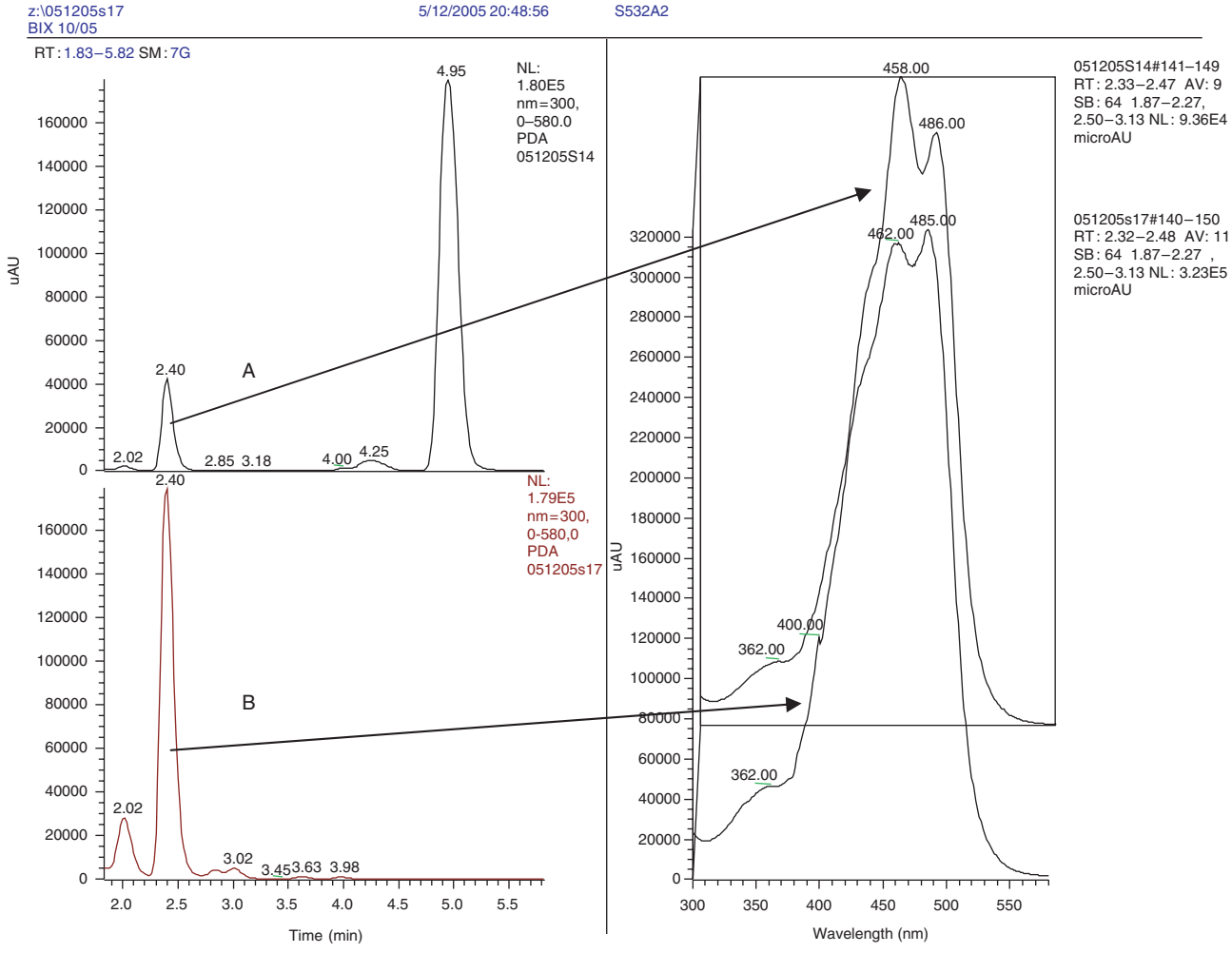

Figure 3. Comparison of PDA spectra of norbixin and bixin (fortified sample at 10 mg kg^{-1}) (A) and of annatto (fortified sample at 67 mg kg^{-1}) (B).

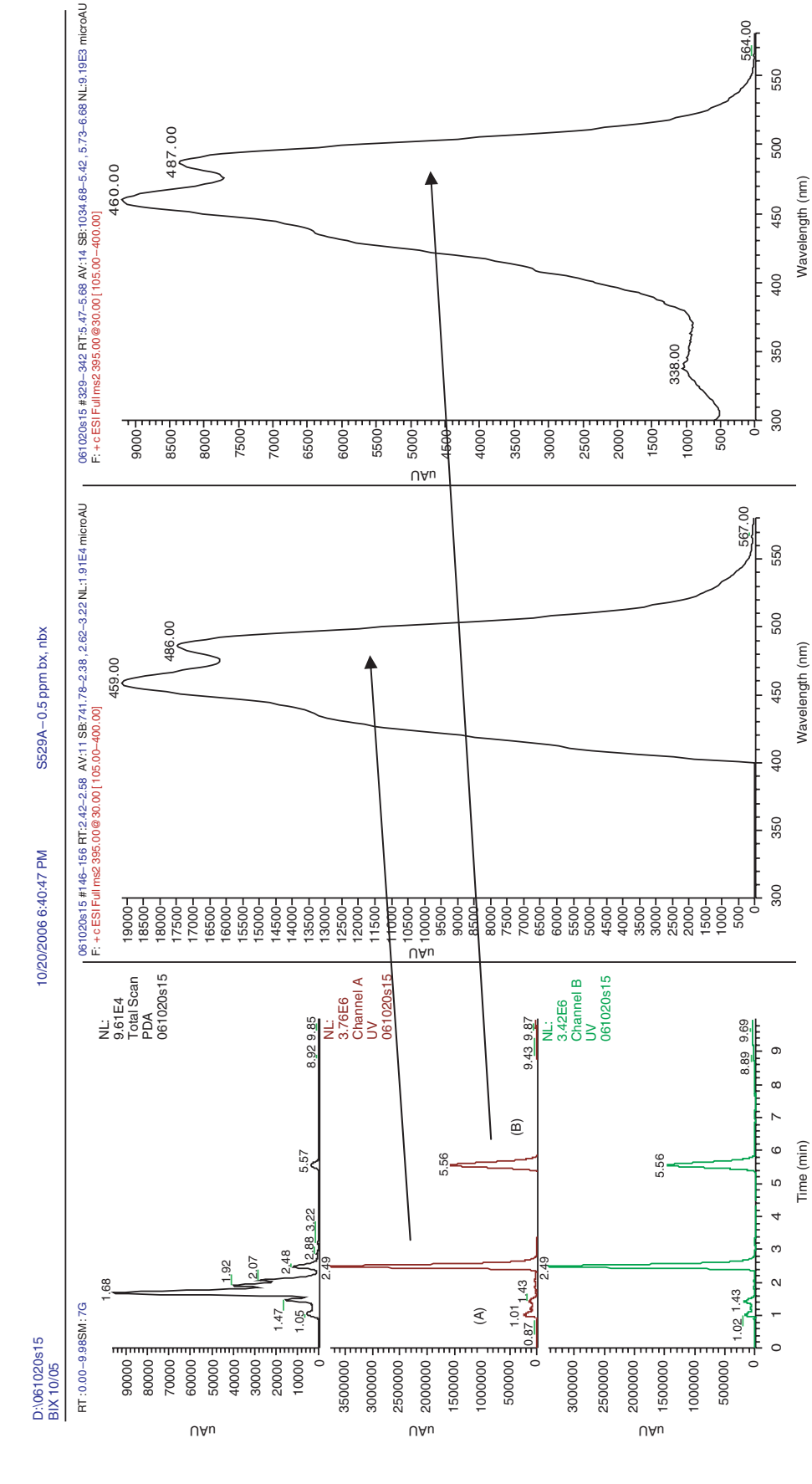

Figure 4. Chromatograms and PDA spectra of a 1-g sample of commercial pork sausage fortified with 0.5 mg kg^{-1} norbixin (A) and bixin (B).

by determining the recovery of additions of known amounts of the analytes to a blank matrix. For this purpose, blank meat tissue was fortified with 0.5, 0.75, 1, 2 and 4 mg kg^{-1} in six replicates and on three different days. All calculated recoveries fulfilled the criterion that recovery should be between 80 and 110% for a mass fraction of, or greater than, 10 μg kg^{-1}, except for norbixin at a level of 2 mg kg^{-1}. The recoveries at this concentration level appeared to be higher for both compounds, although there was no specific explanation. To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. Repeatability was evaluated by calculating the coefficients of variation (CV) using the data of two series of three replicates of fortified samples of an identical matrix at 0.5 up to 4 mg kg^{-1}. These analyses were carried out on two different occasions by the same analyst and under repeatable conditions. According to the Horwitz (HW) equation, the CV for the repeated analysis of fortified samples at a mass fraction of 0.5 and 4 mg kg^{-1} should not exceed 18 and 13%, respectively. All calculated CV values for norbixin and bixin were between 3 and 9%.

Reproducibility

Because no proficiency tests were available for annatto, or more specifically for norbixin or bixin, in matrices of animal origin, to evaluate reproducibility, only the within-reproducibility was considered. Thus, three series of five replicates of fortified samples at 0.5 up to 4 mg kg^{-1} were analysed by different operators and on different days. The calculated CVs for both operators, different days of analysis and different concentrations were all between 3 and 17% for both compounds considered. These values are the same order of magnitude as the CVs calculated using the Horwitz equation.

Ruggedness

To determine the ruggedness of the analytical method, experiments were carried out by changing factors of the sample pre-treatment and clean-up, such as the different SPE sorbents, extraction solvents and volumes. A number of parameters of chromatographic analysis, e.g. injection volume, liquid phases, chromatographic columns and solvent gradient programs, were also tested. For the PDA and MS^2 analysis, different ionization modes and wavelengths were tested, respectively. Evaluation of the ruggedness of our method resulted in restraining the parameters, as described in the Materials and methods section. Above this, a wide range of commercial processed pork meat products (e.g. sausages, non-smoked gammon, smoked gammon and sliced cold meat products) were analysed using the above described method. In all investigated matrices, it was possible to determine annatto, or more specifically norbixin and bixin, at 0.5 mg kg^{-1} using PDA and 1 mg kg^{-1} using MS^2 by spiking the matrix (Figure 4). No problems were encountered with co-extractive interferences, thus demonstrating the robustness of the method with respect to the analysis of processed meat products.

Decision limit (CCα) and detection capability (CCβ)

Finally, the decision limit, which is the concentration level at and above which the sample is non-compliant with 95% certainty, was calculated for the considered coloring agents. This value was defined as the mean concentration of the spiked blank samples at the AL concentration level plus 1.64 times the corresponding standard deviation at this concentration. The detection capability, which is the smallest concentration that can be detected with 95% certainty, was calculated as the CCα plus 1.64 times the abovementioned standard deviation. The CCα values for norbixin and bixin were 0.6 mg kg^{-1}, whereas calculation of the CCβ values for these compounds gave 0.7 mg kg^{-1}.

In routine analysis, quantification of the obtained residues of norbixin and bixin was based on calibration curves of three spikes (0.5, 1 and 5 mg kg^{-1}) in the same matrix of the sample to compensate for matrix effects.

Conclusion

A routine quantitative approach for the determination of annatto, or more specifically norbixin and bixin, in meat tissue in the low mg kg^{-1} concentration range is described. Using this validated method, annatto dye, or more particularly its compounds bixin and norbixin, can be detected in meat tissue from concentration levels of 0.5 mg kg^{-1}, which fits the objectives of the inspection services. The developed method has excellent recovery and good precision. The use of both photodiode array and MS showed large flexibility for the screening and confirmation of norbixin and bixin in routine residue analysis.

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